● RESEARCH ARTICLE

Breviscapine reduces neuronal injury caused by traumatic brain injury insult: partly associated with suppression of interleukin-6 expression

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Graphical Abstract

Breviscapine improves neurobehovior and down-regulates interleukin-6 expression in rats with traumatic brain injury
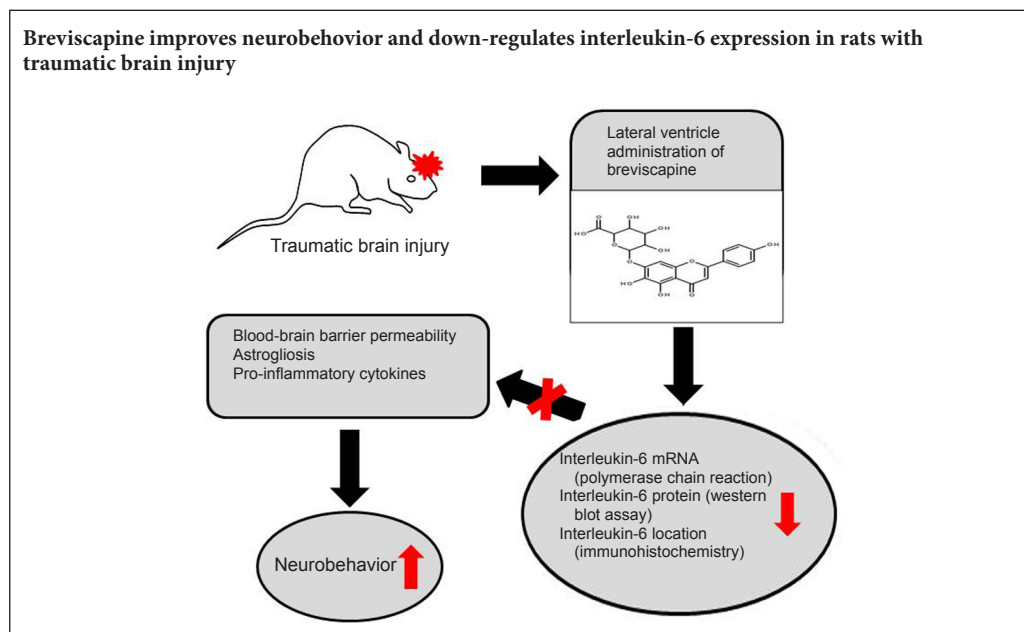
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Abstract

Breviscapine, extracted from the herb *Erigeron breviscapus*, is widely used for the treatment of cardiovascular diseases, cerebral infarct, and stroke, but its mechanism of action remains unclear. This study established a rat model of traumatic brain injury induced by controlled cortical impact, and injected 75 μg breviscapine *via* the right lateral ventricle. We found that breviscapine significantly improved neurobehavioral dysfunction at 6 and 9 days after injection. Meanwhile, interleukin-6 expression was markedly down-regulated following breviscapine treatment. Our results suggest that breviscapine is effective in promoting neurological behavior after traumatic brain injury and the underlying molecular mechanism may be associated with the suppression of interleukin-6.

Key Words: *nerve regeneration; breviscapine; traumatic brain injury; neuroprotective effect; interleukin-6; neural regeneration*

Introduction

Breviscapine is a flavonoid extracted from the herb *Erigeron breviscapus*. Scutellarin is the main active ingredient of breviscapine, and is characterized by the structural formula of 4,5,6-trihydroxyflavone-7-glucuronide (Lou et al., 2015). Clinical trails and experimental studies have shown that breviscapine dilates blood vessels, reduces vascular resistance, improves microcirculation, and suppresses platelet aggregation (Wang et al., 2010; Guo et al., 2014). Accordingly, breviscapine has long been used in clinical practice of patented Chinese medicines to treat cardiovascular and cerebrovascular diseases (Tian et al., 2014). Moreover, breviscapine is considered a routine administration for craniocerebral injury patients.

Traumatic brain injury (TBI) is associated with high disability and mortality, and exhibits a gradually increasing trend with development of society (Zaninotto et al., 2016).

Although many proposed efforts have shown a promising outcome in pre-clinical practice, none have survived the different phases of clinical trials due to the complex pathophysiological process: TBI leads to cerebral structural damage and functional deficits due to instantaneous primary mechanical injury accompanied by delayed secondary injury. Indeed, secondary brain injury plays a crucial role in prognosis of TBI (Kinoshita, 2016), with a cascade of inflammatory processes involved in pathology of secondary damage as a consequence of mitochondrial dysfunction, cerebral hypoxia, and disordered calcium homeostasis (Niklas et al., 2006; Bramlett and Dietrich, 2007). A body of evidence indicates that inflammation plays a dual role in TBI outcome. Inflammation stimulates reparation and regeneration *via* clearance of necrotic and apoptotic cells (Wieloch and Nikolich, 2006; Ziv et al., 2006), while also facilitating secondary injury *via* the release of various inflammatory cytokines, which in turn drives and accelerates additional inflammatory processes (Morganti-Kossman et al., 1997; Zhang et al., 2014). These inflammatory cascades exacerbate brain tissue damage and cause irreversible central nervous system impairment.

Interleukin 6 (IL-6) is an important pro-inflammatory cytokine, and one of the most widely studied molecules in TBI. IL-6 is primarily produced in the central nervous system (Hans et al., 1999; Lau and Yu, 2001), and is markedly up-regulated after injury (Hillman et al., 2007) and also shown to correlate with increased production of other central inflammatory cytokines (Di Santo et al., 1996). Meanwhile, IL-6 expression in cerebrospinal fluid (Singhal et al., 2002; Chiaretti et al., 2008), serum (Arand et al., 2001), and brain parenchyma (Winter et al., 2004) is strongly associated with TBI outcome. Therefore, IL-6 may be a major contributor to the inflammatory response following TBI (Kumar et al., 2015). In neural regeneration, down-regulation of IL-6 ameliorates cell inflammation, apoptosis, and oxidative stress, and may further promote neuronal survival and regeneration (Poulsen et al., 2005; Xu et al., 2014). The relationship between breviscapine and IL-6 expression in neurological repair of neurotrauma has not been reported. Thus, in the current study, we used a rat model of controlled cortical impact to examine the molecular mechanism of the neuroprotective effect of breviscapine on TBI insult.

Materials and Methods

Animals and group assignment

Sixty healthy specific-pathogen-free Sprague-Dawley rats aged 6–8 weeks and weighing 200–240 g were provided by the Laboratory Animal Center of Kunming Medical University in China (license No. SYXK (Dian) K2015-0004). The rats were randomly divided into: sham group, TBI group, and TBI + breviscapine group (**Table 1**). Rats were housed in a 12-hour light-dark cycle and supplied with food and water. All procedures were performed according to the Guide to the Care and Use of Experimental Animals published by the National Institute of Health (NIH publication 85–23, revised 1985), with animal protocols approved by the Animal Ethics Committee of Sichuan University, West China Hospital, China (approval No. ScUEC-145306).

Model preparation and drug treatment

Rats were intraperitoneally anesthetized with 3.6% chloral hydrate ($CCl_3CH(OH)_2$) (10 mL/kg), and placed in the prone position. Following routine disinfection, a midline incision was made through the scalp. A controlled cortical impact model was used to produce TBI in the parietal lobe. A craniectomy was performed on the left anterior frontal area: 2.5 mm from the sagittal suture and 1.5 mm from the coronal suture (Wang et al., 2015). The craniectomy was approximately 5 mm in diameter and was administered using an electric micro drill. After exposure of the dura, a contusion was made using a 3.0 mm convex tip attached to an electromagnetic impactor (Leica, Wetzlar, Germany) mounted to a digitally calibrated manipulator arm. The impact parameters were set at a contusion depth of 2 mm (from dura), constant velocity of 1.9 m/s, and sustained impact of 300 ms. Following controlled cortical impact injury, rats in the TBI + breviscapine group were implanted with a dose of 3 μL (25 μg/μL) breviscapine (batch number 20121203-1; approval number Z20053907; specification 25 mg; Longjing Pharmaceutical Limited Company, Kunming, China). Breviscapine (composed of yellow loose lumps and dissolved in pure water as a 25 μg/μL solution) was implanted into the right lateral ventricle. The scalp was sutured. Finally, rats were placed in a water-heated incubation chamber at 37°C until they fully recovered from anesthesia. Rats in the sham group were treated with the same procedure but without the controlled cortical impact injury. To note, greater attention should be paid to the dura, as rats with disrupted dura were withdrawn from the study.

Neurobehavioral assessment

Severity of neurological deficit was evaluated using the neurological severity score (NSS) system (Chen et al., 2001). Neurobehavioral function is graded on a scale of 0–18 (0, normal score; 18, maximal deficit score). NSS scoring reflects motor, sense, reflex, and balance functions. For injury severity, one point reflects the inability to perform a task or lack of an assessed reflex: 13–18, severe injury; 7–12, moderate injury; and 1–6, mild injury. Thus, more severe injury is reflected by a higher score. Recovery of neurological function was observed and all rats' scores recorded at 1, 3, 6, 9, and 14 days after injury.

Sample harvest

Six days after injury, rats were intraperitoneally anesthetized with 3.6% chloral hydrate (1 mL/100 g) and a thoracotomy made towards the cannula direction. After exposure of the heart, 400 mL normal saline was infused, followed by 500 mL 4% paraformaldehyde fixative. Brains were rapidly removed and post-fixed with 4% paraformaldehyde. Brains were exposed to 15% and 30% sucrose diluted in 4% paraformaldehyde for dehydration. Injured cortical tissue was rapidly removed and stored at −80°C for use.

Table 1 Animal number in each group for each test

Group	Treatment	NSS (1, 3, 6, 9, 14 days)	Immunohistochemistry (6 days)	RT-PCR/ western blot assay (6 days)
Sham	Sham-operated surgery	10	5	5
TBI	TBI-operated surgery	10	5	5
TBI + breviscapine	TBI-operated surgery + breviscapine	10	5	5

NSS: Neurological severity score; RT-PCR: reverse transcription-polymerase chain reaction; TBI: traumatic brain injury.

RT-PCR

Total RNA was extracted from harvested cerebral cortex tissue using Trizol Reagent (SuperfecTRI™, Shanghai, China) prior to cDNA synthesis. To generate cDNA, reverse transcription was performed according to the instructions of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Single-strand cDNA was synthesized by incubating template RNA (2.5 μg) with oligo-(dT) 18 primer (1 μL), and nuclease-free water (to 12 μL) at 65°C for 5 minutes. Next, Revert Aid M-MuLV Reverse Transcriptase (200 U/μL, 1 μL) with 5× reaction buffer (4 μL), Ribo Lock RNase Inhibitor (20 U/μL, 1 μL), and 10 mM dNTP Mix (2 μL) was added (to a final volume of 20 μL), and incubated for 60 minutes at 42°C. Reactions were terminated by heating at 70°C for 5 minutes. PCR was performed using the T100™ Thermal Cycler (BIORAD, Hercules, CA, USA). Five μL of five-fold diluted template cDNA was added in a final volume of 25 μL. The primer sequences were: IL-6, sense 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′ and antisense 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′; annealing temperature: 58°C; and β-actin, sense 5′-GTA AAG ACC TCT ATG CCA ACA-3′ and antisense 5′-GGA CTC ATC GTA CTC CTG CT-3′; annealing temperature: 52.5°C. PCR amplification was performed as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, with elongation at 72°C for 1 minute, followed by elongation at 72°C for 10 minutes. β-Actin was used as the internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates the cycle threshold, with fractional cycle number being the fluorescent signal that reached detection threshold. Normalized ΔCt values for each sample were calculated using β-actin as the endogenous control gene.

Western blot assay

Cortical tissue was harvested, lysed, and sonicated in radioimmune precipitation assay buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland). Protein quantification was performed using the bicinchoninic acid assay kit (Beyotime). Protein samples (100 μg) diluted in sodium dodecyl sulphate loading buffer (Biosharp, Hefei, Anhui Province, China) were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline Tween-20 (TBST) for 2 hours at room temperature, and then incubated overnight at 4°C with IL-6 primary polyclonal antibody (rabbit; 1:200; USCN, Wuhan, Hubei Province, China). Afterwards,

blots were washed three times in TBST for 5 minutes each time. Secondary antibody (goat anti-rabbit IgG; ZSGB-BIO, Beijing, China) was applied at 1:5,000 dilution in TBST, and incubated for 2 hours at room temperature. Finally, samples were developed with enhanced chemiluminescence and analyzed using Alpha Innotech (BIORAD). Optical density values were determined using Image J software (National Institutes of Health, Bethesda, MD, USA), and represented as IL-6 to β-actin ratio.

Immunohistochemistry

After dehydration, brain tissue was sectioned at 20 μm thickness using a freezing microtome (Leica, Wetzlar, Germany). Sections were washed four times in 0.01 M phosphate buffer saline (PBS). To quench non-specific binding, sections were incubated in 10% normal goat serum containing PBS-Triton (0.3%) for 1 hour at room temperature. Sections were incubated with anti-IL-6 antibody (rabbit, 1:100; USCN) overnight at 4°C. Afterwards, tissue sections were washed four times in 0.01 M PBS. Sections were incubated in Alexa Flour 594 secondary antibody (goat anti-rabbit, 1:100; Invitrogen, Carlsbad, CA, USA) at 37°C for 1 hour, and then washed four times in 0.01 M PBS. DAPI-Fluoromount was used to counterstain the nucleus before covering sections with coverslips. Images were acquired using a Leica AF6000 cell station (Leica). For each section, IL-6-positive cell number was counted five times under 400× magnification, with the counter blinded to experimental group. Positive index was expressed by positive cell number per total cells × 100%.

Statistical analysis

Experimental data were expressed as the mean ± SD, and analyzed using SPSS 20.0 software (IBM Corporation, Armonk, NY, USA). One-way analysis of variance was performed to compare three data sets, with Student's *t*-test performed for two sets. A value of *P* < 0.05 was considered statistically significant.

Results

Breviscapine improved neurobehavior in TBI rats

NSS scoring was performed at 1, 3, 6, 9, and 14 days after injury (**Figure 1**). NSS score was significantly higher in the TBI group compared with the sham group at each time point (*P* < 0.01). However, at 6 and 9 days after injury, NSS score was significantly decreased in the TBI + breviscapine group compared with the TBI group (*P* < 0.01, *P* < 0.05). No obvious change was observed at 1, 3, and 14 days after injury.

Breviscapine reversed IL-6 expression in the injured cortex of TBI rats

RT-PCR and western blot assay were used to examine IL-6 in the injured cortex at 6 days after injury. IL-6 gene and protein levels were dramatically up-regulated in the TBI group compared with the sham group ($P < 0.05$; **Figure 2A**). However, breviscapine treatment reversed this trend of up-regulated IL-6 induced by TBI insult, with IL-6 gene and protein expression significantly down-regulated in the TBI + breviscapine group compared with the TBI group ($P < 0.01$, $P < 0.05$; **Figure 2B**).

Breviscapine effect on IL-6 distribution in injured brain tissue of TBI rats

Immunohistochemical staining showed IL-6 was mainly located in the cell membrane, with some in the cytoplasm and extracellular matrix (**Figure 3A**). Moreover, IL-6 expression was increased in the TBI group, but decreased in the cortex following breviscapine treatment. Quantitative analysis confirmed a significantly increased IL-6-positive cell number in the TBI group compared with the sham group ($P < 0.01$). In contrast, breviscapine administration notably decreased IL-6-positive cell number compared with the TBI group ($P < 0.05$; **Figure 3B**).

Discussion

In this study, we successfully established a TBI rat model and then administered breviscapine treatment. We found breviscapine improved neurobehavioral function that had been impaired by TBI insult. Further, both IL-6 mRNA and protein expressions were markedly decreased compared to TBI rats with no breviscapine treatment. These results show that breviscapine plays a neuroprotective role in rats with TBI injury that may be associated with down-regulation of IL-6.

TBI caused by head trauma always results in cognitive and behavioral disabilities (Stoller, 2015; Barman et al., 2016). Here, we found significantly impaired NSS scores with a declining trend. This indicates notable impairment of neurobehavioral function in the early period of TBI, followed by self-rehabilitation in the later stage. The pathological process of TBI involves primary and secondary damage. Secondary damage results from external force after injury, and includes cerebral edema and intracranial hemorrhage (Dardiotis et al., 2014; Hochstadter et al., 2014), which induce significant up-regulation of intracranial pressure in the early period of injury. In clinical practice, increased intracranial pressure is associated with a worsened outcome after TBI insult (Kukreti et al., 2014). Therefore, more severe neurobehavioral dysfunction in the early stage of TBI may be associated with increased intracranial pressure. Conversely, self-rehabilitation in the subsequent period may be associated with decreased intracranial pressure induced by absorption of edema and hemorrhage.

We used breviscapine to treat TBI insult for the first time. In the early stage of TBI, we found breviscapine treatment did not improve any neurological deficits. Contrarily, one day after breviscapine treatment, NSS scores were higher in the TBI + breviscapine group compared with the TBI group. Previous evidence has shown that breviscapine can expand blood vessels, improve articulation, and anti-platelet and red blood cell aggregation, and establish collateral circulation (Zheng et al., 2015). Accordingly, aggravated neuropathology was observed in TBI rats with breviscapine treatment in the early stages, which may be associated with blood vessel dilatation and increased intracranial pressure, which thereby exacerbates neurological deficits. Meanwhile, we found breviscapine improved neurological dysfunction at 6 and 9 days after injection, with no significant effect observed at 14 days. Iadecola et al. (1995) found that breviscapine injection could treat patients with severe brain injury. The protective effect of breviscapine may be associated with its mechanism of improved energy metabolism, free radical scavenging, inhibition of intracellular Ca^{2+} overload, excitatory amino acid toxicity, inflammatory suppression, and regulation of brain blood vessel activity (Wang et al., 2010; Zheng et al., 2015). Thus, after development of the acute stage of TBI, breviscapine may protect from neurological dysfunction. In the later stage, self-rehabilitation and drug metabolism may have resulted in no significant difference in neurobehavioral assessment between rats with and without breviscapine administration.

To examine the underlying molecular mechanism, we investigated expression of IL-6, a critical cytokine controlling the transition from innate to acquired immunity. IL-6 is up-regulated when neuroinflammation is expected, such as following central nervous system infection or injury or central nervous system disease (Erta et al., 2012). Previous reports have shown significantly increased IL-6 expression post-TBI in cerebrospinal fluid, serum (Arand et al., 2001), and brain parenchyma (Winter et al., 2004), which is strongly associated with clinical outcome (Singhal et al., 2002; Chiaretti et al., 2008). These reports confirm our observation that cortical IL-6 mRNA and protein expression is strikingly up-regulated by TBI insult. It has been demonstrated that IL-6 over-expression is associated with neurodegeneration, blood-brain barrier permeability, astrogliosis, and production of other pro-inflammatory cytokines, such as IL-1β and TNF-α (Campbell et al., 1993; Brett et al., 1995; Di Santo et al., 1996; Penkowa et al., 1999). Therefore, suppression of IL-6 can improve neuronal survival and ameliorate neurobehavioral dysfunction.

Here, we show that breviscapine markedly down-regulates IL-6 mRNA and protein levels, which were up-regulated by TBI injury. Except for the ability to dilate blood vessels, inhibit platelet aggregation, and scavenge free oxygen radicals, breviscapine promotes recovery of neurological function, which is associated with a reduction of brain and inflammatory reactions induced by cerebral hemorrhage (Wang et al., 2011; Li et al., 2014). More importantly, Zhang et al. (2007) found that breviscapine decreased IL-6 release associated with inhibition of protein kinase C-alpha mRNA transcription to inhibit the inflammatory cascade. These results firmly support our finding that breviscapine plays a protective role in TBI recovery, which may involve down-regulated IL-6 expression.
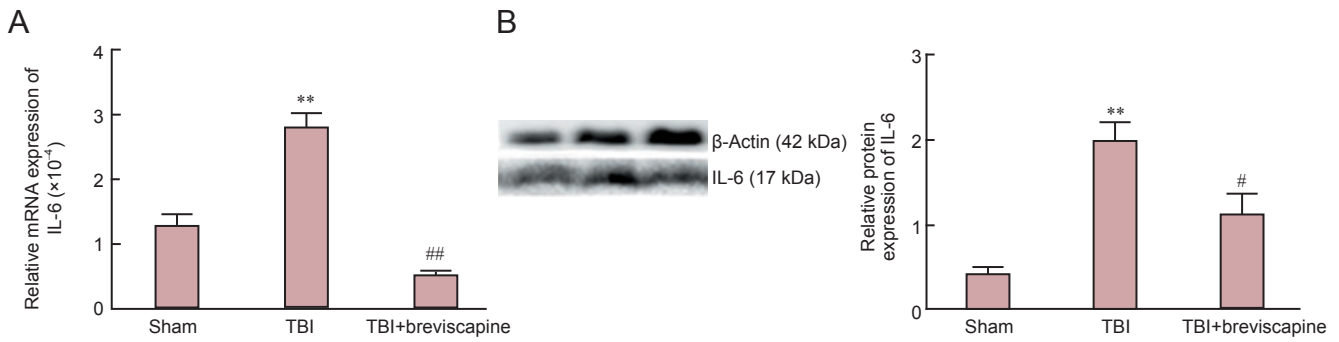
Figure 2 Effect of breviscapine on IL-6 expression in the injured cortex of TBI rats at 6 days after injury.
(A) Reverse transcription-polymerase chain reaction of IL-6 mRNA expression denoted as $2^{-\Delta\Delta Ct}$. (B) Western blot assay of IL-6 protein expression denoted as the optical density ratio of IL-6 to β-actin. Data are expressed as the mean ± SD ($n = 5$), and were analyzed by analysis of variance followed by Student's *t*-test. **$P < 0.01$, *vs.* sham group; #$P < 0.05$, ##$P < 0.01$, *vs.* TBI group. IL-6: Interleukin-6; TBI: traumatic brain injury.
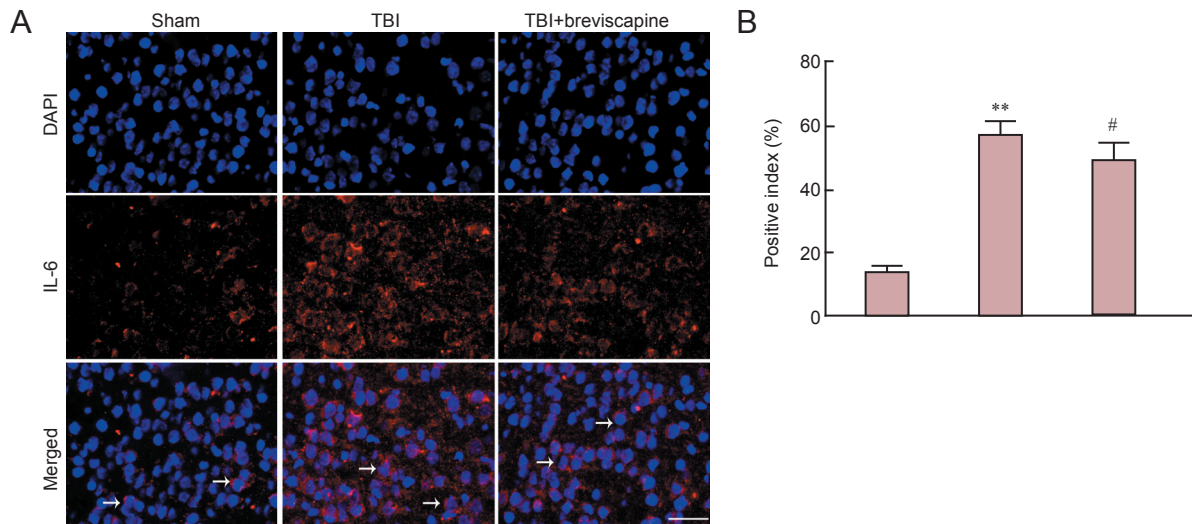
Figure 3 Effect of breviscapine on IL-6 distribution in the injured brain tissue of TBI rats.
(A) IL-6 distribution in brain tissue. Immunofluorescence localization of IL-6 (red) in the injured cortex counterstained with DAPI (blue) to visualize nuclei. Arrows show IL-6-positive cells at 400× magnification. Scale bar: 25 μm. (B) Positive index of IL-6-positive cells expressed as the positive cell number per total cells × 100%. Data are expressed as the mean ± SD ($n = 5$), and were analyzed by analysis of variance followed by Student's *t*-test. **$P < 0.01$, *vs.* sham group; #$P < 0.05$, *vs.* TBI group. DAPI: 4′,6-Diamidino-2-phenylindole; IL-6: interleukin-6; TBI: traumatic brain injury.
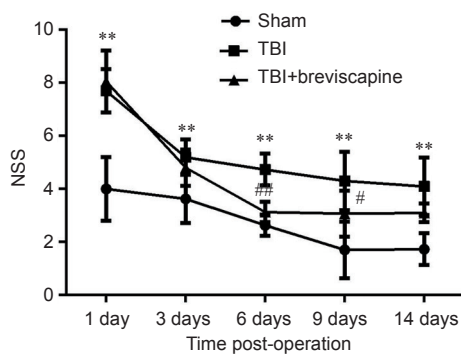

Figure 1 Effect of breviscapine on neurobehavior of TBI rats.
Higher neurological severity scores (NSS) reflect more severe injury. Data are expressed as the mean ± SD ($n = 10$), and were analyzed by analysis of variance followed by Student's *t*-test. **$P < 0.01$, *vs.* sham group; #$P < 0.05$, ##$P < 0.01$, *vs.* TBI group. TBI: Traumatic brain injury.

In summary, we show for the first time that breviscapine can treat TBI, with its neuroprotective effect partly associated with suppression of IL-6 expression.

Our results provide experimental evidence for breviscapine application in the treatment of neurotrauma. Nonetheless, our findings do not support a direct relationship between the therapeutic effect of breviscapine and IL-6 expression. Future investigations should confirm the exact relationship between IL-6 expression and breviscapine treatment for TBI.

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