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·Original Article·

Protective effects of Batroxobin on spinal cord injury in rats

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ABSTRACT

Expansion of the secondary injury following primary spinal cord injury is a major pathological event that increases destruction in the spinal cord, so measures to reduce secondary injury are needed. Our previous study demonstrated that, at the front of the expanding secondary injury in the spinal cord, there is an ischemic area in which many neurons can still be rescued. Therefore, enhancement of blood circulation in the cord may be helpful, and indeed, we found that a traditional Chinese medicine, shu-xue-tong, efficiently reduces the secondary injury. The aim of the present study was to investigate the effect of reducing fibrinogen with Batroxobin, a drug widely used clinically for ischemia, in rats with spinal cord contusion. We found that both 2 and 4 Batroxobin units (BU)/kg efficiently decreased the plasma fibrinogen, and 2 BU/kg significantly increased spinal blood flow, enhanced neuronal survival, mitigated astrocyte and microglia activation, and improved locomotor recovery. However, 4 BU/kg had no effect on the secondary spinal cord injury. These data suggest that Batroxobin has multiple beneficial effects on spinal cord injury, indicating a potential clinical application.

Keywords: spinal cord injury; secondary injury; fibrinogen; Batroxobin

INTRODUCTION

Spinal cord injury (SCI) is a serious problem and has been intensively studied[1]. Secondary injury after primary spinal cord damage plays an important role in the final outcome. Pathological vascular changes and the subsequent events are crucial for the development of secondary injury[2-4]. Destruction of the blood-spinal cord barrier leads to massive leakage of blood components, initiating inflammation and gliosis[5]. Post-traumatic ischemia resulting from tissue compression, thrombosis and vasospasm causes progressive neuronal death, and shows a direct linear relationship with the severity of injury[6-8]. Our previous study has shown that two ischemic zones occur at the front of the expanding secondary injury. In the zone adjacent to the injury, most of the neurons have degenerated or disappeared, whereas in the farther zone, although there are clear signs of ischemia, the morphology of the Nissl bodies appears not much changed, indicating that these neurons can be rescued by improving the blood flow[9]. This explains the finding by Johnson *et al.* that fluarizine, a vasodilator, ameliorates ischemia in the cord and enhances neurological recovery in rabbits[10]. Thus, enhancement of blood flow in the cord could be an efficient strategy for decreasing secondary injury. Our subsequent study using a traditional Chinese medicine, shu-xue-tong, which promotes blood flow, demonstrated the efficacy of this strategy[11]. Therefore, in this study, we further investigated the possibility of making use of innate blood-borne factors.

Fibrinogen is a 340-kDa protein secreted by hepatocytes and is present in the blood at a high concentration. Upon activation of the coagulation cascade, both the α and β chains of fibrinogen are cleaved by

thrombin to form fibrin polymer. The latter, by interacting with platelets, accomplishes the process of blood clotting[12]. Furthermore, recent studies reported that fibrinogen leaking from ruptured blood vessels triggers the activation of astrocytes[13] and microglia[14], suggesting its involvement in the development of secondary SCI.

Batroxobin is a thrombin-like serine protease from the venom of the snake *Bothrops moojeni*. In contrast to thrombin, Batroxobin only cleaves the α chain of fibrinogen, resulting in the production of a fibrin monomer, which has poorer linking ability than the polymer and thus decreases the blood fibrinogen and promotes blood flow[15]. Therefore, Batroxobin has been successfully used in various ischemic disorders, such as stroke, deep-vein thrombosis, myocardial infarction, peripheral arterial thrombosis, and sudden deafness[16-18]. Its effect on SCI, however, has not been studied. Given the important role fibrinogen plays in the pathology of SCI, the present study was designed to investigate whether Batroxobin has a beneficial effect on SCI in rats and its possible clinical application.

MATERIALS AND METHODS

Animal Model

All the animal experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University. Sprague-Dawley rats (200–250 g) were anesthetized with 1% sodium pentobarbital (50 mg/kg). A 30–40 mm dorsal midline incision was made followed by bilateral laminectomy. SCI was made at the T8 vertebra (corresponding to segment T9) by lateral crushing. The widely-used weight-dropping device mimics the clinical situation of SCI, but often causes profuse bleeding in the dorsal funiculus that spreads between the fasciculi to an unpredictable degree. To improve the consistency of the experimental data, Tazlaff developed a manual graded forceps lateral crush SCI model[19]. Because it is difficult to manually ensure vertical orientation of the forceps and equal degrees of compression bilaterally, we designed a mechanical version in which a pair of forceps was mounted on a stereotaxic device and its two blades could be closed simultaneously from both sides. After exposure of the dura mater, the forceps were first pressed until their tips met, then lowered and aligned with the midline of the cord. Then the blades were spread, lowered along both sides of the cord for the desired distance, closed to a preset gap between the blades (0.5 mm), and compression maintained for 20 s.

Batroxobin Administration

According to the supplier's instructions (DF-521, Tobishi), 10 Batroxobin units (BU) is recommended as the regular initial dose, and 5 BU as the maintenance dose. Only in cases with plasma fibrinogen >400 mg/dl or acute hearing loss can 20 BU be used as the initial dose. Considering the differences between human and rat, we used 2 and 4 BU/kg (corresponding to 10.7 and 21.4 BU for a 60-kg patient). Batroxobin is recommended to be administered once every other day. Considering the drastic changes that occur right after the injury, particularly the spread of bleeding, we expected that 12 h after injury was needed for the changes to stabilize. Our pilot experiment showed that by that time the hemorrhage had stopped, with only a little fresh bleeding. Since Batroxobin does not enhance bleeding[15], we injected it at 12 h, 3 days and 5 days after SCI *via* the tail vein. Saline was injected as the control.

Plasma Fibrinogen Assay

Two hours after the last dose of Batroxobin, blood samples were collected for a coagulation test (n = 4/group). The concentration of fibrinogen was determined using an Automated Coagulation Analysis Instrument (STA Compact, Stago Co., France).

Laser-Doppler Flowmetry

Spinal cord blood flow was measured 2 h after the last Batroxobin dose using a MoorLab laser-Doppler flow-meter (wavelength, 780 nm) and an MP4 probe (Moorlab Instruments, Devon, England). The laminectomized animals from the saline or Batroxobin-treated groups were mounted onto a stereotactic instrument and fixed to stabilize the vertebral column. The laser-Doppler probe was affixed to a micromanipulator and placed perpendicular to the cord, barely touching the dorsal surface of the dura mater. The laser-Doppler signals were recorded and analyzed with Moorsoft for Windows Ver. 1.31. The final value for each rat was obtained from the average of 10 consecutive spikes over 1 min of recording.

Immunohistochemistry and Morphological Analysis

At 7 days post-SCI, after behavioral analysis, the

animals were overdose anesthetized with pentobarbital sodium and perfused intracardially with 400 mL 4% cold paraformaldehyde in phosphate buffer (pH 7.4). Then a 2-cm spinal cord segment containing the lesion site at its center was removed and kept in 25% sucrose until it sank**.** Eight sets of serial sagittal sections (14 μm thick) from each cord were prepared on a cryostat. For immunostaining, the slides were blocked with 0.01 mol/L PBS containing 0.3% Triton X-100 and 3% BSA for 30 min (Triton X-100 was omitted from the blocking solution for Neurocan staining), and incubated with rabbit anti-NeuN (1:500, Millipore, Temecula, CA), mouse anti-glial fibrillary acidic protein (GFAP) (1:1 000, Sigma-Aldrich, Saint Louis, MO), rabbit anti-GFAP (1:2 000, Abcam, Cambridge, MA), rabbit anti-Iba-1 (1:1 000, Wako, Osaka), or mouse anti-Neurocan (1:250, Sigma-Aldrich) at room temperature overnight. After washes with PBS, sections were incubated with the corresponding secondary antibody conjugated with Alexa Fluor 594 (donkey anti-rabbit IgG, 1:800, Molecular Probes, Eugene, OR) or Alexa Fluor 488 (donkey anti-mouse IgG, 1:500, Molecular Probes) for 4 h at room temperature in the dark. The nuclei were counterstained with Hoechst 33342 (1:5 000, Sigma). In each animal, five sections separated by 100 μm (a middle section that cut through the central canal, two left and two right of the midline) were mounted on the same slide. NeuN- and Iba-1-stained sections were examined and photographed under a fluorescence microscope (BX51, Olympus). The other immunostained sections were examined and photographed under a confocal microscope (FV1000, Olympus). To quantify the immunostaining, the lesion area was defined by the inner lining of GFAP-stained astrocytes, and the lesion size was calculated by converting the pixels into millimeters using Photoshop CS3. The immunofluorescence intensity (IFI) of GFAP, Neurocan and Iba-1 was measured in the first three zones adjacent to the lesion center by Image-Pro Plus5.0 (IPP5.0), and then divided by the area of the zones respectively.

Basso-Beattie-Bresnahan (BBB) Evaluation of Locomotion

The BBB scale was used to evaluate rat open-field locomotion at 1 day before, and 1, 4, and 7 days after SCI. Animals in a standard open-field were observed for the following criteria: extent of joint movement, weight support, and stepping/walking behavior of the hindlimbs. Functional scores ranging from 0 (no observable hindlimb movement) to 21 (normal locomotion) were assigned for both hindlimbs by two independent observers blinded to the experiments. The mean value was calculated as the main functional outcome. Scoring was performed in a maximum period of 5 min.

Rump-height Index (RHI) Assay

A runway bar was made of a wooden plate (1500 mm long, 120 mm wide, and 16 mm thick). The rats were video-recorded (Samsung S600) at 30 frames/s 1 day prior to and 1, 4, and 7 days post-SCI. Frames in which the animals were in defined phases of locomotion were selected. The RHI is defined as the height of the rump (the vertical distance from the dorsal aspect of the tail base to the beam), normalized to the thickness of the beam measured along the same vertical line. Measurements were performed with Photoshop CS3. To minimize the variations of pre-surgery RHI, the standardized RHI (SRHI, $SRHI = RHI_{post\text{-}SCI}/RHI_{pre\text{-}SCI}$) was used for comparisons.

Footprint Analysis

Footprint analysis was conducted 1 day before and 7 days after SCI. The plantar surface of both hindlimbs of each rat was colored black and the dorsal surface red with non-toxic ink. The rats were then allowed to run on a white paper (89.1 cm × 10.5 cm); the red and/or black ink printed onto the paper was considered as one set of footprints. Stride length (SL) was defined as the distance between the centers of adjacent footprints. The SL of both hindlimbs was measured and the average of 5–8 SLs from each rat was used. The stride length factor (SLF) was calculated as $SLF = (SL_{pre\text{-}SCI} - SL_{post\text{-}SCI})/SL_{pre\text{-}SCI}$ and was used for statistical analysis.

Statistical Analysis

All the morphological measurements were made using Adobe Photoshop CS3, and all data were analyzed by SPSS l6.0 (Chicago, IL). Data are presented as mean ± SEM, and were analyzed with one-way ANOVA, followed by Dunnett's *post hoc* test, except for BBB scoring which was further analyzed by the Bonferroni *post hoc* test as recommended[20]. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Batroxobin on Plasma Fibrinogen and Spinal Cord Blood-Flow

Two hours after the last injection, peripheral blood samples were collected to determine the effects of Batroxobin on plasma fibrinogen. The results showed that 2 BU/kg decreased the fibrinogen by 30.64 ± 4.20% (P <0.05) and 4 BU/kg by 55.42 ± 4.14% (P <0.01) (n = 4 rats, Fig. 1A), demonstrating its efficacy in removing blood fibrinogen. To assess whether spinal cord blood-flow was improved by Batroxobin, laser Doppler scanning was performed at the lesion site 2 h after the last Batroxobin injection. The results showed that 2 BU/kg increased the blood-flow by 43.80 ± 7.70 % (n = 4 rats, P <0.05, Fig. 1B). However, 4 BU/kg did not significantly change the blood-flow (n = 4 rats, Fig. 1B).

Effects of Batroxobin on Neuronal Survival and Lesion Size

NeuN-immunoreactive neurons were counted in animals sacrificed at 7 days after SCI. In areas both rostral and caudal to the lesion, five lines parallel to the astrocytic circumference of the lesion site were drawn, each separated by 250 μm, and the data from both sides of each zone were combined in the subsequent analysis. The NeuN-positive cells were counted in each zone (Fig. 2A–C). The results showed that 2 BU/kg increased the survival of neurons adjacent to the lesion site as evidenced by the

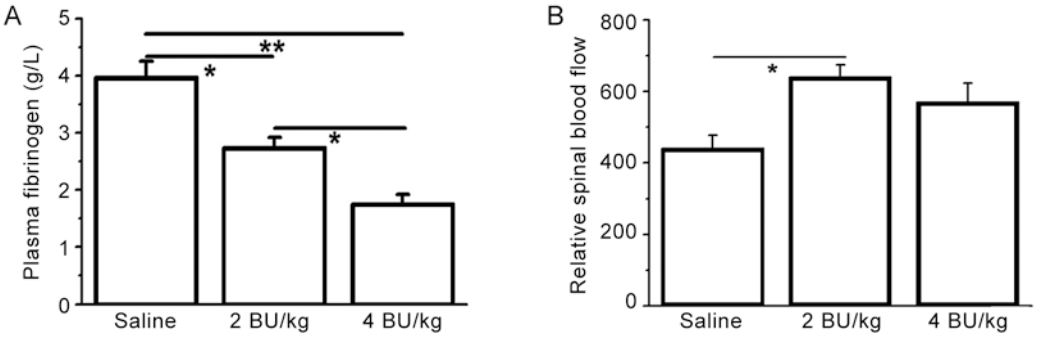

Fig.1. Effects of Batroxobin on plasma fibrinogen and spinal cord blood-flow. A: Reduction of fibrinogen by 2 BU/kg and 4 BU/kg. B: Improvement of spinal cord blood-flow by 2 BU/kg but not 4 BU/kg. n = 4, *P <0.05, **P <0.01.

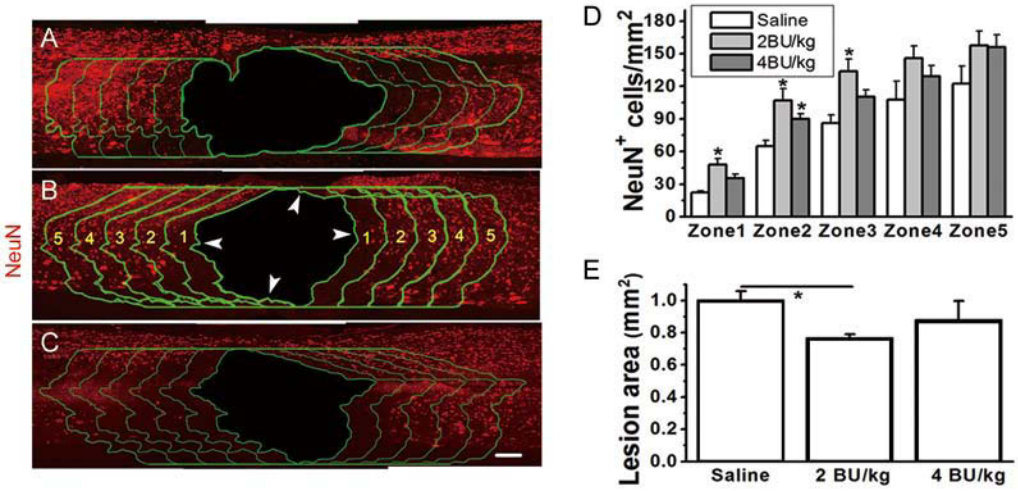

Fig. 2. Effects of Batroxobin on neuron survival and lesion area. A–C: NeuN immunostaining in saline (A), 2 BU/kg (B), and 4 BU/kg (C) groups. Arrowheads indicate the lesion border defined by GFAP immunoreactivity. Scale bar, 200 μm. D: Number of NeuN-positive cells in zones 1–5. E: Lesion areas in each group. n = 6/group. *P <0.05.

increase of NeuN-positive cells by 137.28 ± 36.07% in zone 1, 84.28 ± 27.18% in zone 2, and 80.0 ± 19.03% in zone 3 (n = 6 rats, P <0.05, Fig. 2D). The neurons in zones 4 and 5 appeared normal. No significant increase of NeuN-positive cells was found in the 4 BU/kg group (Fig. 2D). Moreover, 2 BU/kg reduced the lesion area by 23.54 ± 2.12%, while 4 BU/kg did not (Fig. 2E). These results indicated that 2 BU/kg, but not 4 BU/kg, promoted the survival of neurons adjacent to the lesion and reduced the lesion size.

Since the neurons in zones 4 and 5 appeared normal, only zones 1–3 were investigated in the subsequent experiments.

Effects of Batroxobin on Astrocyte Activation

To assess the effects of Batroxobin on the activation of astrocytes, immunohistochemistry for GFAP and Neurocan (another marker of reactive astrocytes to support the results from GFAP staining) was performed. In saline controls, intense GFAP immunoreactivity was seen in zone 1, and this decreased from zones 1–3 (Fig. 3A). But 2 BU/kg decreased the IFI of GFAP by 60.06 ± 4.83% (n = 6 rats, P <0.05, Fig. 3B, D). No significant change of GFAP immunoreactivity was found in the 4 BU/kg group (Fig. 3C, D). Further, the IFI of Neurocan was decreased by 75.22 ± 3.00% (P <0.01) in the 2 BU/kg group, and by 46.50 ± 6.71% (P <0.01) in the 4 BU/kg group (n = 6 rats, Fig. 4). Taken together, these data suggested that 2 BU/kg alleviated the astrocyte activation.

Effects of Batroxobin on Microglia Activation

To define the effects of Batroxobin on the activation of microglia, immunostaining for Iba-1 was performed at 7 days post-SCI. In saline controls, numerous Iba-1 positive cells were found in zone 1 and decreased from zones 1–3 (Fig. 5A). Measurement of the IFI of Iba-1 in the first three adjacent zones showed a reduction of 29.07 ± 7.83% in the 2 BU/kg group (n = 6 rats, P <0.05, Fig 5B, D). But no significant reduction of Iba-1 IFI/area was found in the 4 UB/kg group (Fig. 5C, D). These results suggested that 2 BU/kg effectively alleviated microglia activation.

Effects of Batroxobin on Locomotor Recovery after SCI

To test whether Batroxobin is beneficial to the functional recovery from SCI, we evaluated locomotion by BBB scoring and RHI assay at 1, 4 and 7 days after SCI, and the footprint test at 7 days after SCI. The BBB scores showed that at 4 and 7 days, 2 BU/kg-treated rats showed significant improvement (Fig. 6A). Similarly, the 2 BU/kg-treated rats showed higher lift of the hind limbs at 4 and 7 days as evaluated by the RHI assay (Fig. 6B). The SLF

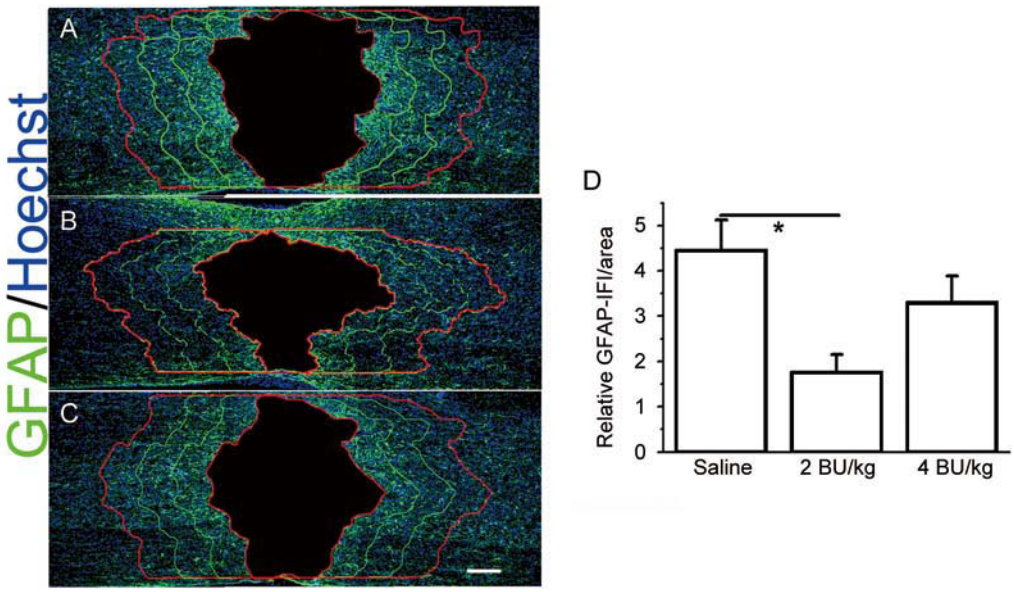

Fig. 3. Effects of Batroxobin on GFAP immunoreactivity at 7 days after injury. A–C: GFAP immunoreactivity in saline (A), 2 BU/kg (B), and 4 BU/kg (C) groups. Scale bar, 200 μm. D: Quantification of the IFI/area of GFAP. n = 6/group. *P <0.05. IFI, immunofluorescence intensity.

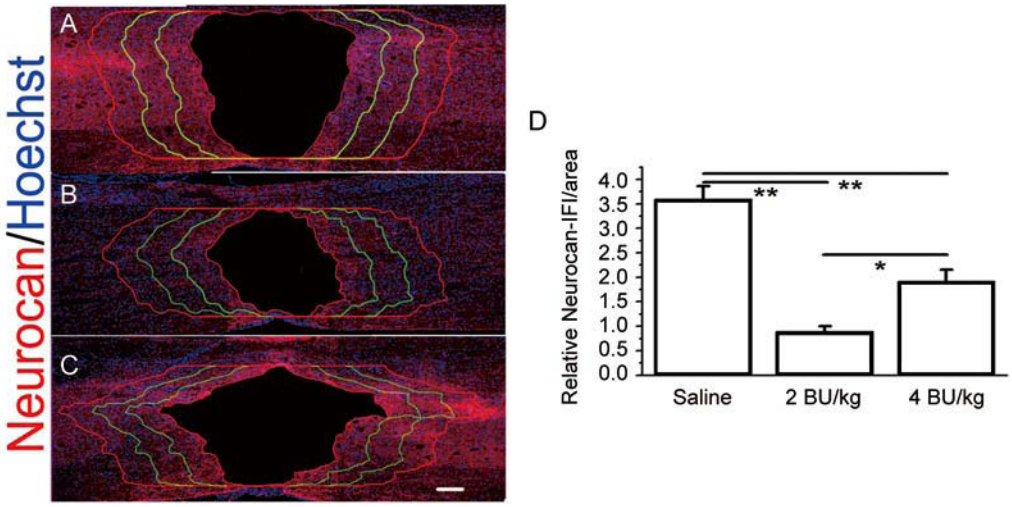

Fig. 4. Effects of Batroxobin on Neurocan immunoreactivity at 7 days after injury. A–C: Neurocan immunoreactivity in saline (A), 2 BU/kg (B), and 4 BU/kg (C) groups. Scale bar, 200 μm. D: Quantification of the IFI/area of Neurocan. *n* = 6/group. **P* <0.05, ***P* <0.01. IFI, immunofluorescence intensity.

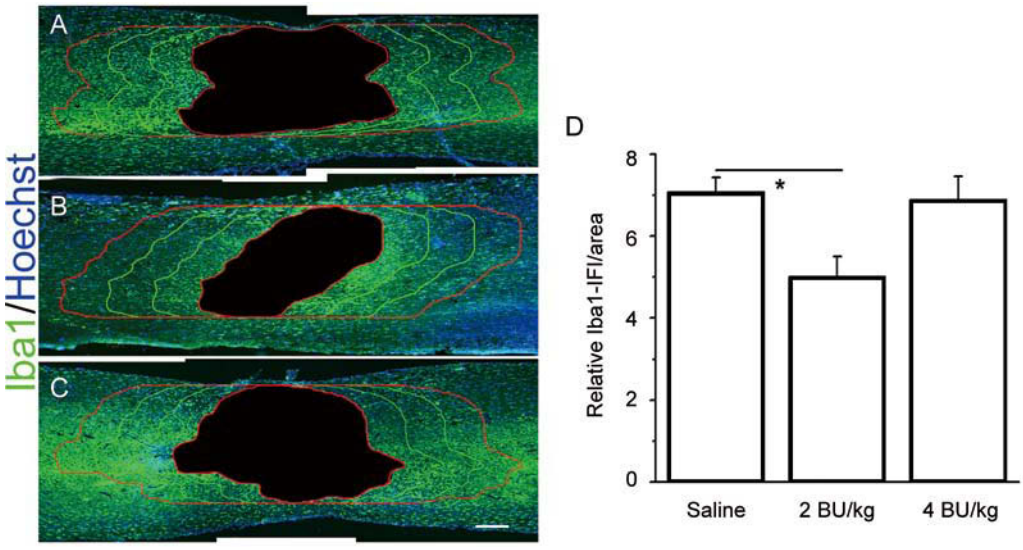

Fig. 5. Effects of Batroxobin on microglia activation at 7 days after injury. A–C: Iba-1 immunoreactivity in saline (A), 2 BU/kg (B), and 4 BU/kg (C) groups. Scale bar, 200 μm. D: Quantification of the IFI/area of Iba-1. *n* = 6/group. **P* <0.05. IFI, immunofluorescence intensity.

in the footprint test showed a 63.67 ± 12.1% decrease at 7 days in the 2 BU/kg group (*n* = 6 rats, *P* <0.05, Fig. 6C). However, 4 BU/kg did not have any significant effects on the BBB scores, RHI and SLF (Fig. 6).

DISCUSSION

SCI has a high incidence and is a major concern for society, patients, and their families. Among the various types of SCI, contusion has the highest incidence. Fortunately, early surgical intervention is very successful[21]. Following the primary spinal cord injury, secondary injury expands continuously for ~4 weeks, so understanding the mechanism and finding measures to control it are of great importance. Our previous study demonstrated that an ischemic zone occurs at the front of the expending secondary injury[9], and this triggers the advance of the

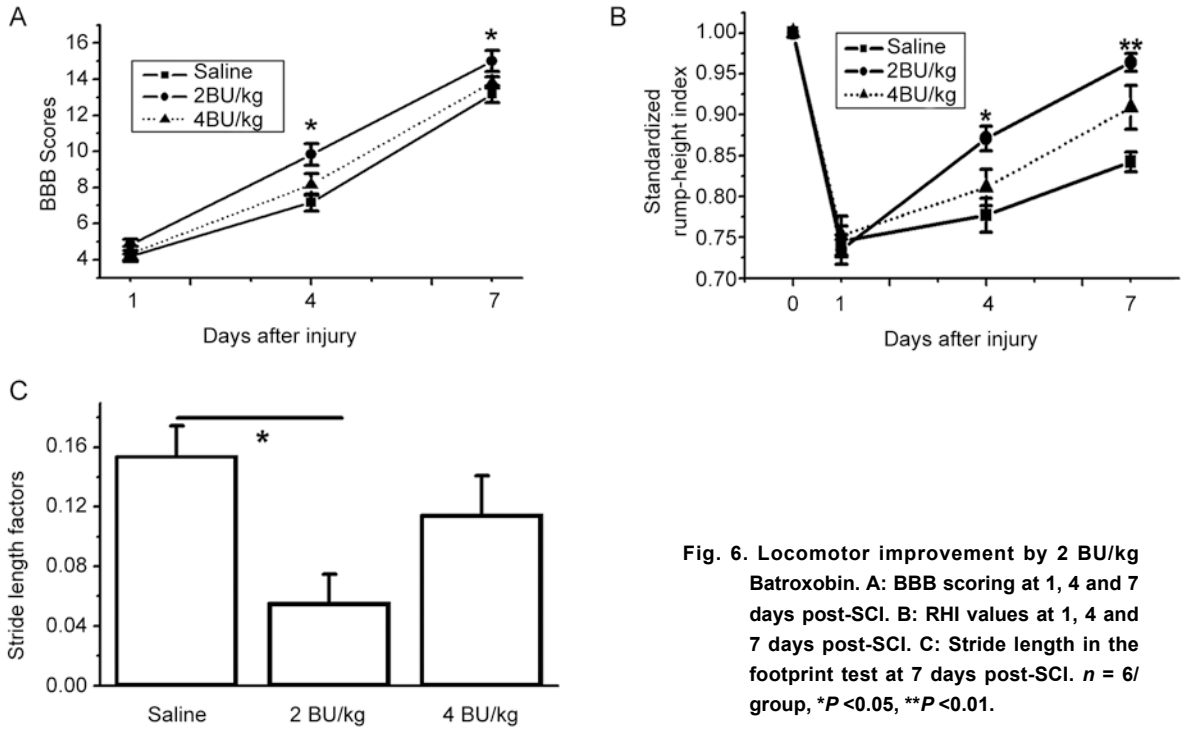

Fig. 6. Locomotor improvement by 2 BU/kg Batroxobin. A: BBB scoring at 1, 4 and 7 days post-SCI. B: RHI values at 1, 4 and 7 days post-SCI. C: Stride length in the footprint test at 7 days post-SCI. *n* = 6/ group, **P* <0.05, ***P* <0.01.

injury. We further showed that enhancing spinal cord blood circulation indeed reduces the secondary injury[11]. Using the same strategy, here we studied the effect of Batroxobin, a drug widely used in various ischemic disorders[16-18], in reducing the secondary SCI. Fibrinogen is a blood-borne molecule involved in both circulation and glial reaction after SCI[12-14], and Batroxobin is a defibrinogating agent. Moreover, Batroxobin decreases blood viscosity without affecting other coagulating proteins such as factors V, VIII and XIII, as well as platelets and endothelial cells[15]. One of the concerns for Batroxobin administration in SCI is the possibility of inducing bleeding in the injured cord. It has been reported that its downstream product, fibrinopeptide-A, forms an unstable clot and even shortens the bleeding time *in vivo*[15,22]. In the present study, we demonstrated that 2 BU/kg promoted neuronal survival, alleviated the activation of astrocytes and microglia, and improved locomotor recovery. But surprisingly, 4 BU/kg had no effect, neither good nor bad. In humans, only one dose is allowed on special occasions. Apparently, 4 BU/kg is an overdose in rats. The mechanisms of the null effect remain to be elucidated.

In conclusion, since Batroxobin is clinically widely used, its beneficial effects in reducing secondary spinal cord injury can be transplanted to the bedside.

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