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Brilliant Blue G Inhibits Inflammasome Activation and Reduces Disruption of Blood–Spinal Cord Barrier Induced by Spinal Cord Injury in Rats

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Background: Brilliant blue G (BBG) is a P2X7 receptor inhibitor that has been reported to improve spinal cord injury (SCI) in previous studies, but the specific mechanism has been unclear. In this study, we investigated the effects of BBG on inflammasomes and blood–spinal cord barrier (BSCB) permeability after SCI.

Material/Methods: The experimental rats were randomly divided into 3 groups: sham, SCI, and SCI+BBG. The expression of P2X7 and inflammasome-related proteins was measured by Western blot and immunohistochemistry, while IL-1 β and IL-18 levels were measured by using an enzyme-linked immunosorbent assay (ELISA) kit. The permeability of the BSCB was evaluated by Evans Blue (EB) exosmosis, and histological alterations were observed by hematoxylin-eosin staining. Motor function recovery was assessed by the Basso, Beattie, Bresnahan (BBB) scale after SCI.

Results: The expression levels of P2X7, NLRP3, ASC, cleaved XIAP, caspase-1, caspase-11, IL-1 β , and IL-18 were increased significantly after SCI, and BBG administration inhibited this increase at 72 h after SCI. BBG administration significantly reduced EB leakage at 24 h after SCI. Furthermore, treatment with BBG significantly attenuated histological alterations and improved motor function recovery after SCI.

Conclusions: BBG administration promoted motor function recovery and alleviated tissue injury, and these effects might be related to the suppression of inflammasomes and the maintenance of BSCB integrity.

MeSH Keywords: **Blood–Spinal Cord Barrier • Inflammasomes • Inflammation • Receptors, Purinergic P2X7 • Spinal Cord Injuries**

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Background

Spinal cord injury (SCI) is a clinically familiar traumatic central nervous system (CNS) disease with gradual degeneration of the nervous system and loss of motor function. The pathophysiological mechanism of SCI includes 2 parts: primary injury and secondary injury. Secondary injury has complex pathophysiological mechanisms that include oxidative stress, ionic homeostasis loss, and local inflammatory reactions, leading to further cell necrosis and apoptosis after SCI [1–3]. The inflammatory response and destruction of the blood–spinal cord barrier (BSCB) can cause neurodegenerative changes, delay neurological function repair, and aggravate neurological dysfunction [4,5]. Therefore, reducing the inflammatory reaction and maintaining the stability of the BSCB are expected to become an effective therapeutic strategy for improving outcomes after SCI.

Inflammasomes, which are multiprotein complexes, play an important role in mediating inflammatory responses during SCI, and mainly consist of 3 parts: NOD-like receptor (NLR), apoptosis-associated speck-like protein containing CARD (ASC), and caspase-1 [6]. When endogenous “danger signals” are sensed, inflammasomes are assembled and activated, leading to the activation of caspase-1 [7]. Activated caspase-1 promotes the production and secretion of the inflammatory cytokines IL-1 β and IL-18, leading to cell necrosis and apoptosis [8]. In past research, the NLRP1 and NLRP3 inflammasomes were shown to be involved in the inflammatory response during SCI, and targeted treatment was proven to exert neuroprotection in SCI rats [9,10].

The BSCB is a naturally formed barrier that separates the CNS from the peripheral circulation to maintain homeostasis and internal environmental stability in the spinal cord tissue [11]. After SCI, the damage to the BSCB will lead to severe pathophysiological reactions and aggravate secondary injury, and the mechanisms involved in BSCB disruption are complex [12]. Previous studies have reported that BSCB permeability can be impaired by certain pro-inflammatory cytokines [13].

The P2X7 receptor is an ATP-gated ion channel that is the main participant in the inflammatory response [14]. Interestingly, P2X7 can affect the activities of both the NLRP1 and NLRP3 inflammasomes [10,15]. Brilliant blue G (BBG) is a low-toxicity and high-selectivity P2X7 receptor antagonist derived from the food dye FD&C blue dye no. 1 [16]. Previous studies have reported that BBG treatment reduces tissue injury and promotes motor function recovery after SCI [17]. However, the influence of BBG on inflammasomes and the BSCB after SCI still needs to be further explored.

Based on the above considerations, we investigated the effect of BBG on inflammasomes and BSCB permeability and assessed the neuroprotective properties of BBG in a rat model of SCI.

Material and Methods

Establishment of an SCI model in rats

Adult male Sprague-Dawley (S.D.) rats (250–300 g) were purchased from Beijing Vital River Laboratory Animal Technology. The animal experiment was approved by the Animal Ethics Committee of China Medical University. To establish the rat SCI model (as previously reported), we performed compression of the aneurysm clamp with 40-g force for 1 min to cause a compression injury in the spinal cord at the T8-T10 segment [4]. All rats were randomly divided into 3 groups: sham, SCI, and SCI+BBG. The sham group rats underwent only the skin incision and laminectomy but not the compression injury. The SCI group rats underwent the compression injury procedure described above. The SCI+BBG group rats received an intraperitoneal injection of BBG (50 mg/kg) 20 min after SCI, and the administration frequency was once every 12 h until 72 h after SCI. In addition, in the SCI group, the rats received an amount of saline equivalent to the BBG injection via an intraperitoneal injection after SCI. After the operation, the rats were given ropivacaine for analgesia and penicillin for infection prevention. All rats were subjected to manual bladder squeezing and urination every 12 h until spontaneous urination was restored.

Evaluation of locomotor function

Locomotion was evaluated by 2 independent investigators using the Basso–Beattie–Bresnahan (BBB) scale at 1, 3, 7, 14, 21, and 28 days after SCI (n=5 for each group), as described previously [9]. This rating scale ranges from 0 to 21 (0: complete paralysis, 21: normal).

Histological study

In all groups, the rats (n=5 in each group) were perfused with 0.9% saline followed by 4% paraformaldehyde 72 h after SCI. The spinal cord tissue was sliced into 10- μ m sections and fixed with paraffin. Some sections were stained with hematoxylin and eosin. The histological score depends on edema, hemorrhage, and neutrophil infiltration and represents the degree of SCI over a range from 0 to 4 points: 0 for none or minor, 1 for limited, 2 for intermediate, 3 for prominent, and 4 for widespread.

Protein extraction and Western blot analysis

Rats (n=5 in each group) were sacrificed 72 h after SCI, and protein homogenates were prepared from samples incubated in pyrolysis liquid for 2 h. The samples were centrifuged at 4°C and 12 000 rpm for 30 min, and the supernatant was collected and stored separately. The protein concentration of the sample supernatant was measured with a BCA kit (Beyotime

Biotechnology). Total protein (60 µg) separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis was transferred to a polyvinylidene fluoride membrane (Beyotime Biotechnology). The membrane was blocked with 5% skim milk powder at room temperature for 2 h and incubated overnight with a primary antibody at 4°C. The primary antibodies included anti-P2X7 (1: 2000, Thermo Fisher Scientific), anti-NLRP1 (1: 1000, Abcam), anti-NLRP3 (1: 2000, Abcam), anti-XIAP (1: 1000, Cell Signaling Technology), anti-ASC (1: 2000, Novus Biologicals), anti-caspase-11 (1: 2000, Cell Signaling Technology), anti-caspase-1 (1: 2000, Abcam), and anti-β-actin (1: 5000, Proteintech Group) antibodies. Then, the membranes were washed with Tris-buffered saline containing Tween and incubated with secondary antibodies (1: 5000, Abcam) at room temperature for 2 h. Subsequently, protein bands were visualized with a chemiluminescence kit (Beyotime Biotechnology, Beijing), and images were collected using sensitive film and chemical imaging software.

Immunohistochemistry

Tissues from rats (n=5 in each group) were perfused and fixed with 4% paraformaldehyde 72 h after SCI. The spinal cord tissue was sliced into 10-µm sections and fixed with paraffin. The slides underwent dewaxing, hydration, and thermal antigen repair. Antigen was inactivated by 3% hydrogen peroxide-methanol for 10 min and the slides were rinsed with PBS 3 times. The samples were blocked with goat serum (50 µl) at room temperature for 20 min and rinsed with PBS 3 times. The slides were incubated with primary antibodies NLRP1 (1: 1000; Abcam), NLRP3 (1: 1000; Abcam), and ASC (1: 1000; Novus Biologicals) overnight at 4°C. After incubation, the slides were rinsed with PBS 3 times and incubated with biotin-conjugated secondary antibodies (1: 1000; Abcam) at 37°C for 30 min. Subsequently, the samples were stained with DAB solution for 10 min, re-dyed with hematoxylin for 5 min, dehydrated with gradient alcohol, soaked in xylene for 10 min, dried, and sealed with neutral resin. The expression of positive cells was observed under a microscope and captured in high-definition pictures.

Measurement of BSCB permeability

The permeability of the BSCB was assessed by an Evans Blue (EB) exosmosis assay, as previously reported [5]. Rats (n=10 in each group) were given a 2% EB saline solution (2 ml/kg) 24 h after SCI, and then all rats were transcardially perfused with normal saline until colorless, transparent fluid flowed out of the right auricle after 2 h of EB circulation. With the lesion as the center, a 1-cm length of spinal cord tissue was dissected, immersed in formamide, and incubated at 70°C for 72 h. The tissue supernatant was collected, and its optical density was measured with Multimode Reader at a wavelength of 620 nm (Thermo Fisher Scientific MultiSkan Go). The EB

content in the spinal cord tissue was calculated using the EB/formamide concentration standard curve.

Half of the rats received a cardiac perfusion with 4% paraformaldehyde at 2 h after EB injection. Spinal cord tissue samples from all groups were cut into 10-µm sections with a cryotome (Thermo Shandon). EB leakage was observed using an Olympus bx-60 fluorescence microscope under 550 nm wavelength green light excitation.

Biochemical analysis

The damaged area of the spinal cord (1 cm, n=5 for each group) was removed 72 h after injury, and the samples were immediately homogenized in normal saline and centrifuged at 4°C for 20 min at 1500 rpm. The concentrations of IL-1β (BOSTER, EK0393) and IL-18 (BOSTER, EK0592) in the supernatant were measured with enzyme-linked immunosorbent assay (ELISA) kits.

Statistical analysis

SPSS 16.0 software was used for statistical analysis, and the results are presented as the mean ± standard. All statistical data were analyzed using an independent-samples *t* test, and *P*<0.05 was considered statistically significant.

Results

Effects of BBG on motor function

BBB scores were used to evaluate the effect of BBG on motor function recovery after SCI in rats. There was no significant difference in the BBB scores between the SCI and SCI+BBG groups at 1 or 3 days after SCI. However, the BBB scores of the treatment group were significantly higher than those of the SCI group from day 7 onward (Figure 1).

Effects of BBG on tissue damage

Histopathological changes in the rat spinal cord were used to evaluate the protective effect of BBG on SCI. The histological scores of the SCI group were significantly higher than those of the sham-operated group. However, compared with saline placebo, BBG administration significantly reduced spinal cord congestion, edema, neutrophil infiltration, and structural damage (Figure 2A–2D).

Effect of BBG on inflammasomes

Western blot analysis was performed to assess the effect of BBG on inflammasome-related proteins after SCI. Immunoblotting indicated that the levels of P2X7, NLRP3, ASC, cleaved XIAP, caspase-11, and caspase-1 in the SCI group were higher than

Figure 1. Basso-Beattie-Bresnahan (BBB) scores at the indicated point time after SCI (n=5). *** $p < 0.001$ BBG+SCI versus SCI. Data are expressed as means $\pm$ S.D.

those in the sham group after SCI. In addition, the P2X7, NLRP3, ASC, cleaved XIAP, caspase-11, and caspase-1 levels were lower in the SCI+BBG group compared with the SCI group. However, the NLRP1 protein levels did not significantly differ among groups (Figure 3). Immunohistochemistry also showed that the expression levels of NLRP3 and ASC were increased after

SCI, while the expression levels of NLRP3 and ASC were decreased in the BBG treatment group. Similarly, there was no significant change in NLRP1 (Figure 4).

Effect of BBG on BSCB permeability in SCI

EB dye was used to evaluate permeability of the BSCB after SCI. Compared with that in the spinal cord tissue of the sham-operated group, the EB content in the spinal cord tissue of the SCI group was significantly increased, which indicated that SCI destroyed the integrity of the BSCB. However, compared with saline placebo, BBG administration significantly reduced the EB dye content in the spinal cord tissue at 24 h after SCI (Figure 5A, 5B). Furthermore, these results were confirmed by an EB dye fluorescence experiment, and the fluorescence intensity of EB in the spinal cord in the BBG intervention group was lower compared with that in the SCI group (Figure 5C, 5D).

Effect of BBG on pro-inflammatory cytokine levels

After SCI, the expression levels of the IL-1 β and IL-18 were significantly increased in the SCI group compared with the

Figure 2. Effects of BBG on tissue damage at 72 h after SCI (n=5). *** $p < 0.001$ SCI versus sham. ### $p < 0.001$ SCI+BBG versus SCI. (A) sham group; (B) SCI group; (C) SCI+BBG group; (D) Histopathologic scores. Data are expressed as means $\pm$ S.D. Scale bar=50 μ m.

Figure 3. Western blot and quantification of P2X7, NLRP1, NLRP3, ASC, XIAP, caspase-11 and caspase-1 in each group 72 h after SCI (n=5). ** p<0.01 and *** p<0.001 SCI versus sham. # p<0.05 and ### p<0.001 SCI+BBG versus SCI. Data are expressed as means ±S.D.

sham group. However, BBG administration reduced the IL-1 β and IL-18 levels in the BBG+SCI group compared with those in the SCI group (Figure 6A, 6B).

Discussion

SCI is a serious CNS disease accompanied by neurodegeneration and motor function loss. The mechanism of SCI is divided into primary injury and secondary injury, and the inflammatory response is an important part of a cascade occurring during secondary injury [1,2]. In the present study, we found that BBG could inhibit the assembly and activation of the NLRP1 and NLRP3 inflammasomes, improve histopathological injuries, and promote the recovery of motor function after SCI. Furthermore, BBG administration could suppress the increase of BSCB permeability after SCI.

Inflammasomes are polyprotein complexes that are involved in the initiation of inflammatory responses and mediate a variety of different systemic diseases. In general, the 3 major domains of each inflammasome are NLRP, ASC, and caspase-1. NLRP is a cytosolic sensor involved in the regulation of host inflammatory responses [7]. ASC is an adaptor protein that enhances the activity of inflammasomes and participates in the activation of caspase-1 [18]. The activated caspase-1 induces the activation of IL-1 β and IL-18. Furthermore, caspase-1 can directly induce cell pyroptosis [8]. XIAP, which can inhibit the activity of caspase-1, is another important component of the NLRP1 inflammasome [19]. After SCI, the cleavage of XIAP produces n-terminal bir1-2 fragments, and the ability of XIAP to inhibit caspases is reduced [20]. In the present study, we found that BBG administration restricted the expression of NLRP1 and NLRP3 inflammasomes-related proteins P2X7, NLRP3, ASC, cleaved XIAP, caspase-11, and caspase-1, but not NLRP1.

Figure 4. Immunohistochemistry and quantification of NLRP1, NLRP3, and ASC in each group 72 h after SCI (n=5). *** $p < 0.001$ SCI versus sham. ## $p < 0.01$ and ### $p < 0.001$ SCI+BBG versus SCI. Data are expressed as means $\pm$ S.D. Scale bar=25 μ m.

Figure 5. Evans Blue (EB) dye extravasation test (n=10). (A) The degree of EB leakage to spinal cord in sham, SCI, and SCI+BBG group. (B) The content of EB in spinal cord tissue in each group. ** $p < 0.01$ SCI versus sham. # $p < 0.05$ SCI+BBG versus SCI. (C, D) EB exosmosis imaging and fluorescence intensity of EB. Data are expressed as means $\pm$ S.D.

Figure 6. Effects of BBG on IL-1 β (A) and IL-18 (B) levels in each group 72 h after SCI (n=5). *** $p < 0.001$ SCI versus sham. # $p < 0.05$ and ## $p < 0.01$ SCI+BBG versus SCI. Data are expressed as means $\pm$ S.D.

IL-1 β plays an important role in CNS damage by exacerbating the infiltration of inflammatory cells, aggravating the local inflammatory response, and triggering the secretion of pro-inflammatory cytokines such as TNF- α and IL-6 [21]. IL-1 β increases vascular endothelial permeability, leading to cell edema and apoptosis [22]. Knocking out the IL-1 receptor in mice can suppress the activation of microglia/macrophages and neuronal death [23], reduce the severity of SCI, and improve motor function recovery after SCI [24]. IL-18, which mediates spinal microglia and astrocyte interactions, is another important pro-inflammatory factor in SCI [25], and blocking IL-18 signaling can reduce neuropathic pain after nerve injury [26]. Consistent with previous studies, we found that IL-1 β and IL-18 expression was significantly increased after SCI and that the expression of these pro-inflammatory cytokines was down-regulated by BBG.

The BSCB is similar to the blood-brain barrier and effectively prevents certain substances, especially harmful ones, in the blood circulation from entering the CNS, and this property is conducive to maintaining the stability of the microenvironment in CNS [12]. After SCI, BSCB destruction and increased microvascular permeability lead to the infiltration of inflammatory cells, such as neutrophils and macrophages, leading to spinal cord tissue edema and cell necrosis [27]. The integrity of the BSCB is further impaired by activation of pro-inflammatory cytokines during secondary injury [28]. Previous studies have reported that inhibiting the NLRP3 inflammasome can

improve traumatic brain injury (TBI) and reduce blood-brain barrier permeability [29]. Other studies have reported that in an *in vitro* blood-brain barrier model, blocking the P2X7 receptor decreased the expression of IL-1 β and MMP-9 and reduced the permeability of the blood-brain barrier [30]. In this study, we found that BBG reduced EB leakage in spinal cord tissue, which proved that BBG reduced BSCB permeability after SCI. This mechanism may be related to down-regulating the expression of IL-1 β and IL-18 by BBG.

Conclusions

In conclusion, the present study demonstrated that BBG promoted motor function recovery, alleviated histological damage, and reduced the increase in BSCB permeability after SCI in rats. Furthermore, BBG reduced inflammation by inhibiting the NLRP3 inflammasome activation and NLRP1 inflammasome assembly process. The present study has certain limitations that should be considered. We conducted only *in vivo* experiments, so the study lacks further explorations in *in vitro* or clinical models, and the protective effect of BBG on the BSCB also requires further in-depth study.

Conflicts of interest

None.

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