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Original Article

Electroacupuncture promotes functional improvement after spinal cord transection injury by inhibiting neuroinflammation

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Abstract: Electroacupuncture (EA) has been used to effectively promote the functional improvement of spinal cord injury (SCI), but the underlying mechanism of EA therapy requires further investigation. Therefore, this study was designed to determine the therapeutic effect of EA on spinal cord transection injury and elucidate the mechanisms involved. Using magnetic resonance imaging (MRI), spinal cord conduction velocity (SCCV), paw withdrawal thresholds (PWT), and tail-flick latency (TFL) tests, we found that EA therapy promotes spinal cord reconstruction and the recovery of sensory and electrophysiological functions in rats after spinal cord transection (SCT). In addition, the SCT-induced microglia activation is suppressed, and the expression of the pro-inflammatory cytokines is reduced following EA treatment. Moreover, EA stimulation depresses the expression of caspase 3 and up-regulates the GAP43 level. Collectively, the present findings demonstrate that EA promotes functional improvement in rats after SCT, accompanied by neuronal regeneration and anti-neuroinflammation activities. These provide biochemical evidence for the mystery of EA efficacy in Chinese traditional therapy and reveal new potential targets for SCT therapy.

Keywords: Spinal cord transection, electroacupuncture, functional improvement, neuronal regeneration, anti-neuroinflammation

Introduction

Spinal cord injury (SCI), a serious impairment of the central nervous system, affects millions of people worldwide by inducing motor, sensory, and autonomic dysfunction [1, 2]. Pathophysiologically, the primary injury-based secondary injury (spreading from the lesions of primary injury) manifests as neuronal apoptosis [3, 4], demyelination [5], and axonal degeneration [6], accelerating the development of SCI. Therefore, therapeutically targeting secondary damage to alleviate the functional deficits has always been considered a potential treatment strategy. Neuroinflammation has been previously proved to be responsible for the deterioration of neurological injuries and the pathological process after SCI [7, 8]. As the markers of neuroinflammation after SCI [9, 10], the activated microglia subsequently release proinflammatory cytokines such as TNF-α which initiates neuronal apoptotic signals [11]. Therefore, the inactivation of microglia for the inhibition of neuroinflammation might be a promising strategy for the treatment of SCI.

Electroacupuncture (EA), a treatment that combines traditional acupuncture with modern stimulation therapy, stimulates the designated acupoints using acupuncture needles with different levels of current. EA has been successfully applied to alleviating pain as a physical therapy for centuries [12-15]. It has been reported that EA exerts a curative effect on attenuating SCI-induced limb spasms and alleviating neurological pain caused by SCI [16]. Accumulating evidence reveals that the therapeutic effects of EA are closely associated with its anti-inflammatory activity [17, 18]. Moreover, traditional Chinese medical theory believes that SCI-caused paralysis is fundamentally correlated with the damage to the *governor vessel*. But whether governor vessel EA therapy can ameliorate the neurological deficits after SCI

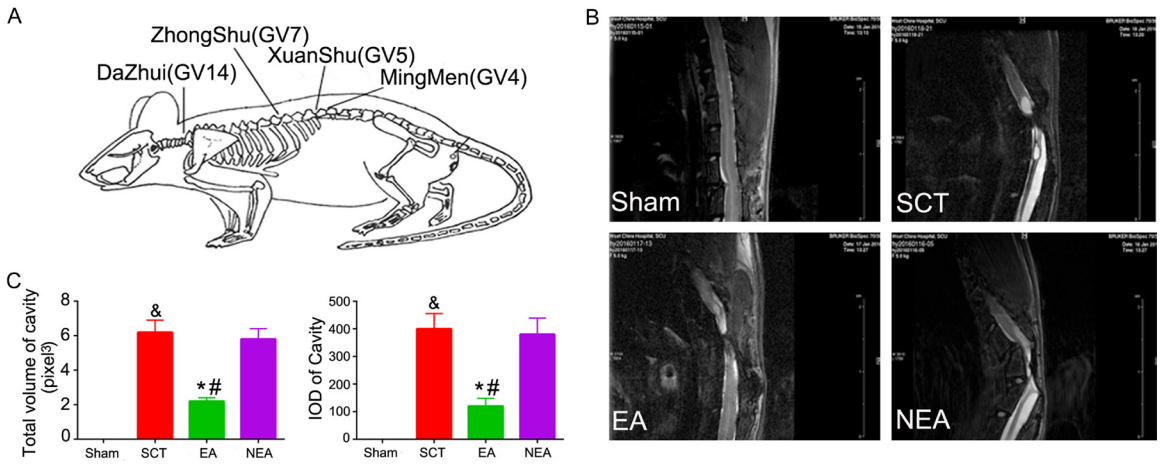

Figure 1. The effects of EA on the reconstruction of the injured spinal cords. A. The locations of the four acupoints selected for the EA stimulation. The black lines represent DaZhui (GV14), ZhongShu (GV7), XuanShu (GV5), and MingMen (GV4), respectively. B. The MRI T2 weighted images of the spinal cords at week 4 after the EA treatment in the sham, SCT, EA, and NEA groups. C. The total volume and IOD of the cavities in the rats in the sham, SCT, EA, and NEA groups at week 4 after SCT. The significance of the total volume and IOD of the cavity were analyzed with one-way ANOVA followed by an SNK post hoc analysis. MRI, magnetic resonance imaging; SCT, spinal cord transection; EA, Electroacupuncture; NEA, non-acupoints Electroacupuncture; IOD: integrated optical density. &P<0.05 vs the sham group; *P<0.05 vs the SCT group; #P<0.05 vs the NEA group; n = 6/group.

and the possible mechanism remains to be further elucidated [19].

The present study reports that EA therapy enhances neuronal regeneration and alleviates the neurological dysfunction caused by SCI. The underlying mechanism might be associated with the inhibition of microglia-mediated neuroinflammation. These findings provide strong implications that EA therapy could be conducive to SCI improvement.

Materials and methods

Subjects

Adult male Sprague-Dawley (SD) rats 6-8 weeks old (200-220 g) were obtained from the Center of Experimental Animals of Southwest Medical University (Luzhou, China). All the experimental operations conformed to the use of laboratory animals promulgated by the Ministry of Science and Technology of the People's Republic of China and were approved by the Animal Care and Use Committee of Southwest Medical University. The animals were housed in plastic cages with soft wood, accessing food and water ad libitum in a temperature-controlled room at 21-25°C with a 12 h light/dark cycle. They were randomly assigned into four groups: the sham group (rats subjected to laminectomy only), the SCT group (rats subjected to complete spinal cord transection), the EA group (SCT rats receiving EA treatment), and the NEA group (SCT rats receiving non-acupoints EA treatment), with 6 rats per group.

Establishment of the spinal cord transected (SCT) injury model

The spinal cord transection was performed as described previously [20]. Briefly, after inducing deep anesthesia in the rats using an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) provided by Southwest Medical University (Luzhou), the exposure of the T10 spinal cord followed. Then, a complete transection of the spinal cord at the T10 level was carried out using ophthalmic scissors. Subsequently, 1-2 mm spinal cord tissue was removed for separation to ensure a complete transection. The animals in the sham group underwent a laminectomy only without further spinal cord damage. After the surgery, all the rats received saturation in layers and daily disinfection with povidone-iodine solution.

Electroacupuncture (EA) treatment

The four acupoints of the governor vessel close to the T10 were chosen as follows: DaZhui (GV14); ZhongShu (GV7); XuanShu (GV5) and MingMen (GV4) (**Figure 1**). On the day after the surgery, the rats were immobilized in a prone

position on a board, and the stainless steel acupuncture needles (0.35 mm in diameter, 40 mm long, Huatuo; Suzhou Medical Appliance Manufactory, Jiangsu, China) were inserted approximately 5 mm deep at the above-mentioned acupoints. The needles were connected to the HANS electrical apparatus and stimulation with an intensity of 2 mA/2 Hz, was performed for 30 min once a day, with 7 days as a treatment course, and lasting for 4 weeks.

Spinal cord conduction velocity (SCCV)

After the SCT operation, SCCV was used to assess the conduction function of the spinal cord as measured by the BIOPAC electrophysiological detection system. Briefly, rats were injected intraperitoneally with 10% chloral hydrate (300 mg/kg) for anesthesia and immobilized in a prone position. Then, incisions of the T7 and L1 vertebrae were made to expose the spinal cord. A stimulating electrode and a recording electrode were connected to the spinal cord at L1 and T7, respectively, and the ground electrode was connected to the rat's tail. The parameters were set as follows: the stimulation waveform is a square wave pulse current (speed: 100 ms/D, sensitivity: 20 uv/D, filter: 2 Hz-2 KHz, width: 10 ms, frequency: 2 Hz, intensity: 0.005 V). The amplitude and latency of the SCCV were recorded by computer.

Paw withdrawal thresholds (PWT)

The rats' paw withdrawal thresholds were assessed using an ascending pressure stimulus with an analgesy-meter (UgoBasile, Comerio, Italy). Following the central region of the plantar surface of the rats, they were pricked using a wedge-shaped probe, and the operator turned on the system and increased the force. The scale of the force was recorded when the rat exhibited the withdrawal response. The final PWT data was ultimately defined as the average of the 3 effective withdrawal responses observed by the two operators blinded to the experiment's design.

Tail-flick latency (TFL)

For the tail-flick test, the rats were placed individually on an elevated iron mesh in a clear plastic cage for 30 min prior to the test. An infrared source was applied to the tail of each rat. The duration till the robust and immediate tail-flick responses to the stimulus were recorded and the upper limit was 22 s. The TFL tests were repeated three times consecutively with 5 min intervals, and the average of three teats was determined as the final data.

Magnetic resonance imaging (MRI) examination

The MRI examinations of the rats' spines were carried out using a 7.0 T magnetic resonance scanner (BrukerBiospec 70/30, Ettlingen, Germany). A 16 cm horizontal gantry and a 40 mm array surface coil were prepared. The anesthesia induction was performed quickly with the inhalation of 4% isoflurane, then the rats were fixed on the scanning bed and kept anaesthetized with a mixture of gases (2% isoflurane-oxygen/nitrogen (30:70). T2 images of the rats were obtained using the T2WI sequence (TR/TE = 2000/15 ms, NAverages = 4, FOV = 50 mm × 50 mm, RareFactor = 8). The total scan time was set at 4 min 16 s. The total volume and integrated optical density (IOD) of the cavity were used to assess the spinal cord injury.

Sample preparation

The rats were deeply anesthetized and quickly sacrificed. For the Western blot and ELISA experiments, the T9-T11 spinal cord segments were dissected and stored at -80°C for later use. For the immunofluorescence staining, the rats were perfused transcardially with 0.9% saline followed by fixation with 4% paraformaldehyde. Their spinal cords were quickly removed and fixed at 4°C overnight.

Immunofluorescence staining

After dehydration for 12 h in 30% sucrose at 4°C, the spinal cord segments were sectioned into 20 um slices. After being routinely de-paraffinized and rehydrated, the slices were blocked with 10% goat serum containing 0.3% Triton X-100 for 1 h at room temperature (RT), followed by incubation with rabbit anti-Iba-1 antibody (1:100; Abcam), anti-NeuN antibody (1:400; Abcam), anti-GAP-43 antibody (1:500; Abcam) overnight at 4°C. After being washed 3 times with PBS, the incubation with FITC-conjugated secondary antibody (1:1000, Abcam) was carried on at RT for 1 h. After being

rinsed 3 times with PBS again, they were subsequently counterstained with diamidino-2-phenylindole dihydrochloride (DAPI) (1:1,000; Solarbio Science and Technology Ltd., Beijing, China). Lastly, the images were taken using a fluorescence microscope (Leica, Germany). The integrated optical density (IOD) of Iba-1 was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Western blot (WB)

WB was performed to determine the protein expressions of Iba-1, Gap-43, and caspase-3 in the injured spinal cord. RIPA (Beyotime, Shanghai, China) was used for the total protein extraction from the spinal cord tissues. After centrifugation and the collection of the supernatants, the protein concentration determination was performed using bicinchoninic acid (BCA) method (Beyotime, Beijing, China). Next, the protein samples were loaded onto SDS-polyacrylamide gels (Beyotime, Shanghai, China), separated and electrically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blockage in 5% skim milk at 37°C for 2 h, the membranes were incubated with anti-Iba-1, anti-Gap-43, anti-caspase-3 and anti-β-actin monoclonal primary antibodies (rabbit, 1:1000, Abcam, USA) overnight at 4°C. After three rinses with TBST for 5 min each, the membranes were subsequently treated with HRP secondary antibody (anti-rabbit, 1:2000, ZSGB-BIO, ORIGENE, Beijing, China) for 2 h at RT. The images were developed using an ECL plus reagent and the intensities of the bands were analyzed using a Gel-Pol analyzer.

Enzyme-linked immunosorbent assay (ELISA)

The samples were harvested, lysed, and homogenized with a RIPA lysis buffer containing phenylmethylsulfonyl fluoride at 4°C. The supernatants were obtained after centrifugation followed by the concentration determination. The expressions of TNF-α and IL-6 were determined using ELISA kits (Shanghai Bridge Club, Shanghai, China) following the manufacturer's instructions. The standard curve was included in each experiment.

Statistical analysis

The data were analyzed using SPSS 17.0 software. The obtained data are presented as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used for the statistical evaluation of the data, followed by an LSD or SNK post hoc test. It was considered statistically significant when $P<0.05$.

Results

EA alleviates spinal cord injuries caused by SCT

The EA therapy was applied to the SCT rats starting the day after the operation, and four acupoints were chosen for the EA stimulation (**Figure 1A**). The protective effect of EA on the spinal cord injury was revealed using MRI (**Figure 1B**). After the EA treatment for 4 weeks, the total volumes of the cavities of the rats in the EA group were significantly reduced compared with the SCT and NEA groups (**Figure 1C**, $P<0.05$), In addition, the relative optical density of the cavity, which was used to evaluate the intensity of the edema in all the rats, showed a marked decrease in the EA rats in comparison to the rats in the SCT and NEA groups (**Figure 1C**, $P<0.05$). These findings demonstrate that EA treatment can significantly reconstruct the injured spinal cord and depress the deterioration of the spinal cord injury.

EA ameliorated the neurological functions in the rats subjected to SCT

To examine the effects of EA on the neurological dysfunction of the SCT rats, PWT and TFL tests at 1-4 weeks after the operations were performed. Compared with the sham group, the PAW withdrawal threshold and the tail flick latency in the SCT group displayed a sustained high level from week 1 to week 4 after injury. The data exhibited that the lower latency and the threshold were observed in the rats treated with EA therapy compared with the rats in the SCT and NEA groups (**Figure 2A**, $P<0.05$). Also, SCCV also was employed to evaluate the rats spinal cord conduction. As shown, the SCT operation prolonged the latency and decreased the SCCV of the rats. But the EA treatment for 4 weeks significantly shortened the latency and increased the SCCV of the SCT rats (**Figure 2B**, **2C**, $P<0.05$). However, there was no significant change of amplitude after the EA therapy. These revealed that EA can effectively prompt a recovery of the neurological deficits in rats after SCT.

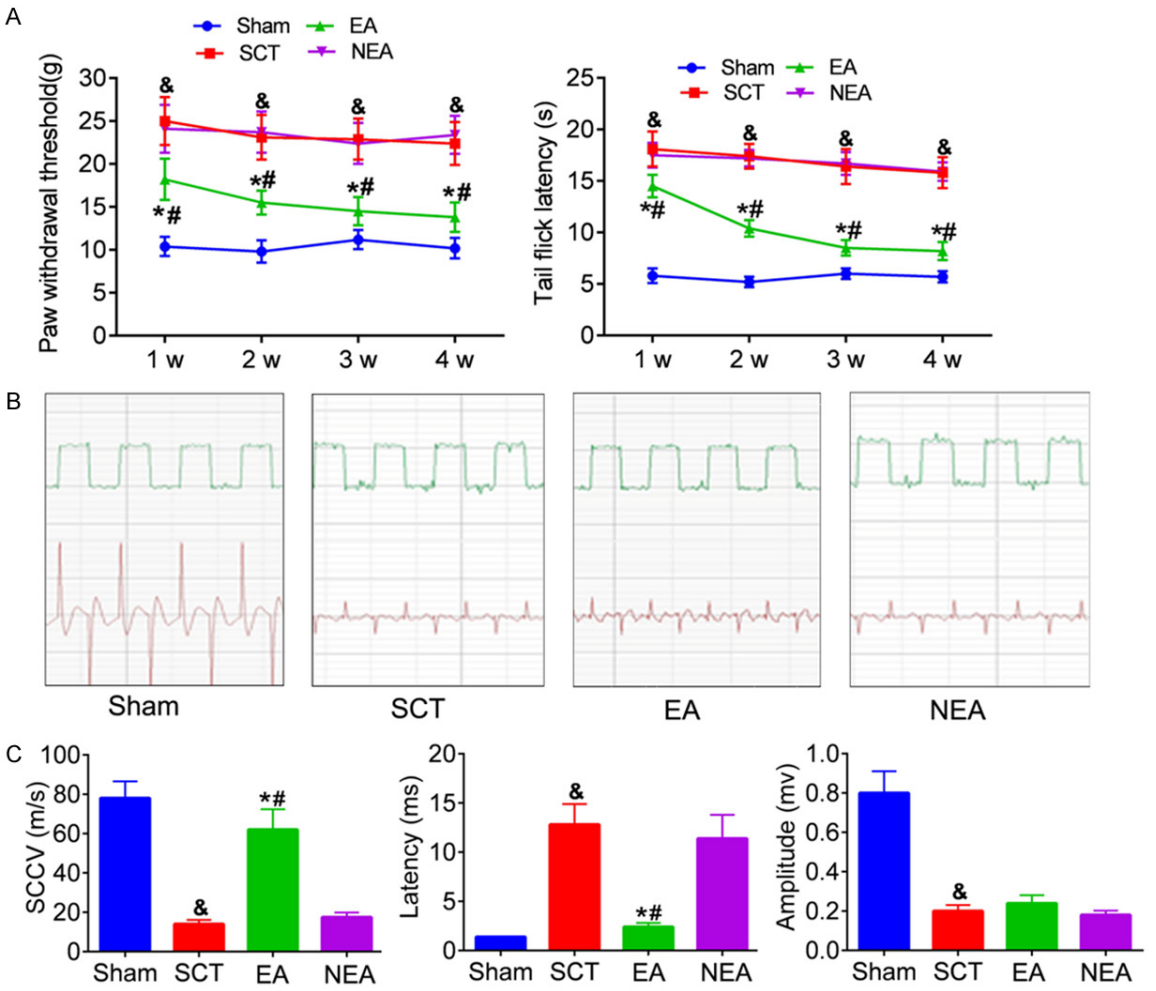

Figure 2. The effect of EA on the functional improvement of the SCT rats. A. The changes in PWT and TFL in the sham, SCT, EA, and NEA groups. B. SCCV waveforms in the sham, SCT, EA, and NEA groups at week 4 after SCT. C. The quantitative bar charts for SCCV, latency, and amplitude of the sham, SCT, EA, and NEA groups at week 4 after SCT. The significance of PWT and TFL were analyzed using two-way ANOVA followed by a Holm-Sidak post hoc analysis. The significance of SCCV was analyzed with one-way ANOVA followed by SNK post hoc analysis. PWT, paw withdrawal thresholds; TFL, tail-flick latency; SCCV, spinal cord conduction velocity; SCT, spinal cord transection; EA, Electroacupuncture; NEA, non-acupoints Electroacupuncture; W, week(s). &P<0.05 vs the sham group; *P<0.05 vs the SCT group; #P<0.05 vs the NEA group; n = 6/group.

The anti-neuroinflammatory effect of EA by inhibiting the activation of microglia

The further injury of the spinal cord is closely associated with the development of neuroinflammation in the injured spinal cord. It is well known that the activation of microglia is the main contributor of neuroinflammation. Therefore, the immunofluorescence staining and western blot of Iba-1 were used to determine the activation of microglia in the spinal cord. The fluorescence density and expression of Iba-1 in the spinal cord were significantly increased in the SCT group at postoperative week 4 in comparison to the sham group (**Figure 3A**, **3B**, P<0.05). After 4 weeks' treatment of EA, the fluorescence density and expression of Iba-1 was significantly decreased compared with the SCT and NEA groups (**Figure 3A**, **3B**, *P*<0.05). These concealed the inhibitory effect of the EA treatment on the activation of microglia induced by SCI. In order to further examine the anti-neuroinflammatory effect of EA, the concentration of proinflammatory cytokines in the spinal cord, such as IL-6, and TNF-α, were quantified using ELISA. The IL-6 and TNF-α levels in the SCT group were highly elevated from week 1 to week 4 after the opera-

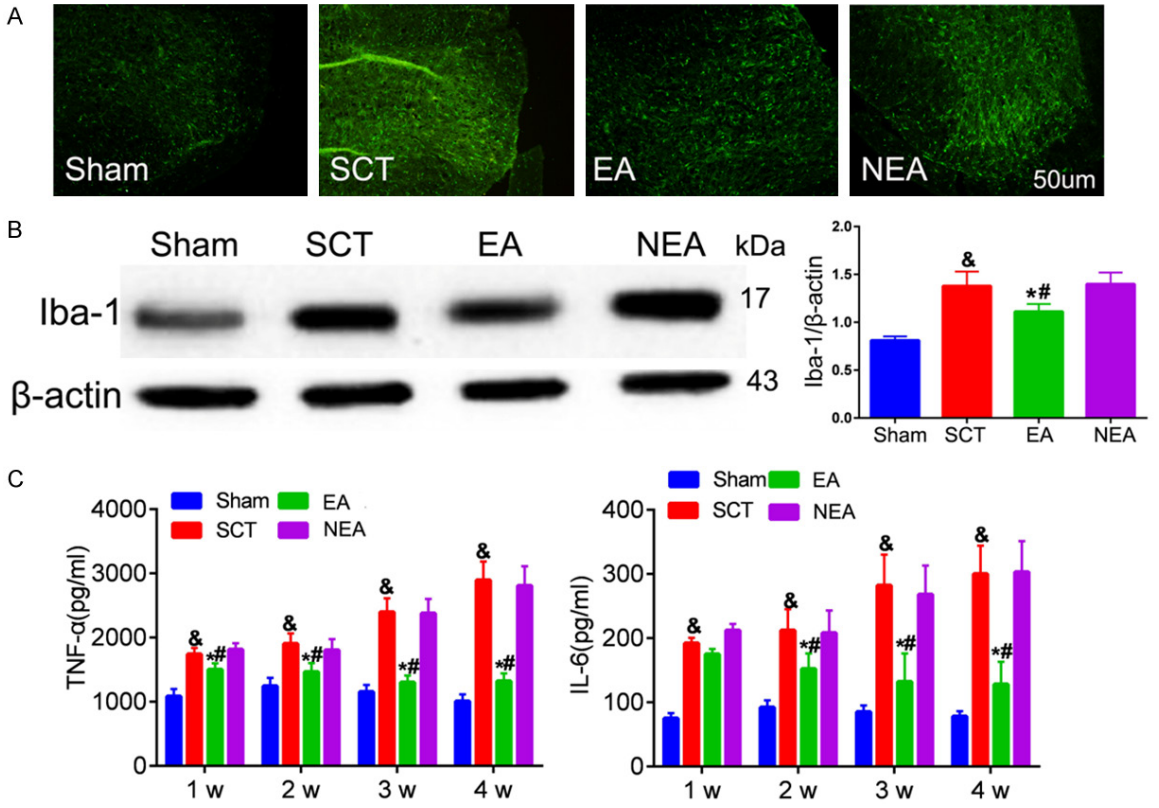

Figure 3. The role of EA in microglia and neuroinflammation. A. The representative immunostaining images show the changes of Iba-1 expression in the T9-11 spinal cord among the sham, SCT, EA, and NEA groups at week 4 after the operation. B. Western blot bands and the relative OD value of Iba-1 in the T9-11 spinal cord in the sham, SCT, EA, and NEA groups at week 4 after the operation. C. The levels of IL-6 and TNF-α in the T9-11 spinal cord of the sham, SCT, EA, and NEA groups. The significance of the relative OD value of Iba-1 was analyzed with one-way ANOVA followed by an SNK post hoc analysis. The significance of the levels of IL-6 and TNF-were analyzed using a two-way ANOVA followed by a Holm-Sidak post hoc analysis. SCT, spinal cord transection; EA, electroacupuncture; NEA, non-acupoints electroacupuncture; OD, optical density; W, week(s). &P<0.05 vs the sham group; *P<0.05 vs the SCT group; #P<0.05 vs the NEA group; n = 6/group.

tions compared with the sham group (**Figure 3C**, *P*<0.05). However, the upregulated proinflammatory cytokines were significantly suppressed by the EA treatment, as indicated by the decreased levels of IL-6 and TNF-α in the EA group compared with the SCT and NEA groups (**Figure 3C**, *P*<0.05).

EA promoted the neuronal regeneration and inhibited the loss of neurons induced by SCI

To determine the effect of EA on the neuronal regeneration of the SCT rats, immunofluorescence staining and Western blot of GAP-43 were performed. The significant up-regulation of GAP-43 in the EA-treated rats was evident when compared with the SCT and NEA groups (**Figure 4A**, **4B**, P<0.05). In addition, It was also found that neuronal loss and the upregulation of caspase-3 were notably induced after the SCT operation (**Figure 4C**, **4D**, P<0.05), which were significantly reversed after 4 weeks' treatment with EA (**Figure 4C**, **4D**, P<0.05). These points uncovered the neuronal growth and anti-apoptotic activities of the EA therapy in the SCI-induced neuronal damage.

Discussion

EA promotes functional improvement in SCT rats

Here, neuroanatomical recovery was determined using MRI. We quantified the cavities of the lesions and the severity of the edema using T2-weighted imaging. It is well known that continued compression caused by bleeding or edema of the trauma spinal cord will subse-

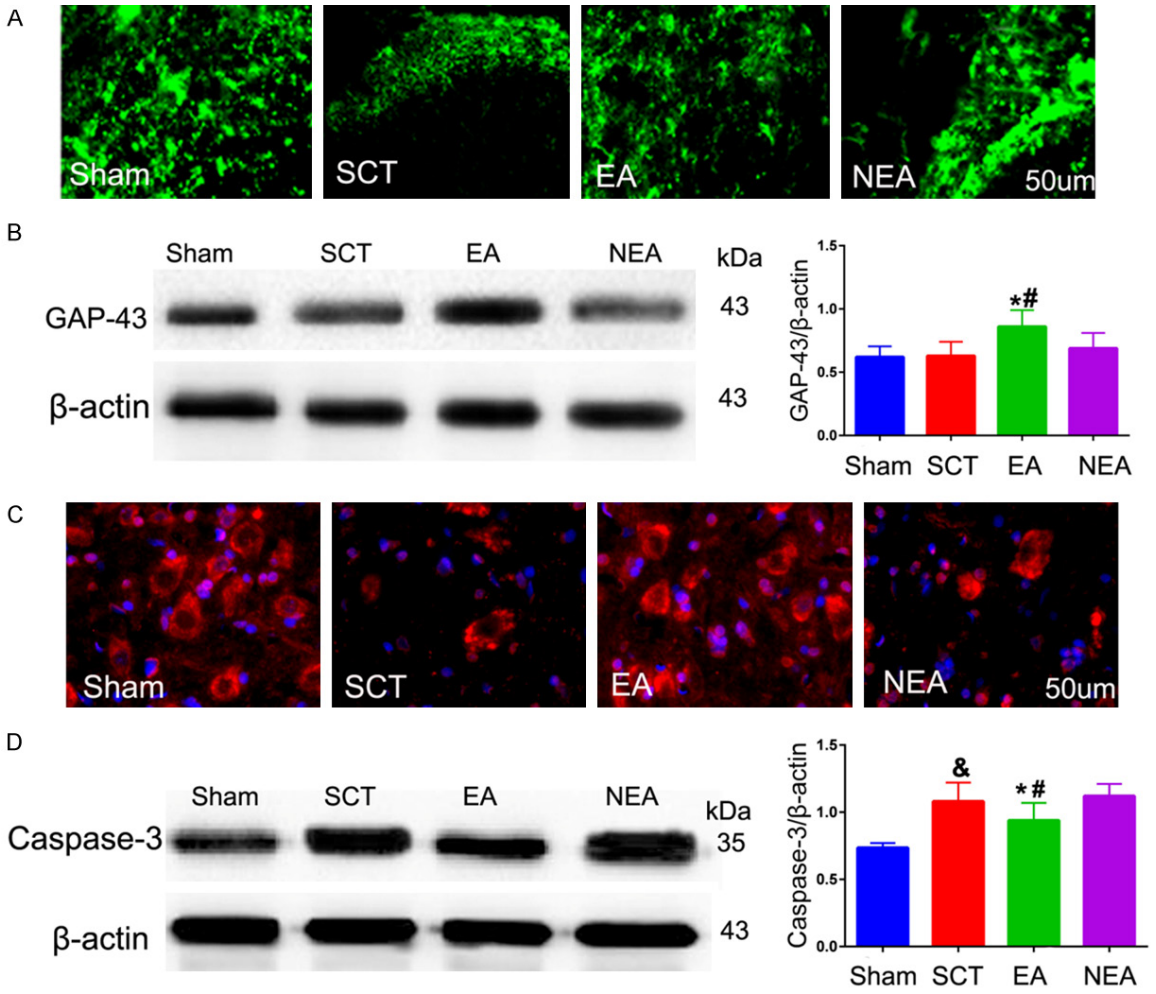

Figure 4. The effect of EA on neuronal regeneration and apoptosis. A. Representative immunostaining images show the changes of GAP-43 expression in the T9-11 spinal cord of the sham, SCT, EA, and NEA groups at week 4 after the operation. B. The Western blot bands and the relative OD Value of GAP-43 in the T9-11 spinal cord of the sham, SCT, EA, and NEA groups at week 4 after the operations. C. Representative immunostaining images show the changes in NeuN/DAPI expression in the T9-11 spinal cord of the sham, SCT, EA, and NEA groups at week 4 after the operation. D. Western blot bands and the relative OD values of caspase-3 in the T9-11 spinal cords of the sham, SCT, EA, and NEA groups at week 4 after the operation. The significance of the relative OD values of GAP-43 and caspase-3 were analyzed with one-way ANOVA followed by an SNK post hoc analysis. SCT, spinal cord transection; EA, electroacupuncture; NEA, non-acupoints electroacupuncture; OD: optical density. &P<0.05 vs the sham group; *P<0.05 vs the SCT group; #P<0.05 vs the NEA group; n = 6/group.

quently trigger a cascade of secondary injury such as GM necrosis, WM damage, and cyst formation [21]. Accordingly, by applying the EA stimulation at DaZhui (GV14), ZhongShu (GV7), XuanShu (GV5), and MingMen (GV4) for 4 weeks, the neuroanatomical analysis showed that the further disruption of the injured site was effectively inhibited in our study. The PWT and TFL tests were designed as methods to assess sensory function in rodents with neurological dysfunction [22-24]. PWT and TFL in the rats were carried out for 4 consecutive weeks, of which the results exhibited significant difference between the sham group and the SCT group, suggesting that the sensory dysfunction was closely associated with spinal cord injury. However, the outcomes of the PWT and TFL tests also demonstrated a significant improvement in the hypoalgesia in the EA group compared with the NEA and SCT groups. To further validate the positive effect of the EA treatment in the recovery of the injured spinal cord, the results of the SCCV revealed that the latency was shortened, and the SCCV was increased in

the SCT rats receiving EA treatment, indicating that EA therapy can effectively improve the spinal conduction in the SCT rats. Taken together, these results suggested that EA treatment significantly eliminated the spinal cord edema and promoted the reconstruction of spinal cord function in the SCT rats.

EA ameliorates the neuroinflammation in the SCT rats by inhibiting the activation of microglia

The pathogenesis of spinal cord injury is complicated, but the secondary injuries are aggravated by the detrimental response of glia activation as well as the infiltration of other inflammatory cells [25-28]. In response to the widespread injury, microglia transferred into stimulus-dependent phenotypes, and the activated microglia exhibited various responses, including morphological changes, innate transcriptional alterations, and proliferation [29, 30]. Consistent with the previous studies, we found that the microglia was significantly activated after SCI using immunofluorescence staining and western blot of Iba-1 (a biomarker of microglia) while they were depressed following the EA treatment. As reported in previous studies, the pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 released after SCI are considered the key factors in the secondary injury of SCI [31-33]. Accordingly, we demonstrated the related proinflammatory cytokines in the lesion parts, such as IL-6 and TNF-α were dramatically up-regulated and accompanied by microglial activation. As expected, EA stimulation at DaZhui (GV14), ZhongShu (GV7), XuanShu (GV5) and MingMen (GV4) significantly inhibited the activation of the microglia and the release of the corresponding proinflammatory cytokines. EA therapy has been regarded as a potential strategy for SCI for centuries, and it also shows significantly positive therapeutic effects on various diseases, including Alzheimer disease [34], ischemic stroke [35], hypertension [36], and pain [37], etc. Recent studies pointed out that EA regulates neuroplasticity [38], autophagy [39], and apoptosis [40], etc. In this study, the inhibition of microglial activation and thus the suppression of neuroinflammation by EA treatment were identified and verified.

EA promotes the neuronal regeneration of SCT rats

The reconstruction of the nerve connection at both ends of the lesion is the key to promoting the functional improvement in the upper and lower spinal cord after SCT [41, 42]. Neuronal apoptosis, the type most commonly observed in spinal cord injury, is closely associated with the functional recovery of the spinal cord [43, 44]. It has been demonstrated that EA treatment functions by suppressing the apoptosis of neurons through its anti-neuroinflammatory effect [45]. Likewise, our results revealed that EA can notably prevent the loss of neurons as shown by the immunofluorescence staining of NeuN and the Western blot of apoptosis-related caspase-3. Indeed, the present study showed that the neuronal loss and caspase-3 expression shared a common variation trend with the expression of Iba-1 and the release of proinflammatory cytokines. Moreover, we also measured the effect of EA on the neuronal regeneration reflected by the GAP-43 immunofluorescence staining. And the up-regulation of GAP-43 after the EA treatment indicated the positive effect of EA therapy on growth of neurites.

Conclusion

Collectively, the present study shows that EA treatment promotes the neurological functional improvement of SCT rats and, dramatically suppresses the activation of microglia and the development of neuroinflammation. Additionally, EA treatment attenuates the SCI-induced neuronal apoptosis and enhances neuronal regeneration. Our findings provide a strong rationale for pursuing EA treatment to alleviate neurological deficits after SCI.

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Disclosure of conflict of interest

None.

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