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RESEARCH ARTICLE

Bee Venom Acupuncture Reduces Interleukin-6, Increases Interleukin-10, and Induces Locomotor Recovery in a Model of Spinal Cord Compression

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Abstract
Spinal cord injuries (SCIs) initiate a series of molecular and cellular events in which inflammatory responses can lead to major neurological dysfunctions. The present study aims to investigate whether bee venom (BV) acupuncture applied at acupoints ST36 (**Zusanli**) and GV3 (Yaoyangquan) could minimize locomotor deficits and the magnitude of neural tissue losses, and change the balance between pro- and anti-inflammatory cytokines after an SCI by compression. Wistar rats were subjected to an SCI model by compression in which a 2-French Fogarty embolectomy catheter was inflated in the extradural space. The effects of BV acupuncture, in which 20 μL of BV diluted in saline (0.08 mg/kg) was injected at acupoints GV3 and ST36 [BV(ST36+GV3)-SCI] was compared with BV injected at nonacupoints [BV(NP)-SCI] and with no treatment [group subjected only to SCI (CTL-SCI)]. The BV(ST36+GV3)-SCI group showed a significant improvement in the locomotor performance and a decrease of lesion size compared with the controls.

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BV acupuncture at the ST36 + GV3 increased the expression of interleukin-10 (anti-inflammatory) at 6 hours and reduced the expression of interleukin-6 (proinflammatory) at 24 hours after SCI compared with the controls. Our results suggest that BV acupuncture can reduce neuroinflammation and induce recovery in the SCI compression model.

1. Introduction

An inflammatory response is one of the secondary events that occur after a traumatic spinal cord injury (SCI). Such responses play an important role in promoting neurological damage [1—3]. The levels of proinflammatory cytokines, such as interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor alpha are increased considerably within the injured nervous tissue [4—6]. Together, these proinflammatory cytokines promote greater activation, proliferation, and attraction of astrocytes and cells from the peripheral immune system to the injury site. On the contrary, the trauma also increases anti-inflammatory cytokines such as IL-10, which modulate the inflammatory response [7]. Previous studies have shown that an injection of IL-10 has a beneficial effect after both traumatic brain injuries and SCIs [8]. IL-10 mediates its effects on most cell populations in the central nervous system and limits inflammation by reducing the synthesis of proinflammatory cytokines IL-1β and tumor necrosis factor alpha [9].

Currently, there is no treatment that is able to reverse the overall consequences caused by SCIs. Thus, it is necessary to search for treatments that can bring benefits and clinical improvements for SCI patients. Bee venom (BV) has long been used as a therapeutic method mainly for inflammatory or painful conditions in Oriental medicine [10]. BV acupuncture is the injection of bee sting or diluted BV into acupoints with the purpose of combining and/or enhancing the effects of BV and acupuncture. BV acupuncture has been shown to be effective in the treatment of experimental models of neurodegenerative diseases such as amyotrophic lateral sclerosis and Parkinson disease [11,12].

Thus, the present study aimed to investigate the effect of BV acupuncture at acupoints ST36 + GV3 (3° acupoint of the Governor Vessel meridian, known to promote functional repair after peripheral nerve injury [13]) on the locomotor recovery, size of the lesion, and balance between pro- (IL-1β and IL-6) and anti-inflammatory (IL-10) cytokines in rats subjected to a model of SCI by compression.

2. Materials and methods

2.1. Animals and procedure for epidural balloon-compression spinal injury

Adult male Wistar rats, weighing between 270 g and 310 g, were kept in 12/12-hour light—dark cycles at a constant temperature, with food and water *ad libitum*. All procedures were approved by the Ethics Committee on Research of the Federal Rural University of Rio de Janeiro (23083.005880/2013).

SCI induced by compression was similar to that described previously by Vanický and colleagues [14]. Briefly, after exposure of the T10-L1 vertebra, a 2-French Fogarty catheter (Edwards Lifesciences, EUA, CA, USA) was inserted into the epidural space through a small hole in the vertebral arch of T10 and advanced cranially until the center of the balloon rested at the T8—T9 level. The balloon catheter was inflated with 15 μL of saline for 5 minutes using a Hamilton syringe (type 1705). A sham-operated group was submitted to mini-laminectomy without insertion of the catheter.

After surgery, the animals were randomly divided into four groups: sham received only a mini-laminectomy; CTL-SCI group was submitted only to the spinal cord compression; BV(ST36+GV3)-SCI group received injection of BV at acupoints ST36 and GV3 at different time points after the SCI; and BV(NP)-SCI group received BV at nonacupuncture points at different time points after the SCI.

2.2. BV acupuncture treatment

BV *Apis mellifera* (catalogue number: V3375; Sigma, St. Louis, MO, USA) at a dose of 0.08 mg/kg was diluted in saline solution and injected subcutaneously (sc) at acupoints ST36 and GV3 (20 μL at each point). Acupoint ST36 is located approximately 5 mm below and lateral to the anterior tubercle of the tibia, and GV3 is located on the dorsal midline at the depression between the spinous processes of the last lumbar and the first sacral vertebrae [15]. The BV(NP)-SCI group was injected with the same dose and volume of BV at nonacupoints located in the same dermatome as the acupoints. For ST36, the nonpoint was located 5 mm lateral to the midline of the posterior face of the hindlimb, and for GV3, approximately 1 cm lateral to the GV3 on the crest of the ilium.

BV treatments were performed in two protocols: (1) given that the peak release of cytokines occurs in the first few hours after traumatic injury [16] for evaluation of the expression pro- and anti-inflammatory cytokines, the BV acupuncture treatment was performed once immediately after the SCI (n = 3—4/group) and the cytokines were evaluated 1 hour, 6 hours, and 24 hours after the SCI; (2) for behavioral and histopathological analysis, the BV acupuncture was carried out at 24 hours, 7 days, and 14 days after the SCI (n = 5—7/group). After the SCI, these animals were kept in controlled temperature and administered an injection of fentanyl (0.032 mg/kg, sc; Fentanil (Janssen Pharmaceutica, Beerse, Belgium)) and a prophylactic dose of antibiotic (pentabiotic, 40,000 IU/kg, sc; Fort Dodge, São Paulo, Brazil). Their bladders were manually emptied daily until animals regained voiding function.

2.3. Chemiluminescent assay for measurement of cytokines

To evaluate the expression of proinflammatory (IL-1β and IL-6) and anti-inflammatory cytokines (IL-10), the rats (n = 3—4 animals/group) were euthanized and

approximately 1 cm of the spinal cord at the lesion site was collected at 1 hour, 6 hours, and 24 hours after the SCI.

Cytokines IL-1β, IL-6, and IL-10 (pro- and anti-inflammatory cytokines) were measured using Rat CVD Panel 1 Kit (Milliplex MAP, Bio-Rad, Hercules, CA, USA) that allows the measurement of different analytes in a single sample. All procedures were performed precisely according to the enclosed guidelines. The cytokine concentrations in the tissues were expressed as picograms per milliliter.

2.4. Functional assessment of locomotor performance

Each animal was placed individually in the center of the field, as described previously [17]. The evaluation was performed on the 1st, 7th, and 14th days postinjury. Scores from 0 to 21 were assigned to the animals, where the number 21 indicates normal locomotion and 0 no spontaneous movement of the hind limbs. Each evaluation was performed by two raters blinded to the experiment, and the values were represented as mean ± standard error ($n = 7$ rats/group).

2.5. Assessment of lesion area

Two weeks after the SCI, the same animals that were submitted to the behavioral test were deeply anesthetized and transcardially perfused with paraformaldehyde. Spinal segments of approximately 1.5 cm containing the epicenter of the lesion was immediately and carefully dissected out and postfixed until histological processing. The spinal segments were embedded in paraffin wax, sliced into 5-μm longitudinal sections, and stained with hematoxylin/eosin. Images of four representative sections of the lesion epicenter were analyzed using the Image J software (MD, USA). To determine the lesion area, the rostrocaudal boundaries of the injury were defined by the presence of inflammatory cells, cavitations, and cyst formation. All the histological analyses were performed by an experienced pathologist blinded to the treatment, and the values were expressed as means of lesion area of four representative sections ($n = 5$ rats/group).

2.6. Statistical analysis

Data were expressed as the mean ± standard error mean. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis for the behavioral test was carried out using repeated measures two-way analysis of variance (ANOVA) followed by the Bonferroni test; for the cytokine expression analysis, one-way ANOVA followed by the Student–Newman–Keuls test or the Kruskal–Wallis test followed by Dunn's test (for data without normal distribution) was used; and for the lesion area, one-way ANOVA followed by the Bonferroni test was applied. Statistical significance was considered when $p < 0.05$.

3. Results

The SCI produced an increased concentration of IL-6, since its expression was higher in all SCI groups in comparison with sham at all time points analyzed ($p < 0.05$). Twenty-four hours after the SCI, IL-6 expression of the BV(ST36+GV3)-SCI group was lower than that of the control groups BV(NP)-SCI and CTL-SCI (one-way ANOVA, $F_{(3,11)} = 18{,}291$, $p < 0.001$; Student–Newman–Keuls test, $p < 0.05$). An increased concentration of IL-1β was also produced 6 hours after the SCI; however, no significant difference was detected in the expression of IL-1β among the BV(ST36+GV3), BV(NP)-SCI, and CTL-SCI groups at all time points evaluated ($p > 0.05$; Fig. 1).

In relation to the expression of anti-inflammatory cytokines, our data showed that the SCI also produced an increase in IL-10, since its expression was higher in all SCI groups in comparison with the sham at 1 hour and 6 hours after the SCI ($p < 0.05$). The expression of IL-10 was higher in the BV(ST36+GV3) group than in the BV(NP)-SCI and CTL-SCI groups at 6 hours after the SCI (one-way ANOVA, $F_{(3,13)} = 12{,}714$, $p < 0.001$; Student–Newman–Keuls test, $p < 0.05$). However, no significant difference was detected between groups at 24 hours after treatment ($p > 0.05$; Fig. 2). In addition, no significant difference was detected in the expression of IL-4 among all groups at all time points evaluated ($p > 0.05$; Fig. 1).

In relation to locomotor performance, on the 1st day after injury, all animals showed paraplegia (BBB score 0), with no significant difference between groups. BBB (Basso, Beattie and Bresnahan test) scores were significantly higher in the BV(ST36+GV3)-SCI group than in the BV(NP)-SCI and CTL-SCI groups on the 7th day and 14th day after the SCI. No differences were detected between the BV(NP)-SCI and CTL-SCI groups (two-way ANOVA for repeated measures, $F_{(8,91)} = 4.692$, $p < 0.0006$; Bonferroni test, $p < 0.05$; Fig. 3). The sham-operated group did not show any motor abnormality and all animals had a score of 21 in the BBB tests. The sham-operated group was omitted in the graph (Fig. 3) and not included in the statistical analysis.

In general, the compression produced significant changes in the spinal structure with numerous inflammatory infiltrates, cystic cavities, and rupture of the spinal tissue. Our results showed that the total lesion area was significantly smaller in the BV(ST36+GV3)-SCI group compared with that in the controls BV(NP)-SCI and CTL-SCI (one-way ANOVA, $F_{(2,17)} = 4.770$, $p = 0.025$; Bonferroni test, $p < 0.05$; Fig. 2). As expected, sham-operated animals had normal histology and were omitted from the statistical analysis and Fig. 2.

4. Discussion

In the present study, BV acupuncture promoted an increase of IL-10 expression in the 1st 6 hours after treatment and a reduction of IL-6 24 hours after treatment. The data revealed that treatment with BV acupuncture had no effect on the expression of IL-1β and IL-4 cytokines. The levels of inflammatory cytokines at the SCI site were analyzed 1 hour, 6 hours, and 24 hours after the SCI considering that its dynamic release at the acute phase can contribute to further tissue damage [18].

Proinflammatory cytokine expressions have already been shown to increase significantly after an SCI and they play a crucial role in neurological worsening [19]. By contrast, the

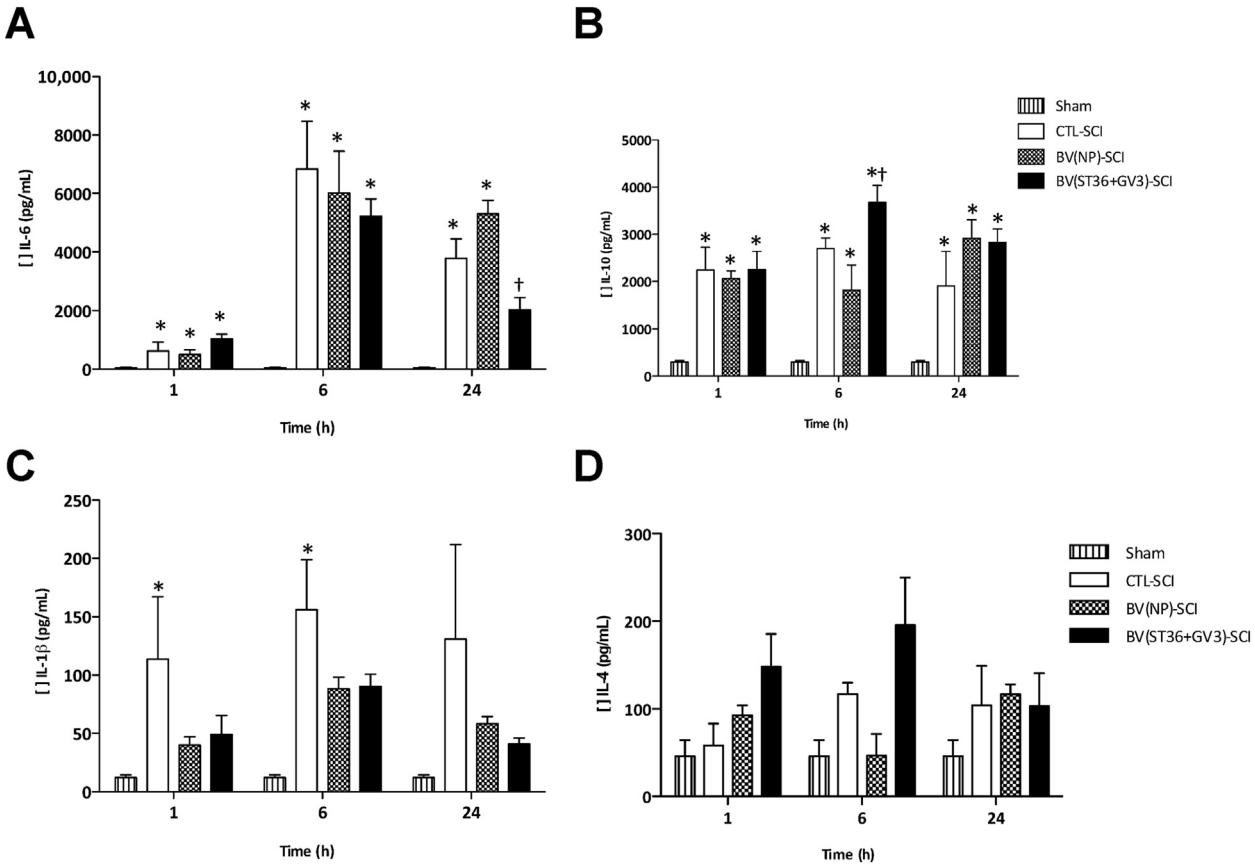

Figure 1 BV acupuncture inhibits expression of proinflammatory cytokines and upregulation expression of anti-inflammatory cytokines in the rat compression SCI model. Comparison of (A) IL-6, (B) IL-10, (C) IL-1β, and (D) IL-4 expressions in rats submitted to the spinal cord injury model by compression at 1 hour, 6 hours, and 24 hours after treatment. The effects of BV acupuncture at acupoints GV3 and ST36 [BV(ST36+GV3)-SCI] were compared with BV injected at nonacupoints [BV(NP)-SCI], control group without manipulation (CTL-SCI), and sham.* Difference from the Sham group. † Difference from the BV(NP)-SCI and CTL-SCI groups ($n = 3–4$ animals/group; $p < 0.05$). BV = bee venom; BV(ST36+GV3)-SCI = group receiving BV at acupoints GV3 and ST36; BV(NP)-SCI = group receiving BV at nonacupoints; CTL-SCI = group subjected only to spinal cord compression; IL = interleukin; SCI = spinal cord injury; sham = group receiving only a mini-laminectomy.

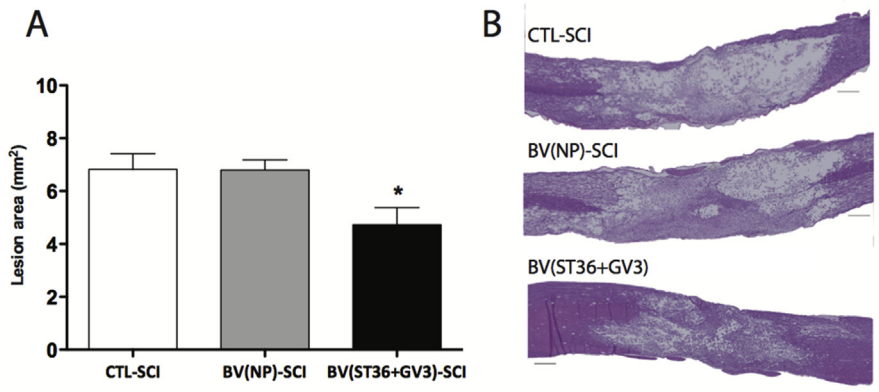

Figure 2 BV acupuncture reduces lesion size after SCI in the rat compression SCI model. (A) Quantitative analysis of lesion area performed 14 days after injury. (B) Representative images of H&E staining of the spinal cord (calibration bars = 500 μm). The effects of BV acupuncture at acupoints GV3 and ST36 [BV(ST36+GV3)-SCI] are compared with BV injected at nonacupoints [BV(NP)-SCI] and control group without manipulation (CTL-SCI).* Difference from CTL-SCI and BV(NP)-SCI. Data are presented as means ± error ($n = 5$/group; $p < 0.05$). BV = bee venom; BV(ST36+GV3)-SCI = group receiving BV at acupoints GV3 and ST36; BV(NP)-SCI = group receiving BV at nonacupoints; CTL-SCI = group subjected only to spinal cord compression; H&E = hematoxylin/eosin; SCI = spinal cord injury.

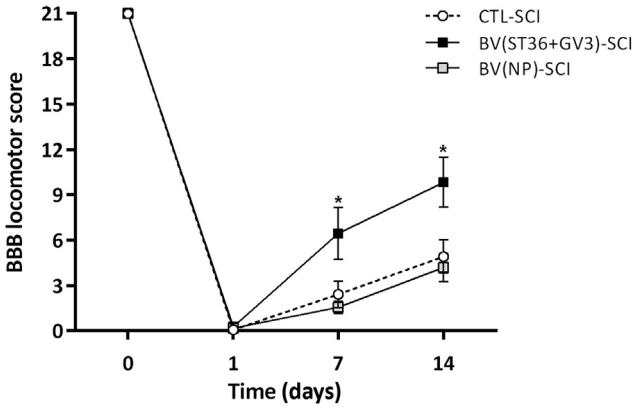

Figure 3 BV acupuncture at acupoints ST36 + GV3 improves locomotor performance in the rat compression SCI model. Functional recovery was assessed with the BBB test on the 1st, 7th, and 14th days after SCI. The effects of BV acupuncture at acupoints GV3 and ST36 [BV(ST36+GV3)-SCI] were compared with BV injected at nonacupoints [BV(NP)-SCI] and control groups without manipulation (CTL-SCI).* Difference in BV-ST36+GV3 in relation to CTL and BV-NP at each time point. The group BV(ST36+GV3)-SCI had a significant improvement in the BBB score on both 7th and 14th days after SCI in comparison with BV(NP)-SCI and CTL-SCI. Data are presented as mean ± error ($n = 7$/group; $p < 0.05$). BBB = Basso, Beattie and Bresnahan test; BV = bee venom; BV(ST36+GV3)-SCI = group receiving BV at acupoints GV3 and ST36; BV(NP)-SCI = group receiving BV at nonacupoints; CTL = control; CTL-SCI = group subjected only to spinal cord compression; SCI = spinal cord injury.

expression of anti-inflammatory cytokine IL-10 increases after an SCI and appears to be involved in the regulation of inflammatory responses, promoting the inhibition of the synthesis and secretion of various proinflammatory cytokines such as IL-1β, IL-2, tumor necrosis factor alpha, and IL-6 to reduce the inflammatory reaction [20,21]. IL-10 is a major anti-inflammatory cytokine, and its receptors are present in various cell types of the nervous system, including neurons and microglia [16,20]. Other anti-inflammatory cytokines such as IL-1ra, IL-4, and TGF-β (Transforming growth factor beta) are also able to suppress the production of proinflammatory cytokines, and this mechanism has a critical effect on the concept of balance between pro- and anti-inflammatory cytokines [16,22]. Disruption of this balance, with high levels of proinflammatory cytokines and low levels of anti-inflammatory cytokines, may lead to an amplification process that can induce cell cycle activation, which further promotes cytotoxicity and neurodegenerative processes [16,22].

In vitro studies have shown that IL-10 may also act directly on motor neurons of the spinal cord and activate a pathway gene transcription through nuclear factor-kB promoting increased expression of antiapoptotic proteins such as Bcl2 and Bclx. Thus, besides acting in cells that participate in the inflammatory response, IL-10 can also directly reduce neuronal death after an SCI.

IL-6, despite having both pro- and anti-inflammatory effects on an SCI, seems to have proinflammatory effects associated with the development of secondary damage [7]. Temporal blocking of IL-6 signaling after an SCI could prevent inflammation and promote functional recovery via activation of the alternative macrophage pathway [19].

In our study, BV acupuncture promoted an increase in IL-10 levels, which may have acted on other inflammatory cells by reducing the production of proinflammatory cytokines such as IL-6, but may also have directly reduced neuronal death in the motor ventral horn [23]. In this regard, our results may also indicate that the initial increase of IL-10 promoted by BV acupuncture at acupoints ST36 and GV3 during the 1st 6 hours may have induced the blocking effect or release of IL-6. Although the pathogenesis of an SCI is known to be multifactorial, we believe that BV acupuncture increased IL-10 and decreased IL-6, which may have contributed to the increased recovery of locomotor function and reduced injury area.

Our data revealed that BV acupuncture did not influence the release of cytokine IL-4. IL-4 is the prototype member of the family of anti-inflammatory cytokines, and currently, very little is known about its role in traumatic spinal injury.

Our results indicated that treatment with BV acupuncture applied to acupoints ST36 and GV3 promoted partial functional recovery of the hind limbs and a decrease in the lesion size after an SCI.

BV acupuncture has been used for many years in traditional Chinese medicine for the treatment of various types of diseases, especially those of an inflammatory nature, such as arthritis and rheumatism, chronic neuralgia, and arthralgia [24]. The main hypothesis is that injection of BV into acupoints can enhance the effects of acupuncture [25] and increase clinical recovery [26]. Chemical stimulation with BV or melittin, which is the major constituent of the BV [27] at the acupoint Zusanli (ST36) was able to produce a significant improvement in motor activity in mice evaluated by the rotarod performance test on the amyotrophic lateral sclerosis model [12,28]. Furthermore, BV at ST36 enhanced motor function and decreased motor neuron death in the spinal cord in hSOD1G93A transgenic mice [25]. Choi and colleagues [6] showed that manual acupuncture (only needle insertion) at acupoints GV26 and GB34 daily for 2 weeks was able to improve the locomotor performance (BBB scores) after an SCI caused by contusion in rats. Moreover, diluted BV (0.25 mg/kg) applied at ST36 induced an attenuation in the neuropathic pain behavior in the rat spinal cord hemisection model [11].

In the present study, BV (diluted, 0.08 mg/kg) was applied at acupoints ST36 and GV3 in low doses for the purpose of causing local irritation [29]. This strategy is different from the BV therapy where BV is used in higher doses (0.25—0.6 mg/kg) [11,30]. The use of high doses of BV can produce systemic effects; therefore, it cannot be determined whether the effect is promoted by stimulation of acupoints or by systemic injection of BV. Regardless of the dose, arthritis animal model studies, in which stimulation with BV was compared between acupoints and non-acupoints, have shown that the injection of BV at acupoints increased the therapeutic activity of BV [31—33]. Similarly, our results also showed that low doses of BV injected into acupoints ST36 and GV3 were able to improve the locomotor performance, since BV injected into nonacupoints had no effect (Fig. 3).

Our data show that the injection of diluted BV at acupoints ST36 + GV3 can improve the functional recovery in

rats after an SCI. This effect appears to be related to the localization of points, since the stimulation of nonacupoints did not produce any significant changes on the functional recovery of rats after the SCI. These results suggest that BV acupuncture at acupoints ST36 + GV3 is able to promote partial recovery of locomotion, and this improvement can be associated with reduced lesion size and an anti-inflammatory action, by modulating the balance of pro- and anti-inflammatory cytokines. Furthermore, the present study suggests that an application of BV acupuncture at acupoints ST36 and GV3 may act as a therapeutic tool in acute spinal injury.

Disclosure statement

The authors have no conflicts of interest and no financial interests related to the material of this manuscript.

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