Recovery of spinal cord injury following electroacupuncture in rats through enhancement of Wnt/β-catenin signaling

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Abstract. Electroacupuncture (EA) has been demonstrated to promote the functional recovery of neurons following spinal cord injury (SCI); however, the mechanisms underlying its effects have yet to be elucidated. The Wnt/β-catenin signaling pathway has been implicated in the regulation of the balance between growth, proliferation and differentiation of neural precursor cells. The present study aimed to investigate the effects of EA therapy on Wnt/β-catenin-regulated gene expression and neuronal recovery in rats with SCI. The Allen method was used to establish SCI in rats, and alterations in Wnt1 and Nestin mRNA and protein expression levels in response to SCI were determined on days 1, 3, 7 and 14 post-injury using reverse transcription-quantitative polymerase chain reaction and western blot analysis. To evaluate the effects of EA treatment on SCI, the following four treatment groups were employed: SCI, SCI + EA, SCI + lithium chloride (LiCl) and SCI + LiCl + EA. The protein expression levels of Wnt1, Nestin and nuclear β -catenin were evaluated on day 3 post-treatment, and neuronal nuclear antigen (NeuN) protein expression levels were evaluated on day 21 post-treatment using western blot analysis. The Basso, Beattie and Bresnahan scoring method was used to evaluate spinal cord recovery on day 28 post-treatment across the four treatment groups. EA therapy at the Dazhui and Mingmen acupuncture points significantly increased the expression levels of Wnt1, Nestin, β-catenin and NeuN, thus suggesting that EA therapy may promote spinal cord recovery following injury. The underlying mechanism was demonstrated to involve enhanced Wnt/β-catenin signaling, which may promote the proliferation and differentiation of neural stem cells. However, further studies are required to elucidate

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the detailed effects and underlying molecular mechanisms of EA therapy on SCI.

Introduction

Spinal cord injury (SCI) is a highly disabling insult of the central nervous system (CNS), which can be caused by traffic accidents, falls, violence or sports-related injuries. It can result in the damage of sensory and motor function, autonomic nervous dysfunction, and can have effects on the mental health of patients (1). Recovery following SCI is difficult, due to the complexity of injury pathogenesis, which can include primary and secondary injuries (2). The secondary injury can last for several weeks or months following the initial spinal cord insult, and disturbances in microcirculation (3), neuronal apoptosis and necrosis (4-6), inflammatory response (7) and glial scar (8) may appear during this period. These complications result from neuronal damage, demyelination of axons and dysfunction of neuroglial cells, as the CNS is characterized by limited capability for repair and regeneration (9).

The Wnt/β-catenin signaling pathway has been identified as a crucial regulator of the growth and differentiation and survival of neurons (10). Wnt proteins are a large family of signaling proteins, which have been implicated in the regulation of axonal growth, including the long axons of spinal cord neurons (10). β -catenin is a critical component of the canonical Wnt signaling pathway, which, when activated, can translocate to the nucleus, where it can interact with transcription factors to induce alterations in the expression of target genes (11). The neuronal nuclear antigen (NeuN) is a protein specifically expressed in post-mitotic neurons. NeuN has been used as a marker of maturing neurons, and has been applied in neuropathological studies to investigate neuronal pathophysiology, as healthy neurons are characterized by strong NeuN expression, whereas weak NeuN expression in indicative of degeneration of differentiated neurons (12).

Novel therapeutic approaches are currently being developed for the treatment of patients with SCI, including stem cell therapy (13,14), and electroacupuncture (EA) treatment (15,16). Acupuncture has a long history of use in traditional Chinese medicine, and EA has been reported to promote the proliferation and differentiation of neuronal stem cells (17). Several acupuncture points for EA therapy have been evaluated for their effects on SCI recovery. Choi *et al* (18) reported that

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acupuncture targeted at Shuigou (GV26) and Yanglingquan (GB34) points significantly alleviated neuronal apoptosis and enhanced their recovery following SCI. Jiang *et al* (16) compared different modalities of acupuncture at Shuigou (DU26) and Fengfu (DU16) points, and reported anti-inflammatory, antioxidative and anti-apoptotic effects for EA.

Although EA has garnered much attention as a potential therapeutic strategy for the treatment of patients with SCI, its use remains limited, as the mechanisms underlying its beneficial effects have yet to be elucidated. Therefore, the present study aimed to investigate the effects of EA therapy on SCI, and explore the involvement of the Wnt/ β -catenin pathway in the molecular mechanisms underlying EA-associated neuronal recovery.

Materials and methods

Animal experiments. Specific pathogen-free male Sprague-Dawley (SD) rats (age, 8 weeks; weight, 250 ± 20 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All rats were acclimated in sterile polypropylene cages under an ambient temperature of $25\pm2^{\circ}$ C, a relative humidity of 50-60% and a 12/12 h- dark/light cycle with free access to food and water. Following 1 week of acclimation, rats were randomized into experimental groups. The study was approved by the Ethics Committee of The Sixth People's Hospital Affiliated to Shanghai Jiaotong University (Shanghai, China).

After acclimation, 54 rats were randomly assigned to three groups (n=18/group): The SCI group, which included rats that underwent injury at the T9-T11 spinal segment; the sham group, which included rats that received a laminectomy; and the normal group, which included rats that were left untreated. SCI group rats were randomly assigned into day 1, 7 and 14 subgroups (n=6/group). Rats in the SCI, sham and normal groups were sacrificed on days 1, 3, 7 or 14 post-SCI surgery, and spinal cord tissue samples were obtained to assess Wnt1 and Nestin expression.

In EA experiments, 96 rats received SCI surgery and were then randomized into four groups (n=24/group): The SCI group, which included rats that were left untreated following SCI surgery; the SCI + EA group, which included rats that received EA treatment lasting 20 min at Dazhui (GV14) and Mingmen (GV4) acupoints every day for 2 weeks following SCI surgery; the SCI + LiCl group, which included rats that received an injection of 0.05 ml lithium chloride (LiCl) (1 mol/l) at the injured spinal segment every 3 days for 2 weeks following SCI surgery; and the SCI + LiCl + EA group, which included rats that received a LiCl injection and EA treatment for 2 weeks following SCI surgery. Tissue samples from the area of SCI were collected from 9 rats in each group and were used to assess the expression of Wnt1, nuclear β-catenin and Nestin on day 3 of treatment. Tissue samples were collected from another 9 rats in each group and were used to assess the expression of Neuronal Nuclei (NeuN) on day 21 of treatment. The remaining 6 rats in each group were used to evaluate the Basso, Beattie and Bresnahan (BBB) score on day 28 of treatment.

SCI. Moderate SCI was established using the modified Allen method, as previously described (19). Briefly, rats were

anesthetized with 10% chloral hydrate (3.5 ml/kg, intraperitoneally), then a laminectomy was performed at the T9-T11 level and the spinal cord was exposed without disrupting the dura. Spinous processes of T9 and T11 were stabilized by clamps, and the exposed dorsal surface of the spinal cord was subjected to contusion injury. The force of contusion was 40 g x cm and was generated by a free drop, using Allen's impactor (Peking Union Medical College Microcirculation Institute, Beijing, China). Rats in the sham group only received laminectomy without further intervention. In order to avoid direct contact of the spinal cord wound with air, the wound was covered with cotton soaked in saline. All surgical interventions and postoperative animal care were in line with the guidelines and rules provided by The Sixth People's Hospital Affiliated to Shanghai Jiaotong University.

EA therapy. Rats belonging to the SCI + EA and SCI + LiCl + EA groups received EA therapy lasting 20 min each day for 2 weeks. The acupuncture points GV14 (Dazhui) and GV4 (Mingmen) were selected on the basis of clinical acupuncture experience and were located as described in Fig. 1. Stainless steel Hwato brand acupuncture needles (0.3x25 mm; Suzhou Medical Appliance Factory, Suzhou, China) were inserted at the selected points to a depth of 5-7 mm and were connected to the output terminals of a G6805-2 EA apparatus (Shanghai Medical Equipment Works Co., Ltd., Shanghai, China). An operating frequency of 2 Hz and a working current of 1 mA were used.

Reverse transcription-quantitative polymerase chain reaction (*RT-qPCR*). Following the last EA treatment, rats were deeply anesthetized with 10% chloral hydrate and sacrificed. T9-T11 spinal cord segments, containing the injury sites, were dissected and maintained at -80°C until used.

Total RNA was extracted from spinal cord samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration of extracted RNA was determined using an ultraviolet spectrophotometer. Total RNA (500 ng) was reverse transcribed into cDNA using PrimeScript[™] RT reagent (Takara Bio, Inc., Otsu, Japan). The reaction volume was 20 µl and the temperature protocol was as follows: Incubation at 25°C for 10 min, at 42°C for 30 min, at 95°C for 5 min and at 4°C for 5 min. The primers used for PCR were as follows: Wnt1, forward 5'-TACCTCCAGTCA CACTCCCC-3', reverse 5'-CCATGGCAGGAGAATAGG AC-3', reverse 5'-AGGCAAGGGGGAAGAGAGAGAGAT GT-3'; and GAPDH, forward 5'-ACAGCAACAGGGTGG TGGAC-3' and reverse 5'-TTTGAGGGTGCAGCGAACTT-3'. qPCR was performed using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc.) with an ABI 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction volume was 25 μ l, and contained 12.5 μ l SYBR-Green mix, 0.5 μ l of each forward and reverse primer, 2μ l cDNA and 9.5 μ l PCR grade sterile water. Thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 15 sec, at 62°C for 1 min, and at 72°C for 1 min. Each experiment was performed three times. The relative expression levels of each gene were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. Total proteins from spinal cord tissue samples were extracted using radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and protein concentrations were determined using bicinchoninic acid assay kits according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 for 2 h at room temperature and then incubated with the following primary antibodies obtained from Abcam (Cambridge, UK): Anti-Wnt1 (cat. no. ab15251; 1:1,000), anti-Nestin (cat. no. ab6142; 1:1,000), anti-β-catenin (cat. no. ab6302; 1:5,000), anti-NeuN (cat. no. ab177487; 1:1,000) and anti-GAPDH (cat. no. ab8245; 1:1,000) for 12 h at 4°C. Membranes were then incubated with goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G secondary antibody (cat. no. BA1055; 1:5,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (Thermo Fisher Scientific, Inc.). The optical densities of the protein bands were semi-quantified using the image analysis system ImageQuant[™] LAS4000mini (GE Healthcare Life Sciences, Chalfont, UK).

BBB score evaluation. In order to examine the functional deficits of SD rats following SCI, the BBB method was employed. Normal male SD rats without functional deficits (BBB score=21), were selected as a control. Following spinal cord surgery, two trained observers who were blind to the experimental conditions evaluated the grade of each rat according to the BBB open field locomotion test (21). Hindlimb movement, body weight support, foot placement and coordination were observed for 5 min to determine the BBB score of each animal.

Statistical analysis. The statistical significance of the difference between groups was assessed by one-way analysis of variance, followed by a post hoc least significant difference range test. Data are expressed as the mean \pm standard error of the mean of at least 3 independent experiments. P<0.05 was considered to indicate a statistically significant difference. The analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Alterations in BBB score, and Wnt1 and Nestin levels following SCI. The BBB score has been widely used as a neurological evaluation method, to assess recovery of functionality following SCI (18,22). BBB scores were significantly lower in SCI-treated rats compared with in control and sham rats (P<0.01), indicating that the rat SCI model was established successfully (Fig. 2).

It has previously been reported that Wnt1 was rapidly induced in the site of injury following SCI, and appeared to serve a role in the regulation of axonal regeneration (23). In the present study, the mRNA and protein expression levels of Wnt1 were assessed on day 1, 7 and 14 following SCI (Fig. 3). When compared with normal and sham rats, the expression of Wnt1 was revealed to be potently and rapidly induced on



Figure 1. Location of the Dazhui and Mingmen acupuncture points in rats. (A) Side view and (B) vertical view of the rat.



Figure 2. Results of the BBB test in normal, sham and SCI-treated groups on day 7 following SCI surgery. Data are expressed as the mean \pm standard error of the mean; n=6 rats/group. **P<0.01 vs. the normal group. BBB, Basso, Beattie and Bresnahan; SCI, spinal cord injury.

day 1 following SCI; however, it returned to baseline during the following 2 weeks (Fig. 3). These results are in agreement with a previously published report by Liu *et al* (23).

Nestin is a member of the intermediate filament protein family, and it has been reported to promote the proliferation and self-renewal of neural stem cells (24). The present study examined whether Nestin mRNA and protein expression levels in spinal tissue were altered in response to SCI. In the normal and sham groups, the expression of Nestin remained at low levels (Fig. 4). However, in the SCI group, the expression of Nestin was significantly increased on day 3 following SCI, then returned to baseline levels during the following 2 weeks (Fig. 4). The aforementioned alterations in BBB score, as well as Wnt1 and Nestin levels, demonstrated that the SCI model was successfully established.

Effects of EA on Wnt1, nuclear β -catenin and Nestin levels on day 3 following SCI. Wnt1 and nuclear β -catenin protein expression levels in rats that received EA therapy were significantly increased compared with in untreated rats following



Figure 3. Relative (A) mRNA and (B) protein expression levels of Wnt1 in spinal tissue from rats in the normal, sham and SCI groups, as assessed on days 1, 7 and 14 following SCI. Data are expressed as the mean \pm standard error of the mean; n=9 rats/group. **P<0.01 vs. the normal group. SCI, spinal cord injury.



Figure 4. Relative (A) mRNA and (B) protein expression levels of Nestin in spinal tissue from rats in the normal, sham and SCI groups, as assessed on days 1, 3, 7 and 14 following SCI. Data are expressed as the mean ± standard error of the mean; n=9 rats/group. **P<0.01 vs. the normal group. SCI, spinal cord injury.

SCI (P<0.01; Fig. 5), thus suggesting that EA may enhance Wnt/ β -catenin signaling. In order to test this hypothesis, SCI rats were treated with LiCl, which inhibits glycogen synthase kinase-3 β and thus can potentiate Wnt signaling. Nuclear β -catenin in the SCI + LiCl group appeared significantly upregulated compared with in the SCI group. Furthermore, Wnt1 and nuclear β -catenin protein levels were significantly higher in SCI rats treated with EA and LiCl compared with in rats treated with LiCl alone (Fig. 5). These results indicated that the mechanism underlying the beneficial effects of EA treatment on SCI may involve the enhancement of Wnt/ β -catenin signaling.

Nestin expression in spinal tissue was significantly increased on day 3 following SCI in rats receiving EA treatment (Fig. 5). Furthermore, when compared with in LiCl-treated rats, Nestin levels were significantly higher in SCI rats that received a combination of LiCl and EA treatment. These results suggested that EA may promote neural recovery following SCI via regulating the expression of Nestin.

Effects of EA on BBB score and NeuN expression on day 21 following SCI. NeuN is a neuron-specific biomarker, and fluctuations in its levels correspond to alterations in neuronal numbers (25). NeuN expression in EA-treated rats was significantly upregulated compared with in SCI untreated rats (Fig. 6A and B). Similarly, rats treated with a combination



Figure 5. Relative protein expression levels of Wnt1, nuclear β -catenin and Nestin in spinal tissue from rats in the SCI, SCI + EA, SCI + LiCl and SCI + LiCl + EA groups, as assessed on day 3 following SCI. Data are expressed as the mean ± standard error of the mean; n=9 rats/group. *P<0.05, **P<0.01 vs. the SCI group; #P<0.05 vs. the SCI + LiCl group, SCI group, rats that received SCI and no treatment; SCI + EA group, rats that received SCI and EA treatment; SCI + LiCl group, rats that received SCI and LiCl treatment; SCI + LiCl + EA rats that received SCI and a combination of EA and LiCl treatment. SCI, spinal cord injury; EA, electroacupuncture; LiCl, lithium chloride.



Figure 6. (A) Relative protein expression levels (B) quantifaction of NeuN in spinal tissue from rats in the SCI, SCI + EA, SCI + LiCl and SCI + LiCl + EA groups, as assessed on day 21 following SCI. Data are expressed as the mean ± standard error of the mean; n=9 rats/group. (C) BBB scores of SCI in rats in the SCI, SCI + EA, SCI + LiCl and SCI + LiCl + EA groups, as assessed on day 28 following SCI. Data are expressed as the mean ± standard error of the mean; n=6 rats/group. *P<0.05, **P<0.01 vs. the SCI group; #*P<0.01 vs. the SCI group; #*P<0.01 vs. the SCI + LiCl group. SCI group, rats that received SCI and no treatment; SCI + EA group, rats that received SCI and EA treatment; SCI + LiCl group, rats that received SCI and LiCl treatment. NeuN, Neuronal Nuclei; SCI, spinal cord injury; EA, electroacupuncture; LiCl, lithium chloride; BBB, Basso, Beattie and Bresnahan.

of LiCl and EA exhibited significantly higher NeuN levels compared with rats treated with LiCl alone following SCI (Fig. 6A and B). These results suggested that the observed NeuN upregulation was induced by EA therapy.

On day 28 following SCI, the BBB score was evaluated across all groups. Rats that received EA treatment exhibited significantly higher BBB scores compared with untreated rats following SCI (Fig. 6C). Notably, the effect of EA treatment on the BBB score appeared significantly greater than the effect of LiCl therapy (Fig. 6C). These results suggested that EA applied at Dazhui and Mingmen points may promote spinal recovery following SCI in rats.

Discussion

The signaling pathways involved in neuronal recovery and regeneration following SCI have garnered much attention. The Wnt/ β -catenin signaling pathway, which is highly conserved among multicellular eukaryotic organisms, has been reported to participate in the regulation of cellular proliferation (26), differentiation (27,28), migration (29) and apoptosis (30,31). Several Wnt genes have been identified, including Wnt1, 2, 2b, 3 and 3a. The Wnt family has been reported to serve an important role in the development of the CNS, since it has previously been demonstrated that the Wnt/ β -catenin signaling pathway promoted the differentiation of neural stem cells into neuronal cells, whereas it inhibited their differentiation into astrocytes (32,33).

In the present study, EA therapy was revealed to upregulate the expression of Wnt1 and nuclear β -catenin, thus suggesting that EA treatment may enhance Wnt/ β -catenin signal transduction pathways. The nuclear accumulation of β -catenin can activate the transcription of genes related to, among others, cellular proliferation and terminal differentiation (34). Nestin is an intermediate filament protein expressed in dividing cells during the early stages of development in the CNS. Upon differentiation, Nestin expression decreases and it is replaced by tissue-specific intermediate filament proteins. In mature organisms, Nestin expression has been revealed to be upregulated under pathological conditions, including glial scar formation following CNS injury (24). In the present study, the expression of Nestin appeared to be upregulated in EA-treated rats compared with in untreated rats following SCI, thus suggesting that EA may promote the proliferation of neural stem cells. In order to further examine whether EA may promote neuronal proliferation, the expression of the neuron-specific marker NeuN (35) was investigated. The present results revealed that EA-treated rats exhibited significantly higher NeuN levels compared with untreated rats following SCI. The increase in Nestin and NeuN expression reported in the present study suggested that EA treatment may promote the differentiation of neural stem cells into neuronal cells in spinal tissue following SCI. Therefore, it may be hypothesized that EA can promote neuronal recovery following SCI, via enhancing Wnt/β-catenin signaling and promoting the differentiation of neural stem cells into spinal neurons.

The increased expression of Nestin and NeuN in spinal tissue reported in the present study following EA therapy did not directly reflect alterations in neural stem cell and neuronal cell numbers. In conclusion, in the present study, EA treatment appeared to promote spinal recovery following SCI, via interfering with the Wnt1/ β -catenin signaling pathway. Furthermore, EA may promote the differentiation of neural stem cells into spinal neurons, via enhancing Wnt1/ β -catenin signaling; however, further studies are required to investigate this hypothesis.

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