Electroacupuncture promotes the proliferation of endogenous neural stem cells and oligodendrocytes in the injured spinal cord of adult rats

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Abstract
A contusive model of spinal cord injury at spinal segment T8-9 was established in rats. Huantiao (GB30) and Huatuojiaji (Ex-B05) were punctured with needles, and endogenous neural stem cells were labeled with 5-bromo-2'-deoxyuridine (BrdU) and NG2. Double immunofluorescence staining showed that electroacupuncture markedly increased the numbers of BrdU+/NG2+ cells at spinal cord tissue 15 mm away from the injury center in the rostral and caudal directions. The results suggest that electroacupuncture promotes the proliferation of endogenous neural stem cells and oligodendrocytes in rats with spinal cord injury.

Key Words
endogenous oligodendrocyte progenitor cells; spinal cord injury; electroacupuncture; proliferation; remyelination; neural regeneration

INTRODUCTION
Demyelination (loss of myelin) and dysmyelination (abnormal myelination) are important contributors to the behavioral deficits associated with models of spinal cord injury (SCI)[1-5]. Oligodendrocyte injury and death play a substantial role in the demyelination/dysmyelination that follows SCI. Although transplantation of stem cell-derived oligodendrocyte progenitor cells (OPCs) may repair the spinal cord, limitations include difficulty in procuring fetal tissue, histocompatibility and ethical concerns for clinical use[4]. Thus, proliferation of endogenous OPCs and their subsequent differentiation into mature myelinating oligodendrocytes are important for remyelination[6-10]. Although some spontaneous remyelination occurs after SCI, it is insufficient to prevent or repair long-term neurological disability. Therefore, augmenting this intrinsic response has possible therapeutic potential. Electrical stimulation has been shown to promote development of OPCs, and subsequent myelination of adjacent dorsal root ganglion axons and neighboring axons in vitro[11-14]. Previous in vitro and in vivo studies have shown that electrical stimulation increases the number of spikes conducted in axons and promotes axonal release of some molecules, such as glutamate[15].
platelet-derived growth factor\(^{[16]}\), adenosine triphosphate\(^{[17-18]}\), and brain-derived neurotrophic factor\(^{[19]}\). These molecules in turn promote the proliferation and differentiation of OPCs, and remyelination of neural axons\(^{[20-22]}\).

Acupuncture was developed in China in around 2000 B.C., and is practiced worldwide\(^{[23]}\). To date, it has already been used to treat SCI in both a clinical setting and in research studies. During electroacupuncture, needles inserted into acupuncture points are attached to a trace pulse current to produce electrical stimulation to alleviate the secondary damage after SCI in both patients and animal models\(^{[24-25]}\). However, the potential mechanism by which electroacupuncture regulates endogenous neural stem cells (eNSCs) after SCI remains unclear. In the present study, we investigated the effects of electroacupuncture on the differentiation of OPCs and on functional recovery of the injured spinal cord.

**RESULTS**

**Quantitative analysis of experimental animals**

Thirty specific pathogen-free adult Sprague-Dawley rats were used to establish the contusion SCI model at spinal segment T\(_9\). The SCI rat models were randomly and evenly divided into SCI and electroacupuncture groups (\(n = 15\) per group). One week after contusive SCI, the electroacupuncture group received electroacupuncture treatment at Huantiao (GB30) and Huatuojiaji (Ex-B05), but the SCI group did not. A total of 30 rats were involved in the final analysis.

**Numbers of 5-bromo-2’-deoxyuridine (BrdU)-positive cells in rats with SCI following electroacupuncture treatment**

BrdU-labeled eNSCs were readily identified by Cy3 immunofluorescent stained nuclei within the parenchyma of all selected spinal cord tissue 4 mm and 15 mm away from the injury center in the rostral (R) and caudal (C) directions. Repeated measures analysis of variance indicated that the number of BrdU-positive cells in rats from the electroacupuncture group was more than those in the SCI group at the spinal level (R-15 mm, and C-15 mm: spinal cord tissue 15 mm away from the injury center in the rostral (R-15 mm) and caudal (C-15 mm) directions) (Figure 1D; \(P < 0.01\); paired sample \(t\)-test). There was no significant difference between R-4 mm and C-4 mm (Figure 1D; \(P > 0.05\)).

**Electroacupuncture intervenes with the proliferation of oligodendrocytes and astrocytes in the rat spinal cord following SCI**

To investigate the phenotypic fate of newborn stem/progenitor cells, oligodendrocytes were identified using NG2. Number of BrdU\(^+\)/NG2\(^+\) cells in the electroacupuncture group was significantly greater than those in the SCI group at remote segments (R-15 mm and C-15 mm) from injury center (\(P < 0.01\); Figure 2).

In double immunostained sections, abundant numbers of BrdU-positive cells expressed glial fibrillary acidic protein (GFAP), a marker for both immature and mature astrocytes. No significant differences in BrdU\(^+\)/GFAP\(^+\) cells at remote segments from the injured spinal cord (R-15 mm, C-15 mm) were detectable between the electroacupuncture and SCI groups (\(P > 0.05\); Figure 3).

**Behavioral changes in rats with SCI following electroacupuncture treatment**

After spinal cord contusion, all rats were paralyzed and moved by pulling themselves forward with their forelimbs. The Basso, Beattie and Bresnahan (BBB) score was higher in the electroacupuncture group than in the SCI group at 2–4 weeks following SCI (\(P < 0.05\), especially at 4 weeks (\(P < 0.01\)).
It has been shown that electroacupuncture at SCI group. It has been shown that electroacupuncture at significant therapeutic direction. augmenting this intrinsic response is a potentially to the clinical use of stem cell-derived OPCs. Therefore, limitations inherent in the procurement of fetal tissue, cord. In addition, electroacupuncture also avoids the and functional electrical stimulation to repair the spinal development of endogenous OPCs. Furthermore, treatment for SCI, has a similar action on the electroacupuncture, a traditional Chinese method of In the present study, we confirmed that Proliferation of endogenous OPCs by electroacupuncture. BrdU: 5-bromo-2′-deoxyuridine; R: rostral; C: caudal.

DISCUSSION

Proliferation of endogenous OPCs by electroacupuncture in the injured spinal cord

In the present study, we confirmed that electroacupuncture, a traditional Chinese method of treatment for SCI, has a similar action on the development of endogenous OPCs. Furthermore, electroacupuncture is cheaper and results in less pain compared with transplantation of stem cell-derived OPCs and functional electrical stimulation to repair the spinal cord. In addition, electroacupuncture also avoids the limitations inherent in the procurement of fetal tissue, including histocompatibility and ethical concerns related to the clinical use of stem cell-derived OPCs. Therefore, augmenting this intrinsic response is a potentially significant therapeutic direction.

It has been shown that electroacupuncture at Huantiao and Huatuojiaji significantly increases proliferation of eNSCs and OPCs. The number of BrdU⁺ cells in the electroacupuncture group was significantly greater than the SCI group.

**Figure 2** EA promoted the proliferation of oligodendrocytes in the rat spinal cord following SCI. NG2⁺/BrdU⁺ cell distribution (arrows in A and B, EA group) and morphology (C–E) in the EA group was consistent with glial progenitor cells. (C) NG2⁺ cell body. (D) BrdU⁺ nucleus. (E) merged. Panels C–E show NG2 staining (C; NG2⁺ cell body, green), BrdU staining (D, BrdU⁺ nucleus, red), and NG2 and BrdU co-localization (E).

(F) EA promoted the proliferation of oligodendrocytes (NG2⁺/BrdU⁺). Data are expressed as the mean ± SD of 15 rats in each group. There were more NG2⁺/BrdU⁺ cells further away (R-15; C-15) from the injury level in the EA group (one-way analysis of variance; *p* < 0.01).

Scale bars (A, B): 100 μm, (C–E): 10 μm.

X-axis: spinal cord tissue 15 mm from the injury center in the rostral and caudal directions (the dotted line represents the SCI position). SCI: Spinal cord injury. EA: electroacupuncture. BrdU: 5-bromo-2′-deoxyuridine; R: rostral; C: caudal.

**Figure 3** EA did not significantly increase the number of BrdU⁺/GFAP⁺ cells in rat spinal cord following SCI. (A) GFAP staining of GFAP⁺/BrdU⁺ cell distribution; (B) BrdU staining of GFAP⁺/BrdU⁺ cell distribution (arrows in A and B, and morphology (C–E) in the EA group was consistent with astrocytes. Panels C–E show GFAP staining (C; green), BrdU staining (D; red), and BrdU and GFAP co-localization (E). (F) There was no significant difference in the number of GFAP⁺/BrdU⁺ cells further away from the injury site, although EA promoted the number of GFAP⁺/BrdU⁺ cells (one-way analysis of variance; *p* > 0.05). Data are expressed as the mean ± SD of 15 rats in each group. Scale bars (A, B): 100 μm, (C–E): 10 μm.

X-axis: spinal cord tissue 15 mm away from the injury center in the rostral and caudal directions (the dotted line represents SCI position). SCI: Spinal cord injury; EA: electro-acupuncture; BrdU: 5-bromo-2′-deoxyuridine; GFAP: glial fibrillary acidic protein; R: rostral; C: caudal.

**Figure 4** Motor function of the hindlimb was analyzed using the BBB open field test. High BBB scores represented better motor function.

The scores gradually increased over time in each group. BBB scores were significantly higher in the EA group as compared with the SCI group from 2 to 4 weeks.

*a* < 0.05, *b* < 0.01, vs. SCI group. Data are expressed as the mean ± SD of 15 rats in each group (one-way analysis of variance). SCI: Spinal cord injury; EA: electroacupuncture; BBB: Basso, Beattie and Bresnahan.
Some of these cells were confirmed as NG2+ OPCs, suggesting that electroacupuncture increased the number of putative myelinating glia in the spinal cord. We used double labeling (BrdU/GFAP) and confocal microscopy to rule out the possibility that increased NG2 expression was due to reactive astrogliosis caused by SCI or electroacupuncture. The present findings provide evidence for an effect of electroacupuncture on oligodendrogenesis, and showed that electroacupuncture did not promote an increase in GFAP protein levels following SCI. In other words, electroacupuncture did not significantly affect astrogliosis in the SCI group. Immature oligodendrocytes were present both dorsally and ventrally in the outer circumference of the spinal cord in the electroacupuncture group. It is possible that these immature oligodendrocytes were migrating outwards to reach the ventrodorsal regions26-27. Electrical activity, by stimulating the motor fibers and circuits in the spinal cord, may be the cause of the increase in immature oligodendrocytes in the border of the ventrodorsal areas. However, the molecular and cellular mechanisms of these results are still under investigation.

Implications for recovery after SCI
This report provides the first demonstration that electroacupuncture can enhance cell generation in the injured adult rat. The dramatic effect of electroacupuncture on endogenous cell regeneration in the injured spinal cord may partially mediate such recovery. The results demonstrate the need for further studies on activity-based recovery/restoration therapies in animal models and for individuals with SCI, given that the physical benefits of active electroacupuncture exercise are a sufficient rationale for treatment initiation. These results raise the exciting possibility that in addition to the physical and rehabilitative benefits of electroacupuncture, electroacupuncture may offer a pragmatic approach to augmentation of spontaneous repair and perhaps recovery of neurological function. In summary, the present experiments show that electroacupuncture increased BrdU-labeled eNSCs and promoted the proliferation of OPCs, but not astrocytes. Thus, our data suggests that electroacupuncture may enhance spontaneous regeneration after neurological injuries, and that electroacupuncture could be used to promote remyelination and recovery of function after SCI.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
This study was performed at Yunnan Province Key Laboratory, Neurosciences Institute of Kunming Medical University, Kunming University, China, between May and October 2011.

Materials
Thirty specific pathogen free adult Sprague-Dawley rats weighing 225–250 g were provided by the Experimental Animal Center, Kunming Medical University, China (SCXK (Dian) 2011-0004). Rats were housed in a 12-hour light/dark cycle room at 23 ± 2°C with free access to food and water. Prior to experimental manipulation, rats were allowed to acclimatize to the housing facilities and were handled daily at least for 3 days. All experimental protocols and animal handling procedures were approved by the Animal Care and Use Committee of Kunming Medical University, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People’s Republic of China29.

Methods

Establishment of the rat SCI model
All animals underwent contusion SCI injury at spinal level T929-30. The hindlimb locomotor activity of rats in both groups was evaluated by the BBB open field test at five time points between 1 day and 4 weeks post-lesion. Bladders of all SCI animals were pressed three times a day until recovery of reflex urination. All rats were handled for 5 minutes per day and housed individually with absorbent bedding. Animals were anesthetized with 75 mg/kg ketamine and 0.6 mg/kg medetomidine intraperitoneally (i.p.) to provide sufficient analgesia. A dorsal laminectomy was performed at the T9-10 vertebra level. The dorsal surface of the T9 spinal cord was exposed (keeping the dura intact), and subjected to an Infinite Horizon Impactor (Precision System, Kentucky) with a force of 200 kdyn. A piece of Gelform was placed on the surface of the injured cord to protect the tissue. Muscle and skin incisions were closed with silk sutures and wound clips, respectively. Successful models were demonstrated by rats exhibiting the following traits: subdural hemorrhage; transient contraction of lower limbs, and head and neck muscles several times; and severe swing of rat tail for several seconds. This injury was moderate and caused incomplete paraplegia in rats. After surgery, animals were given 0.06 mL antisedan intramuscularly to reverse the effects of the anesthetic, and placed in a heating
incubator (36.5°C) until full recovery was achieved. Animals also received a subcutaneous injection of 15 mL sterile saline and 0.05 mL of gentamycin.

**Electroacupuncture treatment**

Two pairs of acupoints were utilized during the electroacupuncture treatment: **Huantiao** (located laterally 1/3 and medially 2/3 of the distance between the sacral hiatus and greater trochanter of the femur, corresponding to the sciatic nerve) and **Huatuojiaji** (3 mm lateral to the midline of the back at the T₆ level, corresponding to the dorsal ramus of the T₆ spinal nerve). Two pairs of needles (0.5 in. long) connected to the output terminals of a Multi-Purpose Electrical Stimulator (KWD-808-I, Jiangsu, China) were inserted into **Huantiao** and **Huatuojiaji**. Stimulation parameters were: current intensity 3 mA, alternative dense wave 100 Hz/2 s and disperse wave 2 Hz/2 s, with a needle depth 5 mm, vertical to the skin plane. During electroacupuncture, animals remained awake and showed no observable signs of distress or pain. Control animals stayed in similar cages without acupuncture.

**BrdU injection for labeling eNSCs and BBB behavioral analysis to monitor motor function of the hindlimb**

A week after contusive SCI, electroacupuncture was delivered twice a day (each session was 30 minutes) for 3 weeks. BrdU (Shanghai Boshang Biotechnology, Shanghai, China) was injected (i.p.) daily during the last week of the electroacupuncture treatment and survival period. Animals received daily injections of BrdU (0.1 M in saline, 50 mg/kg; i.p) during the last week of survival (7 days). The BBB locomotors rating scale[31] was used to analyze and scoring of behaviors involving the trunk, tail and hindlimb. Each session lasted 4 minutes. Scores from both examiners were averaged for each rat. Scores ranged from 0 to 21 (0, no movement; 21, normal movement). The second test qualitatively assessed the accuracy of foot placement and coordination, which differentiated local reflex activity from voluntary movement. All animals underwent behavioral testing every week post-surgery for 4 weeks. Both examiners were blind to each group when they participated in behavioral evaluation.

**BrdU+/NG2+ and BrdU+/GFAP+ immunocytochemistry staining**

At the end of week 4, following behavioral testing, all animals were deeply anesthetized with sodium pentobarbital and then perfused intracardially with 200 mL saline with 50 units heparin. This was followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffered saline. After perfusion, spinal cord tissues were fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2–3 days. Frozen transverse sections were cut at 40 μm and stored at –20°C with cryoprotective solution for BrdU immunocytochemistry. The sections were incubated in 2N HCl at 37°C for 60 minutes and rinsed twice in 0.1 M borate buffer (pH 8.5; 15 minutes for each). After 2 × 10 minutes washes in PBS, nonspecific antigen binding was blocked in solution containing 0.1 M PBS, 10% (v/v) normal goat serum, and 0.1% (v/v) Triton X-100 for 1 hour. Double-fluorescent immunolabeling was performed using rat anti-mouse BrdU (1:600; Chemicon, OX51GE, UK) with rabbit anti-rat NG2 polyclonal antibody (Immunoreactivity purified NG2 Chondroitin Sulfate Proteoglycan from rat; 1:200; Chemicon, MA, USA) or rabbit-antibovine polyclonal antibody (GFAP isolated from bovine spinal cord; 1:4; ImmunoStar, WI, USA). Sections were incubated in primary antibodies at 4°C for 48 hours. After 3 × 10 minutes rinsing with PBS, Cy3-conjugated AffiniPure goat anti-rat IgG (red; 1:500; Jackson ImmunoResearch, PA, USA) was used to stain BrdU+ nuclei, and goat anti-mouse IgG conjugate (green; 1:500; Molecular Probes, NY, USA) was used to stain the cytoplasm (NG2, GFAP). After 3 × 10 minutes rinsing in PBS, sections were mounted onto slides, allowed to half-dry, and coverslipped using antifade reagent.

**Confocal microscopy and quantification of BrdU-positive cells**

The numbers of BrdU-labeled cells in the spinal cord were quantified as an index of “newly born cells”, and the numbers of BrdU-labeled cells co-labeled with the phenotypic markers were also monitored. Scanning laser confocal microscopy was used to identify double-labeled cells. Cells in 40 μm frozen sections were quantified using an unbiased optical fractionator method with the assistance of a semiautomatic stereology system (Stereoinvestigator™, Microbrightfield Inc., Brattleboro, VT, USA) driving a Ludi X-Y motorized stage (Ludi Electronics Products, Ltd., Hawthorn, NY, USA). Fluorescent images were acquired with a Magnafire camera. Rough boundaries of each cord cross-section were drawn under low magnification. Samples were then marked under higher magnification (20×; UplanApo, 0.8N.A.). The software randomly superimposed a sampling grid with an unbiased counting frame (X = 122 μm, Y = 110 μm) and moved in a raster scanning.
pattern to provide 12–15 counting frames within the cross-section of the cord. The top of the section was marked and a guard focus height of 2 μm was set using the software. The optical thickness of each image was approximately 0.7 μm and the total optical dissector height was 20 μm. Cells meeting the sampling criteria were marked interactively during the session. For each BrdU-positive cell, the complete cell nucleus was followed through the Z-axis, and only cells with a well-circumscribed, labeled nucleus and/or immunopositive cell body contained entirely within the counting volume frame were considered positive. Based on the numbers of marked cells and the volume of the slices, the software (Sigma Stat v3.5, Baltimore, MD, USA) calculated the total numbers and densities of BrdU$^+$ nuclei. Six slices per spinal level were quantified per animal in each procedure. The mean value of each animal was calculated, and the final results were used in statistical analyses. To avoid the confusion associated with immunolabeled cells within and immediately surrounding the SCI site, we performed our analyses on spinal levels more distant from the injury, where tissue necrosis and immune cell infiltration were minimized, and where the total numbers of cells and cord circumference were unchanged from uninjured conditions.

**Statistical analysis**
The data were statistically analyzed used SigmaStat v3.5 software (Baltimore, MD, USA) and were expressed as the mean ± SD. Comparison between experimental groups was conducted by one-way analysis of variance and repeated measures analysis of variance. Comparisons were conducted for the total number of BrdU$^+$ cells and phenotypic markers between groups and within groups at the spinal cord level. For all analyses, significance was accepted at $P < 0.05$ with Bonferroni correction for multiple tests.

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**Conflicts of interest:** None declared.

**Ethical approval:** All surgical interventions and postoperative animal care followed the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committees of Kunming Medical University, China.

**REFERENCES**


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