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Original Article

Electro-acupuncture at Governor Vessel Improves Neurological Function in Rats with Spinal Cord Injury*

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ABSTRACT **Objective:** To determine the effects of electro-acupuncture (EA) at Governor Vessel (GV) on the locomotor function in spinal cord injury (SCI) rats and explore the underlying mechanism. **Methods:** Thirty-two male Sprague-Dawley rats were randomly divided into four groups namely: the sham group (with sham operation); the untreated group (without treatment after spinal cord impact); the EA-1 group [EA applied at Baihui (GV 20) and Fengfu (GV 16) after spinal cord impact] and the EA-2 group [with EA applied at Dazhui (GV 14) and Mingmen (GV 4) after spinal cord impact]. Real-time quantitative-polymerase chain reaction (RT-PCR) and Western Blotting were used to assess changes in the mRNA and protein expression levels of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) at 7 weeks following EA administration. In addition, the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale was assessed at 1 day, 1 week, 3 weeks and 7 weeks post-injury. **Results:** The results showed that EA stimulation induced neuroprotective effects after SCI correlated with the up-regulation of BDNF and NT-3 ($P<0.05$). Furthermore, EA stimulation at GV 14 and GV 4 could significantly promote the recovery of locomotor function and this may be linked to the up-regulation of BDNF and NT-3 ($P<0.05$). **Conclusions:** EA treatment applied at GV acupoints either within the injury site or adjacent undamaged regions near the brain can improve functional recovery, which may be correlated with the up-regulation of BDNF and NT-3. In addition, it would be more effective to administer EA at GV 14 and GV 4 near the injury site of the SCI rats.

KEYWORDS electro-acupuncture, spinal cord injury, neuroprotection, brain-derived neurotrophic factor, neurotrophin-3

Spinal cord injury (SCI) produces a series complicated challenges to the recovery of locomotor function.[1,2] At present, there is still a lack of effective treatments for spinal cord injuries. The pathophysiology of SCI can be divided into two phases. The initial injury constitutes the primary phase and the secondary phase begins hours after the initial insult, which is marked by a number of cellular and molecular changes in and around the injured area.[3,4]

Acupuncture is a therapeutic technique of Chinese medicine. Electro-acupuncture (EA) is a type of therapy that utilizes a trace pulse current that is attached to a needle inserted into an acupoint with the purpose of producing synthetic electric and needling stimulation.[5] The EA application at Governor Vessel (GV) shows alleviation of secondary damage after SCI in both patients and animal models.[6-8]

There are 28 acupoints on GV and whether or not the stimulation on all acupoints of GV can create the similar effect on SCI is not yet clear. In other words, it is difficult to select the more effective acupoints for the SCI treatment between the acupoints near the injury site and adjacent undamaged regions of spinal cord. Our paper discusses the initiated studies to determine the effect of EA at different GV acupoints on the improvement of locomotor function after SCI.

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Neurotrophic factors are key nervous system regulatory proteins that modulate neuronal survival, axonal growth, synaptic plasticity and neurotransmission.[9] As a member of the nerve growth factor family, brain-derived neurotrophic factor (BDNF) plays a crucial role in development and maturation of the physiological function of the nervous system, which is necessary for the survival and function of neurons.[10-12] It is well documented that neurotrophin-3 (NT-3) is indispensible in neuronal plasticity particularly in the recovery processes after SCI.[13,14]

In this study, we investigated the effect of EA at different GV acupoints on the improvement of locomotor function following SCI and explored the underlying mechanism that would be correlated with the up-regulation of BDNF and NT-3.

METHODS

Animal and Experimental Groups

All experiments were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. A total of 32 male Sprague-Dawley rats (180 to 220 g) were assigned to four groups: (i) the sham group received a laminectomy only (sham, *n*=8), (ii) the untreated group received a spinal cord impact without treatment (untreated, *n*=8), (iii) the EA-1 group, received EA treatment at Baihui (GV 20) and Fengfu (GV 16) after spinal cord impact (EA-1, *n*=8), (iv) the EA-2 group received EA treatment at Dazhui (GV 14) and Mingmen (GV 4) after spinal cord impact (EA-2, *n*=8). All rats were kept in separated cages with free access to food and water. Room temperature was set at 25±3 ℃.

Spinal Cord Injury

All rats were anesthetized with chloral hydrate (500 mg/kg), and a laminectomy was performed at the T10 level, exposing the cord beneath without disrupting the dura mater. The spinous processes of T8 and T11 were then clamped to stabilize the spine and the exposed dorsal surface of the cord was subjected to contusion injury (10 g×25 mm) using a New York University Impactor except the sham group rats.[15-17] The wound was covered with cotton soaked in saline to avoid direct contact of the spinal cord with air. Bladders were manually emptied twice daily for a week (if full). To prevent postoperative infection, gentamicin injection was used with 2.5 mg/kg every 12 h for 3 days.

Acupuncture Application

An immobilization apparatus designed by our laboratory (Patent application No.: 201110021482.5, State Intellectual Property Office, Figure 1) was applied to reduce stress without anesthesia during EA stimulation, which was both convenient for acupuncture research and comfortable for experimental rats.[18] The rats in the EA-1 group received EA treatment at GV 20 and GV 16, while that in EA-2 group received EA treatment at GV 14 and GV 4. Those acupoints were located on GV the posterior midline, and a rat immobilization apparatus was designed by our laboratory to reduce stress during acupuncture administration (Figure 2).

Figure 1. Rat Immobilization Apparatus

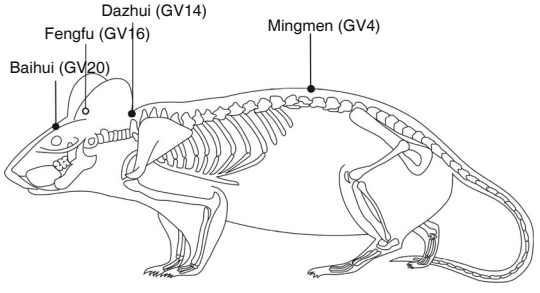

Figure 2. Locations of the Four Acupoints

In the EA-1 and EA-2 group, each pair of stainless needles of 0.25 mm in diameter was applied to inserte into the acupoints. The needles were

connected to the output terminals of an EA apparatus (HANS-200E, Jisheng Medical Instruments, China) and stimulated by continuous-wave of 2 Hz frequency, 0.2 mA intensity, for 30 min. EA was administered once every 2 days for 6 weeks after 1 week post-surgery.

Basso-Beattie-Bresnahan Test

The Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale was used to evaluate locomotor recovery averaged across both the right and left hindlimbs.[19, 20] The scale evaluated the movements of the rats on a 21 point rating scale, with a score of 21 representing normal movement and 0 representing complete paralysis of the hindlimb, including: joint movements, stepping ability, coordination and trunk stability. Behavioral tests were performed at the same time at 1 day, 1, 3 and 7 weeks after operation and graded by the same two observers blinded to treatment conditions, according to the BBB scale.

Western Blotting

A 5-mm length of the spinal cord centered at the injury epicenter was quickly dissected on a chilled stage at 7 weeks after SCI. The samples were centrifuged (12,000 r/min, 20 min, 4 ℃) and the supernatants assayed for total protein. Western blotting analysis was performed to investigate the expression of the BDNF and NT-3 protein. Protein lysates were prepared 15 μL/lane each sample and then fractioned on 10% SDS-polyacrylamide gels. Electroblotting proteins were transferred to nitrocellulose membranes (Solarbio, Inc. HATF00010). The blots were then incubated with rabbit polyclonal antibody anti-BDNF (1:200, Santa Cruz, Inc. sc-546) and rabbit polyclonal antibody anti-NT-3 (1:500, Abcam Biotechnology, Inc. ab65804) for 24 h at 4 ℃. The membranes were then incubated with a dilution of horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (1:5000, Beytime, BL003A). The bands on the immunoblots were visualized using the Chemi Imager 5500 V2.03 software and integrated density values (IDVs) were calculated by Fluor Chen 2.0 software and normalized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real-Time PCR

Quantitative RT-PCR was conducted to determine the changes of the expression levels of BDNF and NT-3 mRNA in each group respectively at 7 weeks following SCI. Total RNA was isolated from a 0.5 cm-long spinal cord fragment that contained the wound epicentre using the TRIZOL Reagent (Invitrogen, 15596-026). Using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, K1622) and specific primers as previously described, cDNA synthesis and RT-PCR was carried out. For relative quantification, each gene of interest was first subjected to a serial dilution assay to determine the optimum detection range of Ct values, with a Ct threshold of 35 for undetectable levels of expression. Using 10 ng of reverse-transcribed total RNA, 20 pmol/mL of both sense and antisense primers and the Fast SYBR Premix Ex Taq™ (TaKaRa Code:DRR420A) in a final reaction volume of 20 μL. The reactions were run on an ABI PRISM 7900 Fast Sequence Detection System instrument and software (Applied Biosystem) according to the manufacturer's protocol. The primers used for BDNF and NT-3 were synthesized by the Genotech (Daejeon, Korea) and GAPDH was used as an internal control. The sequences of the primers were: BDNF: 5'-GGACCCTGAGTTCCACCA-3' (sense)', 5'-CAAAAGTGTCAGCCAGGGA-3' (antisense); NT-3: 5'-GCGAGACTGAATGACCGAACT-3' (sense), 5'-GCCACGGAGATAAGCAAGAAA-3' (antisense); GAPDH: 5'-AGAGGGAAATCGTGCGTGAC-3' (sense), 5'-AGAGGTCTTTACGGATGTCAACG-3' (antisense).

Statistical Analysis

SPSS 13.0 software was used for the statistical analysis. Data are presented as the mean ± standard deviation ($\bar{x} \pm s$). The BBB Scale score was analyzed using repeated-measures analysis of variance (ANOVA). Other data were analyzed using one-way ANOVA. If equal variances were found, Fisher's least significant difference test was performed. Otherwise, the Kruskal-Wallis Test and Dunnett's T3 were used. The statistical significance level was set at $P<0.05$.

RESULTS

Effects of EA on Neural Function Evaluation

Evaluation of neural function showed that the BBB Locomotor Rating Scale in the sham group at 1 day, 1, 3 and 7 weeks post-surgery were 20.5 ± 0.46, 20.56 ± 0.42, 20.44 ± 0.42, 20.44 ± 0.32 respectively, which were similar to the normal values (Figure 3). SCI caused a severe injury in neuronal function in the untreated group indicated by dramatically decreased

BBB scores at 1 day, 1, 3 and 7 weeks post-injury (1.56 ± 0.62, 2.62 ± 0.52, 7.13 ± 0.44, 9.75 ± 0.47) compared with the sham group (P<0.05). In contrast to the untreated group, BBB scores gradually increased over time in the EA-1 group (1.88 ± 0.35, 3.13 ± 0.58, 10.25 ± 0.60, 12.38 ± 0.69) and EA-2 group (1.75 ± 0.71, 3.13 ± 0.58, 13.38 ± 0.58, 16.88 ± 0.64), suggesting that both the two EA treatments improved recovery of neuronal function after acute SCI (P<0.05). Furthermore, the EA-2 group showed a significant high score after 3 weeks and notably higher at 7 weeks following the operation compared with the EA-1 group at the same time (P<0.05).

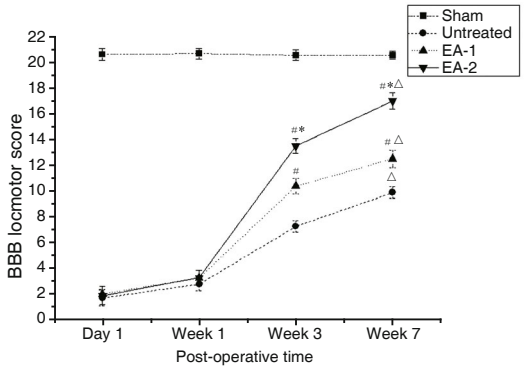

Figure 3. The BBB Locomotor Scores of the Four Groups at 1 Day, 1 Week, 3 Weeks and 7 Weeks Post-injury ($\bar{x} \pm s$, n=8)

Notes: $^{\#}P$<0.05, versus the untreated group; $^{*}P$<0.05, versus the EA-1 group; $^{\triangle}P$<0.05, versus the same group at 3 weeks respectively

EA Differentially Regulate Protein Expression of BDNF and NT-3 Following SCI

As shown in Figure 4A, it revealed the changes of the protein expression of BDNF and NT-3 in each group. Figure 4B illustrated the optical density analysis of BDNF and NT-3 proteins in the spinal cord tissues. The IDVs of BDNF with GAPDH in the sham, untreated, EA-1 and EA-2 groups were: 0.0663 ± 0.0033, 0.1291 ± 0.0047, 0.1828 ± 0.0041 and 0.2943 ± 0.0162 respectively at 7 weeks after SCI and the IDVs of NT-3 with GAPDH in the sham, untreated, EA-1 and EA-2 groups were 0.0242 ± 0.0123, 0.0617 ± 0.0059, 0.1167 ± 0.0040 and 0.2438 ± 0.0130 respectively. The protein expression of BDNF and NT-3 of the spinal cords in the untreated group increased compared with that in the sham group at 7 weeks post-surgery (P<0.05). Treatment with either EA-1 or EA-2 significantly promoted the SCI-induced activation of BDNF and NT-3 expression compared with the untreated group

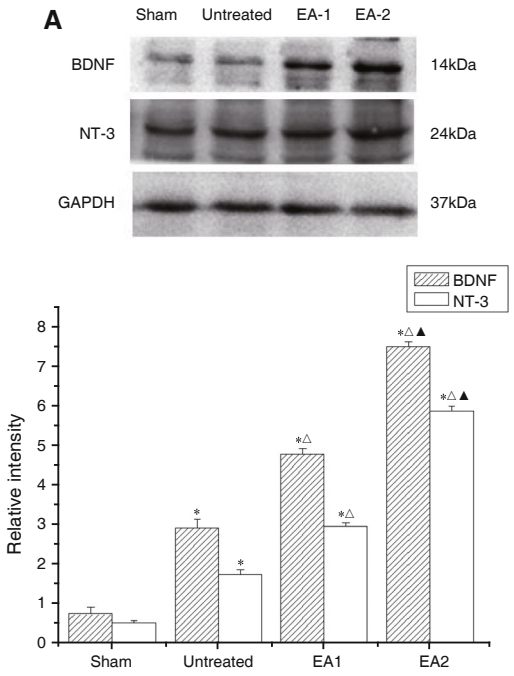

Figure 4. Protein Expression Levels of BDNF and NT-3 Protein in Different Groups ($\bar{x} \pm s$, n=8)

Notes: $^{*}P$<0.05, versus the sham group; $^{\triangle}P$<0.05, versus the untreated group; $^{\blacktriangle}P$<0.05, versus the EA-2 group

(P<0.05). Furthermore, IDVs of BDNF and NT-3 protein expression in the EA-2 group were significantly higher than those in the EA-1 group at 7 weeks post-surgey (P<0.05).

EA Differentially Regulate Gene Expression of BDNF and NT-3 mRNA after SCI

The data illustrated that the levels of BDNF and NT-3 mRNA in the spinal cord tissues increased in the untreated group compared with that in the sham group at 7 weeks post-surgery (P<0.05), as illustrated in Figure 5. Meanwhile, both EA-1 and EA-2 treatments significantly enhanced expression of BDNF and NT-3 compared with those in the untreated group. Furthermore, IDVs of BDNF and NT-3 mRNA in the EA-2 group were significantly higher than those in the EA-1 group at 7 weeks post-surgery (P<0.05).

DISCUSSION

SCI is a serious injury of the central nervous system and up until now there is no evident effective treatment for SCI. Axonal regeneration is the significant way to restore functions after serious SCI that interrupts the long tracts mediating motor and sensory function.[21,22] Several obstacles are known to prevent recovery of spinal cord function after SCI. These include: neurite growth-inhibiting constituents

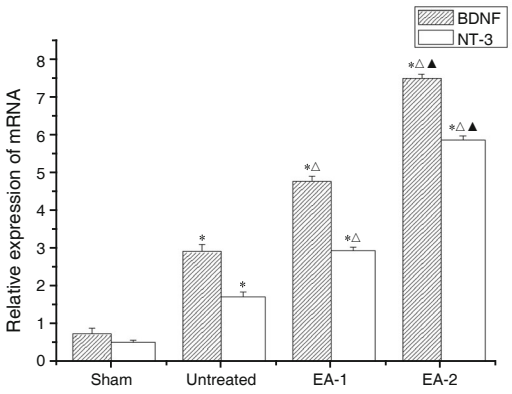

Figure 5. Gene Expression Levels of BDNF and NT-3 mRNA in Different Groups ($\bar{x} \pm s$, n=8)

Notes: $^{*}P$<0.05, versus the sham group; $^{\triangle}P$<0.05, versus the untreated group; $^{\blacktriangle}P$<0.05, versus the EA-1 group

of myelin, scar-associated inhibitory factors and a lack of sufficient neurotrophic support.[23,24] Neurotrophic factor delivery to injured axons or neuronal cell bodies is one means to promote neuronal survival, prevent neuronal atrophy and enhance axonal growth after spinal cord injury.[25,26] Axonal regeneration can be guided along a gradient of neurotrophic factors established by neurotrophin gene transfer distal to a spinal cord lesion site.[27]

EA has been widely used in many clinical scenarios such as pain management, addiction treatment and alleviating symptoms of menopause. Several animal studies of SCI have reported that acupuncture induces neuronal function recovery and anti-inflammatory responses.[28] In our present study, the hindlimbs locomotor functions of rats in the EA-1 and EA-2 groups have been found to be improved at 1, 3 and 7 weeks respectively following EA therapy.

Our data suggested that the levels of BDNF, NT-3 mRNA and protein expressions in spinal cord increased after EA treatment at 7 weeks following SCI. BDNF was discovered in the early 1980's which seemed to be a target-derived trophic factor for many placode-derived sensory neurons.[29-31] An intensive interest in exploring BDNF's potential in treating SCI have been spurred because of its role as a promoter of cell survival and neurite outgrowt.[32] Additionally, as a third member of the neurotrophin family, NT-3 has been shown to enhance regenerative sprouting of lesioned corticospinal, dorsal root and reticulospinal fibers in the injured spinal cord.[13,33] Previous studies have illustrated that injured dorsal column sensory axons extend across a spinal cord lesion site if axons are guided by a gradient of NT-3 rostral to the lesion.[34]

Furthermore, we found that EA application at GV 4 and GV 14 was more effective than GV 20 and GV 16 on neural function recovery. The expressions of BDNF, NT-3 mRNA and protein in the EA-2 group were significantly higher than those in the EA-1 group. Above all, clinical principles of acupoint selection may be the key points for acupuncture to achieve optimum treatment effects.

GV 20 and GV 16 located near the brain. Beyond the proximal consequences of SCI at the spinal level, SCI leaded to topographic and neuronal reorganizations in the cerebral cortex as well as cortical atrophy which indicated that the brain responds to a distant SCI. Increasing evidence has shown that loss of spinal cord input to the brain could promote profound functional modifications in the brain center, such as rapid changes in the spontaneous electrophysiological activity of neuronal networks.[35,36] In addition, new research is starting to reveal that lesion to the spinal cord can impact molecular systems, which are important for synaptic plasticity in the brain.[37,38] A recent study showed that cervical dorsal rhizotomy, a model known to induce cortical reorganizations similar to those observed after SCI, also induced reactive neurogenesis in the primary sensorimotor cortex of adult monkeys.[39] Our data has shown that a certain degree of therapeutic effects could also be achieved after EA was applied at GV 20 and GV 16 during the chronic post-injury period. In our study, spinal cord injuries were incomplete, leaving spared neural pathways to motor neurons which initiated and coordinated movement. Thus, we estimated that EA was able to improve the disruption of the connection between the brain and spinal cord after a spinal injury and induce functional motor recovery to harness plasticity in these spared neural pathways.

In addition, GV 4 and GV 14 located near the lesion. Previous studies have shown that electric fields play a role in nerve growth and reduction of injured nerve degeneration.[40] Extracellular stimulation of spinal cord showed that action potentials in most of the electrically treated preparations were conducted in both directions across the lesion.[41] In our study, the electrical current passed through the injuried tissue directly though GV 4 and GV 14. It was reported that in most of the electrically treated animals, processes from giant axons with swollen irregular tips indicating

active growth in or across the lesion.[42] It is possible that these facilitated regenerative responses are mediated by the effects of the artificially applied electric fields on the natural steady current of injury entering the spinal lesion.

In conclusion, EA treatment applied either within the injury site or adjacent undamaged regions near the brain at GV acupoints improves neurological function in rats with spinal cord injury. It would be more effective to administer EA at the GV acupoints near the injury site of the SCI rats.

Our work demonstrates that EA treatment applied at GV acupoints both near the brain and near the site of trauma can improve functional recovery, which may be correlated with the up-regulation of BDNF and NT-3. Furthermore, EA stimulation at GV 14 and GV 4 can partial promote axonal regeneration and locomotor functional recovery, which indicates a promising avenue of treatment of spinal cord injury.

Conflict of interest

The authors have no conflicts of interest to disclose.

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