Inflammation Research (2019) 68:311–323
https://doi.org/10.1007/s00011-019-01217-z

Inflammation Research

ORIGINAL RESEARCH PAPER

Bazedoxifene protects cerebral autoregulation after traumatic brain injury and attenuates impairments in blood–brain barrier damage: involvement of anti-inflammatory pathways by blocking MAPK signaling

Yu-Long Lan[1,2,3,4] · Xun Wang[1,2] · Yu-Jie Zou[5] · Jin-Shan Xing[1,2] · Jia-Cheng Lou[1,2] · Shuang Zou[4] · Bin-Bin Ma[1,2] · Yan Ding[6] · Bo Zhang[1,2]

Received: 8 October 2018 / Revised: 4 January 2019 / Accepted: 25 January 2019 / Published online: 31 January 2019

Abstract

Objective Traumatic brain injury (TBI) is a significant cause of death and long-term deficits in motor and cognitive functions for which there are currently no effective chemotherapeutic drugs. Bazedoxifene (BZA) is a third-generation selective estrogen receptor modulator (SERM) and has been investigated as a treatment for postmenopausal osteoporosis. It is generally safe and well tolerated, with favorable endometrial and breast safety profiles. Recent findings have shown that SERMs may have therapeutic benefits; however, the role of BZA in the treatment of TBI and its molecular and cellular mechanisms remain poorly understood. The aim of the present study was to examine the neuroprotective effects of BZA on early TBI in rats and to explore the underlying mechanisms of these effects.

Materials and methods TBI was induced using a modified weight-drop method. Neurological deficits were evaluated according to the neurological severity score (NSS). Morris water maze and open-field behavioral tests were used to test cognitive functions. Brain edema was measured by brain water content, and impairments in the blood–brain barrier (BBB) were evaluated by expression analysis of tight junction-associated proteins, such as occludin and zonula occludens-1 (ZO-1). Neuronal injury was assessed by hematoxylin and eosin (H&E) staining. LC–MS/MS analysis was performed to determine the ability of BZA to cross the BBB.

Results Our results indicated that BZA attenuated the impaired cognitive functions and the increased BBB permeability of rats subjected to TBI through activation of inflammatory cascades. In vivo experiments further revealed that BZA provided this neuroprotection by suppressing TBI-induced activation of the MAPK/NF-κB signaling pathway. Thus, mechanically, the anti-inflammatory effects of BZA in TBI may be partially mediated by blocking the MAPK signaling pathway.

Conclusions These findings suggest that BZA might attenuate neurological deficits and BBB damage to protect against TBI by blocking the MAPK/NF-κB signaling pathway.

Keywords Bazedoxifene · Traumatic brain injury · Inflammation · Neuroprotection

Introduction

Traumatic brain injury (TBI) is currently the most important cause of death or disability among adolescents and children [1]. TBI includes both primary and secondary injury [2]; primary brain damage is mainly caused by mechanical attacks and is incurable [3]. The primary injury is often accompanied by delayed secondary injury, which occurs within minutes or hours to days after the initial injury. Secondary injury often leads to neuronal loss and central nervous system dysfunction, such as blood–brain barrier (BBB) damage, brain

Responsible Editor: Thiago Mattar Cunha.

Yu-Long Lan, Xun Wang and Yu-Jie Zou contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00011-019-01217-z) contains supplementary material, which is available to authorized users.

✉ Bo Zhang
zhangbodlykdx@126.com

Extended author information available on the last page of the article

edema, neurodegenerative lesions, neurological dysfunction, and cerebral ischemia [4, 5]. However, the time-dependent progression of TBI offers a window for intervention to reduce the delayed secondary injury, although therapeutic efficacy is limited by the BBB. Thus, developing chemotherapeutic drugs that can penetrate the BBB is of great significance for improving the prognosis of TBI patients.

The BBB is primarily composed of brain endothelial cells, a basal lamina, astrocytic foot processes, and sealed tight junctions (TJs), including claudins, zonula occludens (ZO) and occludin [6]. The breakdown of the BBB is an important step in the progression of TBI. Various mechanisms have been recently proposed to explain the secondary injury following BBB disruption. It has been reported that the expression levels of TJ-associated proteins, such as occluding and ZO-1, are significantly reduced in TBI animal models [7–9]. In addition, BBB damage can arise as a part of the brain's response to injury or neurological pathologies [10]. Brain edema is inevitable following BBB destruction and is usually caused by increased permeability after endothelial destruction; vascular contents infiltrate through dilated endothelial TJs to ultimately lead to outward penetration [11]. Subsequently, the accumulation of liquid further increases pressure in the brain and aggravates TBI. In addition, BBB damage can also lead to long-term complications, such as cognitive impairment. Therefore, elucidating the mechanism of brain damage caused by BBB damage will aid in the discovery of therapeutic targets for TBI.

Although not an inflammatory disease, TBI may cause increased expression of cerebral cytokines, including interleukin (IL)-1β, IL-6, cyclooxygenase-2 (COX-2), and tumor necrosis factor-a (TNF-a), which are produced by supporting microglia and astroglial cells, endothelial cells and neurons [12]. These major proinflammatory molecules also play supplementary roles in BBB disruption [13], as demonstrated in animal models, which have revealed increased IL-1β-dependent BBB permeability related to occludin/ZO-1 loss and TJ redistribution [14]. In addition, other inflammatory factors, including bradykinins and matrix metalloproteinases (MMPs), could also promote BBB disruption [15].

NF-κB, a central regulator of the inflammatory process, plays a critical role in inflammation and is an optimal therapeutic target for the pathogenesis of inflammation. This molecule regulates the expression of various proinflammatory mediators [16]. Various studies have suggested that NF-κB nuclear translocation is an active inflammatory response, suggesting that it could be possibility manipulated in anti-inflammatory drug development [17]. It is known that microglia can be activated in large quantities after brain injury, and microglia are closely associated with the formation of secondary brain edema [18, 19]. The release of proinflammatory mediators by activated microglia is regulated by multiple signaling pathways, among which the mitogen-activated protein kinase (MAPK) signaling pathway is the most important [20, 21]. MAPK signaling pathways, such as those mediated by p38, JNK, and extracellular signal-regulated kinase (ERK), are also important for NF-κB transactivation or translocation [22]. Elucidating the precise mechanisms involved in targeted inhibition of the major pathways involved in the inflammatory cascades may help effectively control functional impairments in the early stages after severe TBI.

Selective estrogen receptor modulators (SERMs) are compounds that act as an estrogen in certain tissues, such as bone tissue, and as an anti-estrogen in other tissues, such as the uterus or the breast, based on the distribution of subtypes of estrogen receptor α (ERα) and ERβ and on the activity of ER-associated coregulatory proteins [23]. Currently, tamoxifen and raloxifene are the two most widely used SERMs in the clinic. However, both are also associated with an undesirable risk of hot flushes and venous thromboembolism [24]. Additionally, tamoxifen can increase the risk of endometrial hyperplasia and may increase the risk of cancer [25]. Bazedoxifene (BZA), a new molecule with unique structural characteristics compared with raloxifene and tamoxifen, is a novel SERM developed for the treatment of osteoporosis for postmenopausal women [26]. BZA was developed using stringent preclinical screening parameters, including requirements for favorable effects on lipid metabolism and the skeleton and, more importantly, breast and uterine safety [27]. We sought to explore the neuroprotective effects of BZA treatment on early TBI in rats and to explore the underlying mechanisms of these effects.

Materials and methods

Chemicals

Bazedoxifene (purity > 98%) was purchased from GrowingChem Co., LTD. (Shanghai, China). Antibodies specific to occludin, ZO-1, NF-κB p65 (p65), phospho-NF-κB p65 (p-p65), phospho-IκB-α (p-IκB-α), p-ERK, p-JNK, p-p38, β-actin, and Lamin B1 and all secondary antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Inc., USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified. The remaining reagents were used according to the analyses performed.

Animals

Male Sprague–Dawley (SD) rats (pathogen-free grade males weighing 230–280 g; aged 7 weeks) were purchased from the Animal Experimental Centre of Dalian Medical University, China (Certificate no. SCXK (Liao) 2008-0002). The

study was approved by the Animal Research Ethics Committee of Dalian Medical University. All experimental procedures were performed according to the Guidance Suggestions for the Care and Use of Laboratory Animals published by the Ministry of Science and Technology of the People's Republic of China.

Fifty SD rats were randomly divided into the following five groups for the efficacy experiment, and each group was assessed at 2 time points (24 and 48 h): (1) Sham: rats only underwent a craniotomy, but their brain tissue was not damaged. After the operation, sham rats received the same amount of vehicle by intraperitoneal injection every 24 h ($n=10$); (2) Sham + BZA (3 mg/kg): After the operation, rats received 3 mg/kg BZA by an intraperitoneal injection every 24 h ($n=10$); (3) TBI: After TBI, rats received the same amount of vehicle by intraperitoneal injection every 24 h ($n=10$); (4) TBI + BZA (3 mg/kg): After TBI, rats received 3 mg/kg BZA by intraperitoneal injection every 24 h ($n=10$); (5) TBI + BZA (6 mg/kg): After TBI, rats received 6 mg/kg BZA by intraperitoneal injection every 24 h ($n=10$).

Another fifty SD rats, including males and females, were used to explore possible gender effects and to investigate the potential long-term neuroprotective effects of BZA. The sham animals underwent the same craniotomy, but were not injured ($n=5$ males; $n=5$ females). The TBI animals were divided into four groups: (1) M-saline group (TBI + saline, male, $n=12$), (2) M-BZA group (TBI + BZA, male, $n=12$), (3) F-saline group (TBI + saline, female, $n=8$) and (4) F-BZA group (TBI + BZA, female, $n=8$).

TBI model

The TBI model used in the present study was based on the controlled cortical impact (CCI) model with some modifications (Supplementary Fig. S1). Briefly, (1) Rats were anesthetized by inhalation of 4% isoflurane mixed with 2:1 N_2O/O_2 in a closed bottle for 60 s. (2) Rats were fixed on a stereotactic frame, and a mechanical ventilation device was used to assist breathing. Anesthesia was maintained with 4% isoflurane mixed with 2:1 N_2O/O_2. (3) The hair was removed from the top of the head, and the skin was disinfected with iodophor. The skin was incised from the right side of the centerline and opened, the soft tissue was removed from the periosteum, and the skull was exposed. (4) A pneumatic grinding drill was used for skull fenestration to create a cranial window of 4 mm in size with an oval shape; the coronal seam and the herringbone seam were the front and back boundaries, and the sagittal joint and the coronal ridge were the left and right boundaries. The dura was retained completely intact. (5) The height of the dropping weight, i.e., the distance between the dropping weight and the cortical surface, was adjusted. (6) The impact depth of the attack was set to 1 mm, and the impact speed was set to 2 m/s. The other parameters were as follows: impact tip diameter, 3 mm; compression time, 85 ms. (7) Anesthesia was stopped, and silk sutures were used to close the skin with the bone flap removed. The rats were removed from the stereotactic instrument and placed in their cage for observation.

Neurological severity test

Neurological scores were assessed 24 h and 48 h after TBI using the neurological severity score (NSS) system. Two observers independently assessed the ability of each rat ($N=6$) to perform 10 different tasks. Rats that failed to perform the tasks received one or no points (Supplementary Table S1).

Brain water content

The water content in the brain was measured using the wet-dry weight method, as previously described [28]. In brief, the contusion cortex and the surrounding injured brain tissues were rapidly harvested, quickly weighed to obtain the wet weight, dried in an incubator at 120 °C for 24 h and reweighed (dry weight). The brain water content was calculated by the following formula: ratio = [(wet weight − dry weight)/wet weight] × 100%.

Morris water maze test

To assess the cognitive changes after radiation, rats received certain treatments in different groups, and the Morris water maze test was then conducted. The test was performed in a circular black pool (width: 127 cm, height: 60 cm) with a black platform. The pool was filled with milk-diluted water at room temperature (21.0 ± 1.0 °C). The platform was set at approximately 1.5 cm below the water surface.

Facing the wall of the pool, the rats were placed in the water at one of four possible locations, and a camera was used to record movement. Each mouse was given up to 60 s to find the platform. The trial was terminated when the mouse located the platform within 60 s; if the mouse failed to find the platform within 60 s, it was guided to the platform by a researcher and was allowed to remain on the platform for 2–3 s. To permit the rats to adapt to the pool environment, each mouse was three practice trials per day for 2 days and was then tested 3 times per day for 5 days to locate the hidden platform. The latency (the time taken to locate the platform), distance traveled, and other data were recorded by automated video tracking software (EthoVision 2.3.19, Noldus, Wageningen, The Netherland). The pool was divided into four quadrants for further analysis. In the first part, the platform was moved to a different quadrant in each session for visual training. In the second part, the platform

was located in the center of the target quadrant and remained in the same position for hidden platform training. The behavior of the rats was recorded using EthoVision 3.0, and the escape latency was quantified.

Open-field assessment

The rats were removed from their home cage and placed individually in clear Perspex tracking arenas (Coulburn TruScan, USA). The total number of movements over a 60-min period was recorded and analyzed.

Foot fault test

To evaluate sensorimotor function, the foot fault test was carried out before TBI and at 1, 4, 7, 14, 21, 28, and 35 days after TBI or sham surgery by an investigator blinded to the treatment groups. The rats were allowed to walk on a grid (12 cm × 57 cm with 1.3 cm × 1.3 cm diameter openings). With each weight-bearing step, a paw might fall or slip between the wires [29], and a slip was recorded as a foot fault. A total of 50 steps were recorded for each right forelimb and hindlimb.

Tissue processing

The rats were rapidly killed 24 h post-TBI, and their ipsilateral cortexes were collected. The tissue was positioned directly over the center of the injury site and included both contusions and penumbra. The samples were immediately frozen in liquid nitrogen and stored at − 80 °C until use. For the immunohistochemistry analysis, the ipsilateral brain tissue was removed 24 h after TBI, immersed in 4% paraformaldehyde overnight and measured by a fluorescence-activated cell sorter (FACS) using an Annexin V-FITC Apoptosis Detection Kit (Nanjing KeyGEN Biotech. CO., LTD.).

Enzyme-linked immunosorbent assay (ELISA)

Total protein was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, China). The levels of inflammatory cytokines in the brain tissue were quantified using rat-specific ELISA kits (IL-1β, IL-6, COX-2 and TNF-α from Shanghai Saimo Biotechnology, Shanghai, China) according to the manufacturer's instructions. The inflammatory cytokine contents in the brain tissues are expressed as nanograms per gram protein.

Western blotting analysis

Total and nuclear proteins were extracted from the ipsilateral cerebral cortical tissue using a Total and Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. Proteins were separated by electrophoresis on 7.5–12% sodium dodecyl sulfate-polyacrylamide minigels (SDS-PAGE) and then electrophoretically transferred to PVDF membranes. The western blots were probed with specific antibodies, and protein bands were detected by enhanced chemiluminescence. Similar experiments were performed at least three times. The total protein concentration was determined using a BCA protein assay kit.

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues using TRIzol reagent according to the kit protocol (TaKaRa Bio, Dalian, China). cDNA was reverse-transcribed using a PrimeScript RT Reagent Kit (TaKaRa Bio, Dalian, China) according to the manufacturer's instructions. Q-PCR was performed following the kit protocol (TaKaRa Bio, Dalian, China), and amplification was performed using an Mx3005P Real-Time PCR System (Agilent, CA, USA). The relative mRNA expression of each gene was normalized to GAPDH RNA levels and analyzed using the $2^{-\Delta\Delta C_T}$ method. The primers were synthesized by Invitrogen (Shanghai, China).

Immunohistochemical staining

The brain tissue samples were fixed with 10% neutral formalin and embedded in paraffin. For the immunohistochemical examination, the tissue sections (4 mm) were incubated with occludin, ZO-1 or p-p65 NF-κB (1:50) antibodies and examined under a light microscope. The images were examined under a Leica DM 4000B microscope equipped with a digital camera.

Detection of BZA through the BBB

Adult SD rats were intraperitoneally injected with BZA (3 mg/kg), and after 1 h, the rats were anesthetized with 4% chloral hydrate. Cerebrospinal fluid (CSF) was collected from the cerebellomedullary cistern by puncturing the foramen magnum. Next, the CSF was extracted twice using an equal volume of acetonitrile. The supernatant was dried in a nitrogen blowing instrument and reconstituted in 50 µl mobile phase (acetonitrile:pure water = 45:55). Finally, the reconstituted sample and BZA standard solution were analyzed by LC–MS/MS.

Statistical analysis

All experiments were repeated three times. Data are represented as the mean ± standard deviation (SD). Analysis of

variance and Student's *t* test were used to compare the values of the test and control samples in vitro and in vivo. $P<0.05$ was considered to be a statistically significant difference. SPSS 17.0 software was used for all statistical analyses.

Results

BZA attenuated the neurological deficits, brain edema, impaired cognitive functions and neuronal injury caused by TBI

The NSS includes motor, sensory and reflex tests. The mean NSS for each group is shown in Fig. 1a. The NSS for the TBI group at 24 h and 48 h post-TBI was lower than that of the sham group, indicating that TBI caused significant

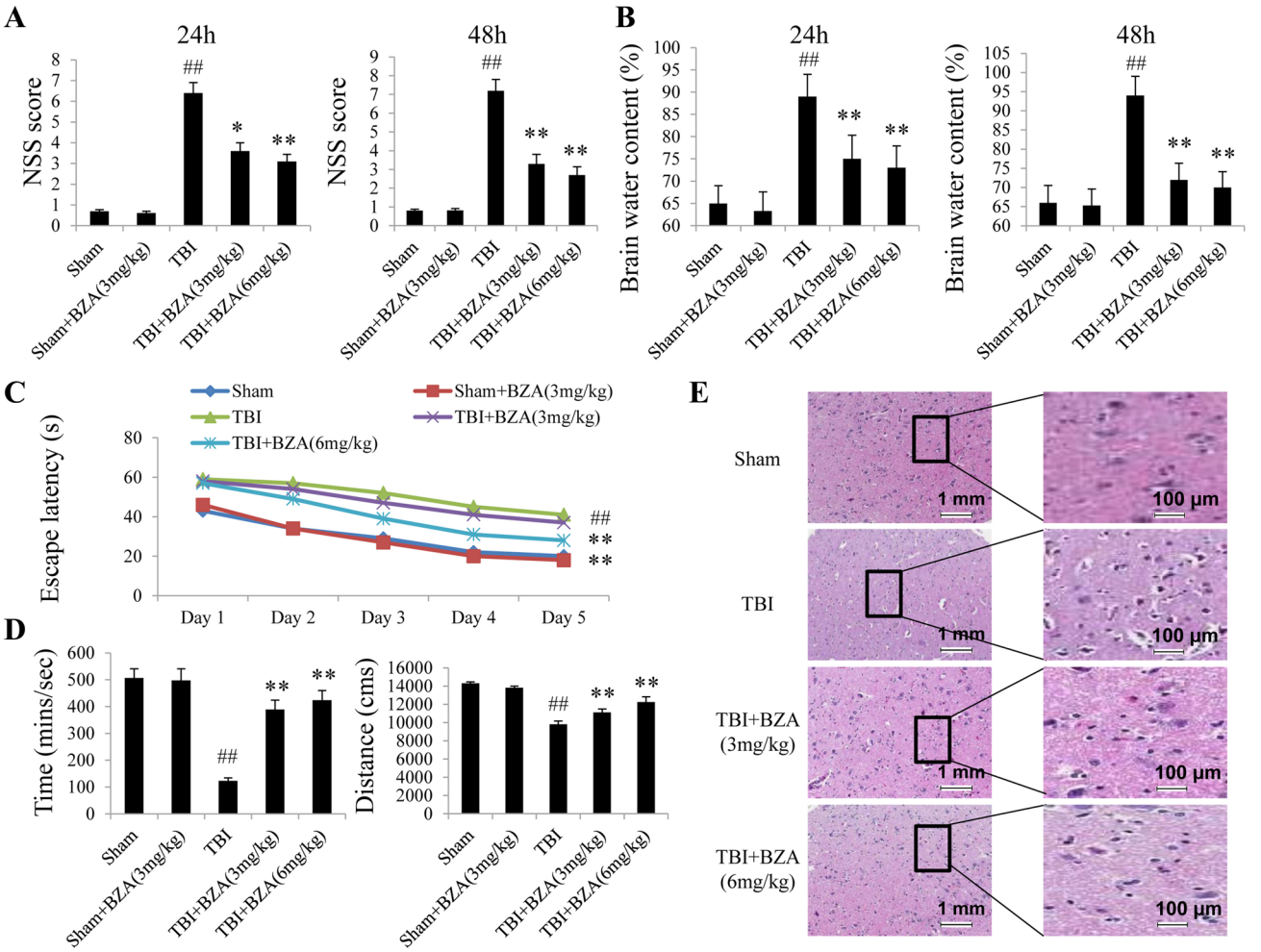

Fig. 1 BZA attenuated the neurological deficits, brain edema, impaired cognitive functions and neuronal injury caused by TBI. **a** BZA decreased the NSS after TBI in rats and reduced the water content in the injured tissue at 24 h and 48 h. $^{\#\#}P<0.01$ vs. sham group. $^{*}P<0.05$, $^{**}P<0.01$, vs. control group. **b** BZA reduced the water content in the injured tissue at 24 h and 48 h. $^{\#\#}P<0.01$ vs. sham group. $^{*}P<0.05$ $^{**}P<0.01$ vs. control group. **c**–**d** BZA attenuated TBI-induced impaired cognitive functions. **c** The effect of BZA on impaired spatial learning of rats (escape latency in the training trials) was observed by the Morris water maze test. $^{\#\#}P<0.01$ vs. sham group. $^{**}P<0.01$ vs. control group. **d** BZA-treated rats showed significantly enhanced willingness to explore in the open-field activity test. Treated rats showed significant increases in (left panel) ambulatory time and (right panel) ambulatory distance ($^{\#\#}P<0.01$ vs. sham group. $^{**}P<0.01$ vs. control group.). **e** Representative images of H&E-stained sections at 24 h post-TBI. Cells with brown-stained cytoplasm are considered positive. Neurons with round or oval shapes and a clear nucleolus were regularly arranged in the sham group. Compared with the sham group, severe degenerative changes were observed in the TBI group, including shrunken cytoplasm and extensively pyknotic nuclei in neurons. Treatment with BZA significantly decreased the intensity of nuclear staining compared with the TBI group. Scale bars = 100 μm

neurological deficits in the rats. However, significant improvement was observed in the BZA treatment group compared with the TBI group at both 24 h and 48 h, suggesting that BZA treatment effectively attenuated the neurological deficits in rats with TBI. Brain water content was also measured to assess the severity of brain edema, which is associated with the integrity of the BBB. The results indicated that the brain water content in the TBI group was significantly higher than that in the sham group. However, the BZA treatment group showed a significant decrease compared with the TBI group (Fig. 1b).

The Morris water maze test was used to evaluate spatial learning. The rats in the TBI group did not appear to learn during the 5-day training phase of the water maze, which may reflect cognitive deficits without treatment. Similarly, sham rats did not show significant improvement during the training phase of the trial. BZA-treated rats demonstrated overall improved learning of the task and improved recall of the task compared with controls (Fig. 1c). The open-field behavioral assessment indicated a significant increase in exploration activity for the TBI plus BZA-treated group compared with the TBI group, and there was no significant difference between the sham group and the sham plus BZA-treated group (Fig. 1d). The long-term effects of TBI and the neuroprotective effects of BZA have also been explored, and the changes in body weight in both genders before and after TBI are shown in Supplementary Table S2. The results showed that body weight was significantly decreased at days 1 and 4 postinjury in the saline-treated rats ($P < 0.001$) and at days 1, 4 and 7 in the BZA-treated rats ($P < 0.001$). However, there were no significant differences in body weight between the BZA- and saline-treated groups for either males or females, nor were there significant differences between males and females in either the saline-treated or BZA-treated group ($P < 0.05$). At 14 days after injury, body weight had almost returned to preinjury levels and continued to increase slowly afterwards. Possible gender effects of BZA were also investigated (Supplementary Fig. S2). TBI impaired spatial learning without a significant gender difference (Supplementary Fig. S2B). BZA treatment significantly improved spatial learning in males at days 33, 34 and 35 and in females at days 34 and 35 compared with the saline-treated group ($P < 0.05$). The sensorimotor function test (Supplementary Fig. S2C) further indicated that TBI caused a significant increase in the number of right forelimb foot faults contralateral to the TBI from 1 to 21 days postinjury compared with the preinjury baseline for both genders. However, male TBI rats exhibited a significantly higher frequency of foot faults than female TBI rats on days 1, 4 and 7 postinjury ($P < 0.05$). Treatment with BZA significantly reduced the number of contralateral forelimb foot faults from 1 to 28 days in the males after TBI compared with treatment with saline ($P < 0.05$). Similar effects were observed from 7 to 21 days after TBI in the female TBI rats treated with BZA compared with the female saline-treated rats ($P < 0.05$). Furthermore, hematoxylin and eosin (H&E) staining was used to evaluate the effect of BZA on histopathological changes at 24 h after TBI. The morphology of neurons stained with H&E in the sham group (Fig. 1e) was normal; these neurons had round or oval morphologies and a clear nucleolus with a regular arrangement. The TBI group demonstrated severe degenerative changes, such as decreased cytoplasm and the presence of dark pyknotic nuclei. However, the BZA-treated group exhibited a lower intensity of TBI-related changes than the TBI group.

BZA treatment alleviated TBI-induced loss of TJ proteins in the ipsilateral cortex

Relative mRNA expression and protein levels of occludin and ZO-1 in ipsilateral cortex tissues were further examined. The results indicated that the mRNA expression (Fig. 2A) and protein levels (Fig. 2b, c) of occludin and ZO-1 were significantly lower in TBI rats than in sham-operated rats. Furthermore, the mRNA and protein levels of occludin and ZO-1 were significantly higher in BZA-treated TBI rats than in TBI rats. The results of the immunohistochemical examination are shown in Fig. 2d ($n = 5$/group). Decreased occludin and ZO-1 expression was observed in the TBI group compared with the sham group at 24 h. The BZA treatments (3 mg/kg and 6 mg/kg) increased occludin and ZO-1 expression. These results indicated that BZA treatment could alleviate TBI-induced loss of TJ proteins.

BZA induced significant reductions in multiplex cytokine levels after TBI

Inflammatory processes are considered major components of the secondary injury cascade following TBI. As shown in Fig. 3, the expression levels of proinflammatory cytokines (i.e., IL-1β, IL-6, COX-2 and TNF-α) were low in the brains of rats in the sham group, but were significantly increased following TBI ($^{\#\#}P < 0.01$); however, the administration of BZA significantly decreased the expression levels of these cytokines compared with the levels in the TBI control group ($^{*}P < 0.05$, $^{**}P < 0.01$). These indicators did not significantly differ between the sham and sham + BZA groups.

BZA inhibited activation of the NF-κB signaling pathway and NF-κB translocation into the nucleus induced by TBI

Activation of the NF-κB signaling pathway is closely related to the overexpression of the abovementioned various proinflammatory cytokines [30, 31]. Thus, we examined the effect of BZA on NF-κB expression and NF-κB

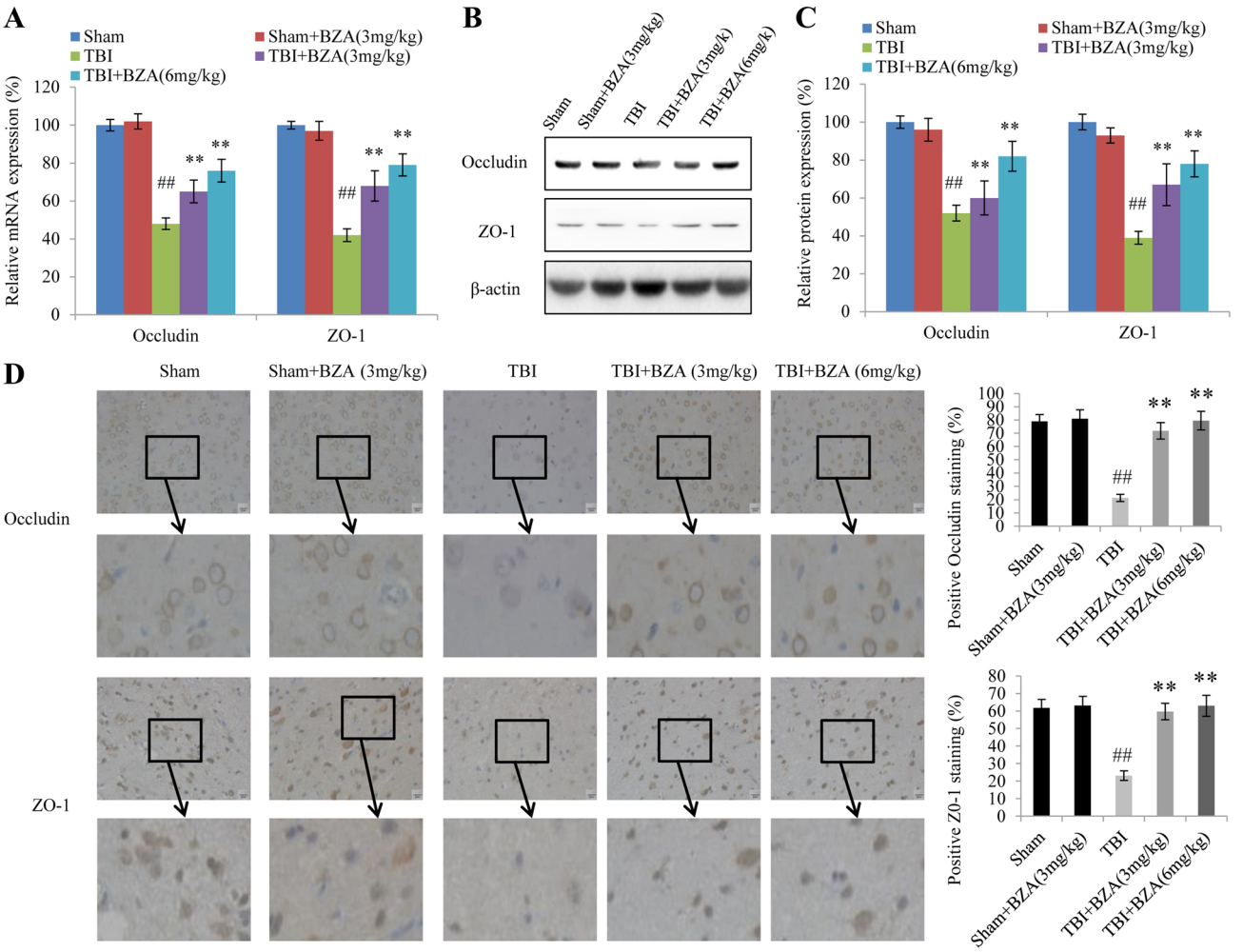

Fig. 2 BZA treatments (3 mg/kg or 6 mg/kg) alleviated TBI-induced loss of junction proteins in the ipsilateral cortex of experimental animals. **a** Relative mRNA expression levels of occludin and ZO-1 were analyzed by RT-qPCR 24 h postinjury. **b** Protein expression levels of occludin and ZO-1 were examined by western blotting analysis 24 h postinjury. β-actin was used as a loading control. **c** Quantitative analysis of occludin and ZO-1 protein expression. Data are presented as the mean ± SD. Experiments were repeated in triplicate. $^{\#\#}P < 0.01$ vs. sham group. $^{**}P < 0.01$ vs. control group. **d** Representative images of immunohistochemical (IHC) analysis of occludin and ZO-1 protein expression (original magnification, ×400). Scale bars = 20 μm

translocation. According to the results of western blotting (Fig. 4a, b), the expression levels of p-p65 and p-IκB-α in the TBI control group were higher than those in the sham group. However, these proteins were obviously decreased in the BZA treatment group compared with in the TBI control group ($^{**}P < 0.01$) (Fig. 4a). Then, we extracted the nuclear protein, and western blot analysis further indicated that the expression levels of NF-κB p65 and p50 were significantly upregulated in the TBI control group, while the protein content was markedly lower in the BZA treatment group than in the TBI control group ($^{**}P < 0.01$) (Fig. 4b, left panel). In addition, the cytosolic protein expression levels of p65 and p50 were significantly downregulated in the TBI control group, while they were markedly increased in the BZA treatment group compared with in the TBI control group ($^{**}P < 0.01$) (Fig. 4b, right panel). Immunohistochemical analysis indicated that the expression of phosphorylated p65 (p-p65) was increased significantly, while BZA treatment suppressed the p-p65 expression level (Fig. 4c). These results indicated that BZA could attenuate the increased activation of p65 induced by TBI.

BZA suppressed the phosphorylation of MAPKs

The phosphorylation status of MAPKs was investigated by western blotting analysis, and the results indicated that BZA significantly suppressed the phosphorylation of ERK, p38 and JNK MAPKs (Fig. 5a). These results indicate that BZA suppresses the inflammatory response through partial regulation of MAPK signaling by

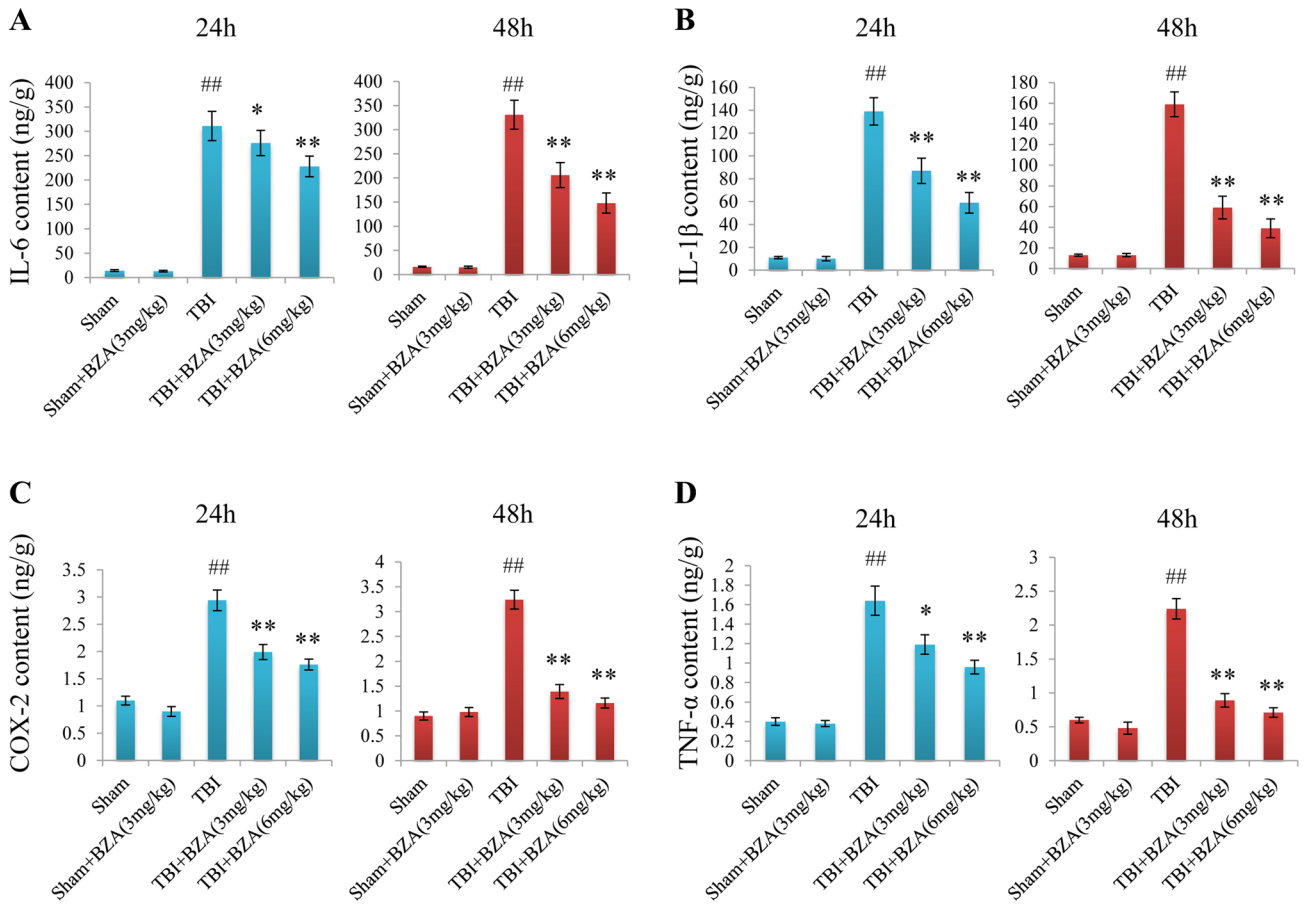

Fig. 3 BZA ameliorated TBI-induced inflammation. ELISA of the expression levels of proinflammatory genes, including **a** IL-6, **b** IL-1β, **c** COX-2 and **d** TNF-α 24 h after TBI. $^{\#\#}P < 0.01$ vs. sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group

targeting p-ERK, p-JNK and p-p38. The anti-inflammatory mechanisms of BZA regarding its effect on inflammatory signaling pathways are illustrated in a graphical representation (Fig. 5b).

BZA can traverse the BBB

Since the BBB is the greatest barrier between the blood and the brain for nearly 98% of small molecules [32], we next explored whether BZA could penetrate the BBB. After treatment of BZA via intraperitoneal injection or intravascular injection (data not shown), we collected CSF for assessment by LC-MS/MS assay. As shown in Fig. 6, a high chromatographic peak was observed at 1.34 min in the CSF samples, which was consistent with the BZA standard sample. This result indicated that BZA could penetrate the BBB, which may lead to a better therapeutic effect in treating TBI or other intracranial lesions.

Discussion

In the current study, we confirmed that BZA attenuated TBI-induced BBB damage, functional impairments and activation of inflammatory cascades. In vivo experiments revealed that BZA provided this neuroprotection by suppressing TBI-induced activation of the MAPK/NF-κB signaling pathway. These results suggest that BZA could be used to treat patients with TBI and improve prognosis. These findings could have broad application prospects in the preparation of neuroprotective drugs for the treatment of brain injury.

No pharmacotherapy has been approved by the US Food and Drug Administration (FDA) for the treatment of TBI in humans [33]. Estrogen has been demonstrated to alleviate secondary injury associated with TBI and could be important for functional recovery after TBI [34]. Four natural estrogens are biosynthesized in women: estrone,

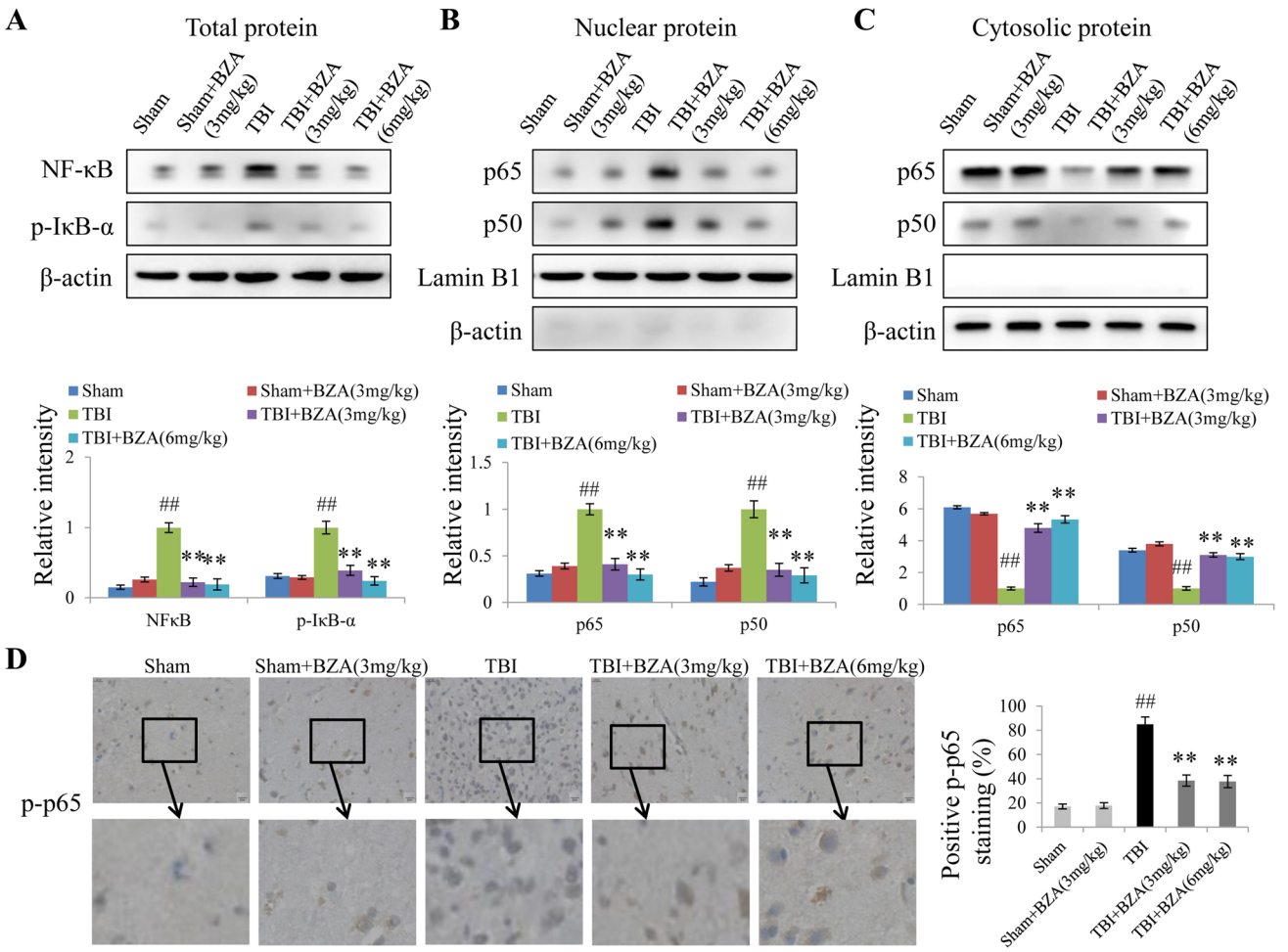

Fig. 4 BZA inhibited the activation of the NF-κB signaling pathway and its translocation into the nucleus induced by TBI. **a** Total protein expression of NF-κB and p-IκB-α by western blotting analysis. **b** Nuclear protein expression of NF-κB p65 and p50 by western blotting analysis. **c** Cytosolic protein expression of NF-κB p65 and p50 by western blotting analysis. $^{\#\#}P<0.01$ vs. sham group. $^{**}P<0.01$ vs. control group. **d** Protein expression of p-p65 by IHC (original magnification, ×400). Scale bars = 20 μm. $^{\#\#}P<0.01$ vs. sham group. $^{**}P<0.01$ vs. control group

estradiol, estriol, and estetrol. Among them, estradiol is the strongest form [35]. Estradiol could have significant physiological roles in both females and males [34]. However, elucidating the precise mechanisms of the neuroprotective effects of estradiol requires further research. Estradiol and other ligands, such as various SERMs, which bind to ERs, are known as ER agonists and are highly regarded for their roles in attenuating neuroinflammation [36, 37]. Conjugated estrogen treatment has been demonstrated to obviously reduce cell death [38]. Thus, it is proposed that BZA may also exert a neuroprotective effect against TBI similar to estrogen.

Other SERMs, such as tamoxifen and raloxifene, have also been previously demonstrated to improve functional outcomes following TBI [34, 39, 40]. BZA is a third-generation SERM that was developed from raloxifene by replacing its benzothiopenic core with an indolic ring [26]. Compared with other SERMs, BZA is generally well tolerated and has a favorable safety profile with respect to reproductive tissue. Furthermore, the effects of BZA on the endometrium appear more favorable than raloxifene [41]. Studies have also suggested that BZA, but not raloxifene, reduced the rate of nonvertebral fractures in high-risk postmenopausal women [42]. Thus, there are great differences in the pharmacological activities between BZA and raloxifene or other SERMs, and clinical studies have indicated that BZA is significantly superior to raloxifene in many ways. However, the specific mechanism of BZA requires further study. More importantly, if patients with TBI do not tolerate the side effects of raloxifene, BZA may be a superior alternative. In addition, rational combined use of various drugs has also become a new trend. Because progressive pathogenesis in TBI occurs via multiple cellular and molecular mechanisms, a multi-active compound or in combination with another suitable

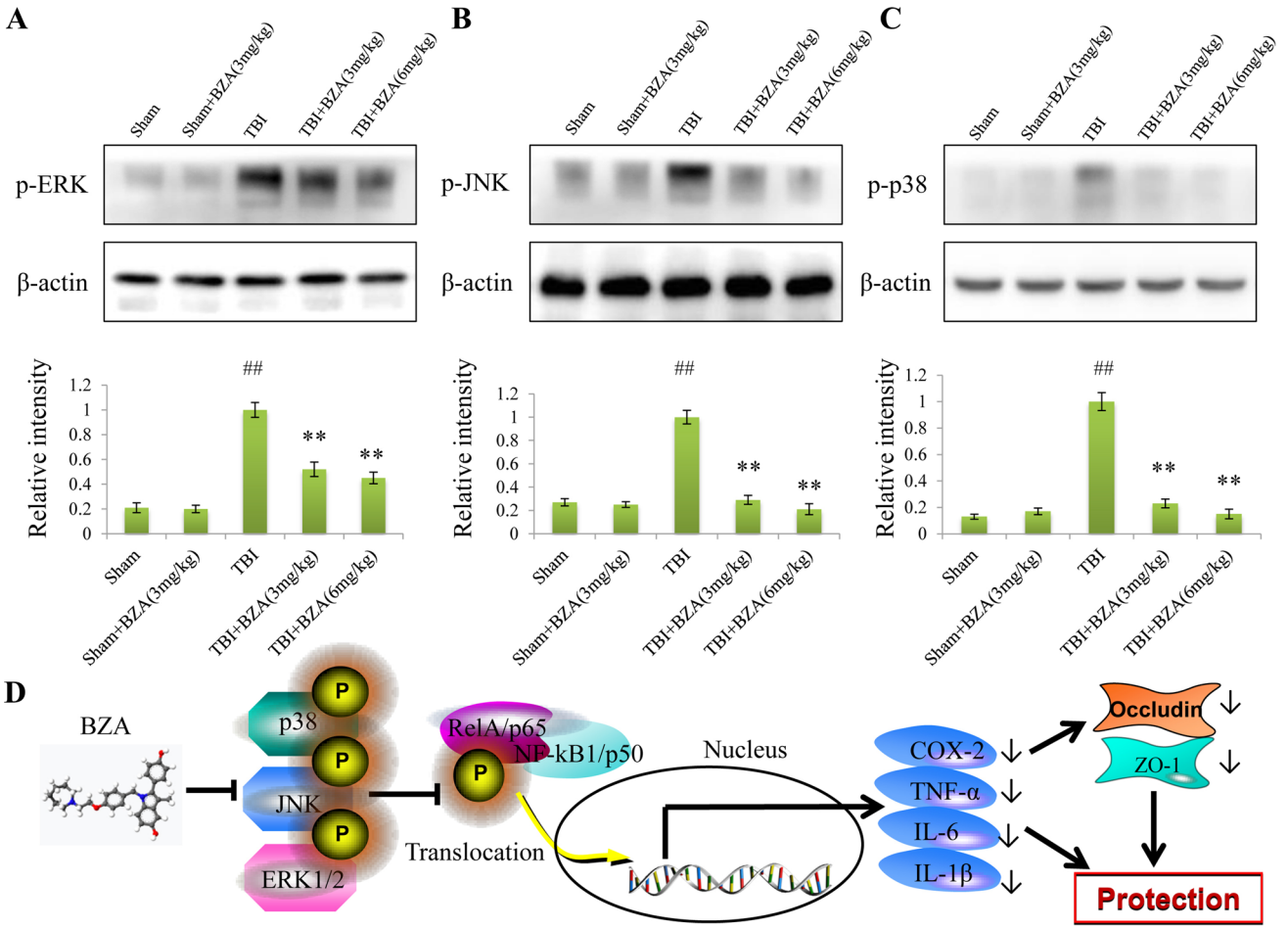

Fig. 5 BZA attenuated the increased phosphorylation of ERK, JNK and p38 MAPKs induced by TBI. **a–c** Western blotting and quantitative analysis further revealed that BZA specifically targets p-ERK, p-JNK and p-p38. $n=3$. $^{**}P<0.01$ compared with the control group; $^{\#\#}P<0.01$ compared with the indicated treatment group. **d** Schematic depiction of the mechanism by which BZA may protect cerebral autoregulation after TBI and attenuate impairments in BBB damage via the MAPK/NF-κB signaling pathway

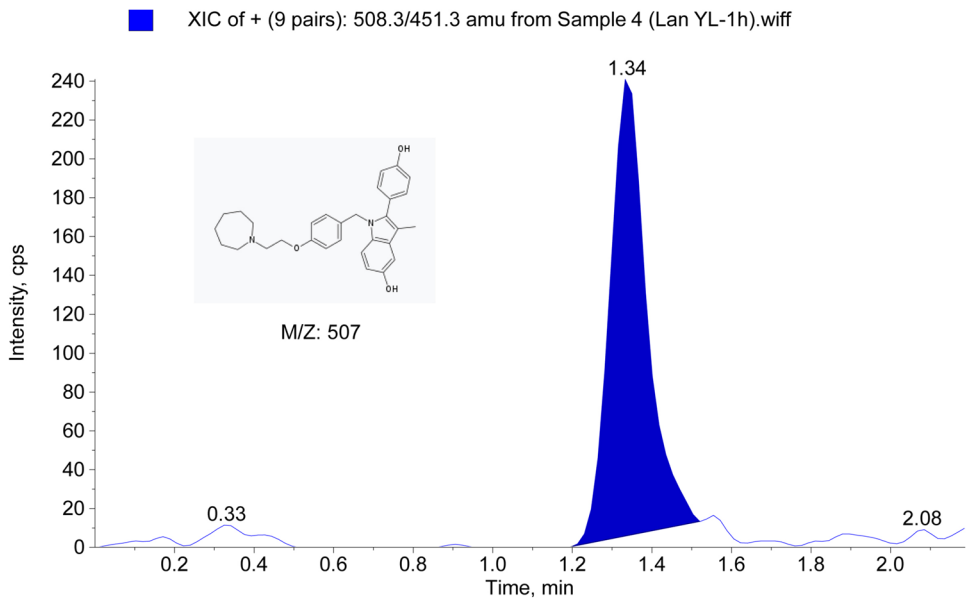

Fig. 6 BZA can penetrate the BBB. LC-MS spectrum of a CSF sample from a TBI rat after pretreatment with BZA (1 h)

therapeutic agent may be used to simultaneously target the multiple mechanisms of pathogenesis. Thus, the combination of BZA with other effective SERMs could provide greater therapeutic efficacy than any individual drug for the treatment of TBI, which we plan to study in future research.

It is known that the therapeutic efficacy of TBI is often hindered by the BBB [43]. Intriguingly, the current study indicated that BZA could penetrate the BBB, which suggests a better therapeutic effect and more promising application prospects of this drug for treating TBI. The current study also indicated that the BBB is disrupted within 24 h after TBI in animal models, but BZA treatment could reduce BBB disruption. It is widely known that TJs, including cytoplasmic (ZO-1, ZO-2, and ZO-3) and transmembrane proteins (claudins and occludin), have critical effects in regulating BBB integrity [44], and the upregulation of TJs has been confirmed to be beneficial for maintaining the integrity of the BBB [45]. BBB dysfunction could lead to brain edema and neuronal death and cause subsequent long-term TBI complications, such as cognitive and psychological impairments. Thus, we explored the effect of BZA on the levels of both occludin and ZO-1 in animal models of TBI. We found that both occludin and ZO-1 expression levels were decreased within 24 h after TBI and reversed by BZA, suggesting that BZA could partially restore BBB impairment resulting from TBI. Considering that BBB damage is associated with brain edema, neuronal death, and neurological impairments after TBI [43], BZA treatment exerted at least partial protective effects against TBI by attenuating BBB disruption.

There are various mechanisms for the beneficial effects of BZA administration observed following injury. To explore the precise mechanisms regarding the neuroprotective effects of BZA, we examined the changes in NF-κB protein expression. Our results indicated that BZA significantly suppressed the nuclear translocation of NF-κB p65 and p50 from the cytosol during the acute phase of TBI. In addition, MAPKs are a family of serine/threonine protein kinases that could play important roles in signal transduction in TBI [46]. The roles of three MAPKs—ERK, JNK and p38—in neuronal survival and death have been found to be altered by various insults [47, 48]. In the current study, we confirmed that BZA inhibited the nuclear translocation of NF-κB p65 and p50 and the phosphorylation of ERK, JNK and p38 MAPKs. These results suggested that the MAPK signaling pathway is also involved in inflammatory responses after TBI. Thus, BZA could alleviate TBI-induced inflammation through inhibition of the MAPK/NF-κB signaling pathway.

Blood–brain barrier is a major barrier that reduces the treatment efficacy of anti-cancer drugs for glioblastoma multiforme (GBM) [49]. Studies have confirmed that the CSF brain barrier is a more imperfect region of the BBB and can allow communication between the CSF and the extracellular fluid of brain tissue [32]. If a substance can enter the CSF from the blood, it can then diffuse freely into the brain tissue. Therefore, detecting the drug content in the CSF could be an important method for evaluating drug entry into the brain tissue [50]. The results of LC–MS/MS analysis indicated that BZA was detected in CSF collected from living rats. Thus, BZA could pass through the BBB, indicating its great potential for treating central nervous system (CNS) diseases.

Furthermore, TBI is a chronic condition [51] that is highly gender specific. For example, most moderate to severe brain injuries occur in men and typically result from engagement in high-risk activities, behaviors, and occupations historically considered more suitable for men than women, such as professional contact sports, construction, or military occupations [52]. Consequently, the effects of gender on etiology and outcomes have typically not been assessed in TBI research. However, current epidemiological studies [53] have reported that women sustain approximately one-third of TBIs according to hospitalization data. Furthermore, a growing body of literature suggests that neuroprotective agents or treatments proven in males may not work effectively in females after TBI [54, 55]. Therefore, it is increasingly necessary to address the effect of gender on all aspects of TBI to increase scientific knowledge. Interestingly, our present findings suggested that BZA significantly improves histological outcome, spatial learning recovery and sensorimotor function in both genders, indicating that BZA is a promising neuroprotective agent for TBI in both male and female patients and warrants further investigation. However, further studies are needed to clarify the differences between males and females regarding the neuroprotective effects of BZA in rodent TBI models.

Taken together, we demonstrated for the first time that BZA confers neuroprotective effects in a rat model of TBI. The neuroprotective effects of BZA may be associated with the inhibition of inflammatory responses via blocking the MAPK/NF-κB signaling pathway.

Acknowledgements This work is supported by grants from National Natural Science Foundation of China (nos. 81372714, 81672480), Liaoning Provincial Natural Science Foundation of China (no. 201602244), Distinguished Professor Project of Liaoning Province, Special Grant for Translational Medicine, Dalian Medical University (no. 2015002), Basic research projects in colleges and universities of Liaoning Province (no. LQ2017033).

Author contributions Conceptualization, YLL; Funding acquisition, BZ; Investigation, YLL, XW, YJZ, JSX, JCL, and SZ; Methodology, YLL and XW; Writing-original draft, YLL; Writing-review and editing, YLL, XW, JCLL, JSX, SZ, BBM, YD, and BZ.

Funding This work is supported by grants from National Natural Science Foundation of China (nos. 81372714, 81672480), Liaoning Provincial Natural Science Foundation of China (no. 201602244), Distinguished Professor Project of Liaoning Province, Special Grant

for Translational Medicine, Dalian Medical University (no. 2015002), Basic research projects in colleges and universities of Liaoning Province (no. LQ2017033).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Affiliations

Yu-Long Lan[1,2,3,4] · Xun Wang[1,2] · Yu-Jie Zou[5] · Jin-Shan Xing[1,2] · Jia-Cheng Lou[1,2] · Shuang Zou[4] · Bin-Bin Ma[1,2] · Yan Ding[6] · Bo Zhang[1,2]

1 Department of Neurosurgery, The Second Affiliated Hospital of Dalian Medical University, Dalian, China

2 Department of Neurosurgery, Shenzhen People's Hospital, Shenzhen, China

3 Department of Pharmacy, Dalian Medical University, Dalian 116044, China

4 Department of Physiology, Dalian Medical University, Dalian 116044, China

5 Department of Nursing, The First Affiliated Hospital of Dalian Medical University, Dalian 116023, China

6 Department of Pediatrics, Children's Hospital of Boston, Harvard Medical School, Boston, MA 02115, USA