

Original Research Article

Neuroprotective effects of electro-acupuncture in spinal cord injury rats via up-regulation of DUSP14

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Abstract

Purpose: To study the effect and mechanism of action of electro-acupuncture (EA) on nerve regeneration by analyzing the behavior, inflammation and cell death in spinal cord injury (SCI) rat model.

Methods: SCI model was established according to Allen's falling strike method. Electroacupuncture was performed on Jiaji (EX-B2)/Mingmen (GV4) acupoint with a 1 mA current intermittent wave at a frequency of 2Hz for 20 min daily. Interleukin (IL-6) and tumor necrosis factor- α (TNF- α) levels were measured using ELISA kits. Apoptosis-induced DNA strand breaks were evaluated by TUNEL assay while relative mRNA expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Protein levels were measured by western blot.

Results: Relative mRNA and protein expressions of DUSP14 decreased in SCI rats with time but increased by EA treatment. Further, partial locomotor functional recovery was presented in SCI rats by EA treatment. Moreover, intraspinal injection of DUSP14 over-expression viral supernatants/EA treatment ameliorated inflammation and apoptosis in SCI rats. Meanwhile, the protein levels of NF- κ B p65 (nucleus) and phosphorylated TGF-activated kinase 1 (p-TAK1) increased in SCI rats following EA treatment but were decreased by EA treatment and intraspinal injection of DUSP14 over-expression viral supernatants.

Conclusion: EA acupoint treatment exerts neuroprotective effects in SCI rats via the reduction of inflammation and apoptosis, and induction of DUSP14.

Keywords: Electro-acupuncture, Spinal cord injury, Inflammation, TUNEL index, DUSP14

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INTRODUCTION

Spinal cord injury (SCI) is devastating to humans and affects millions of people around the world [1]. The common causes include recreational and sports accidents (9 %), violence (13.5 %), falls (>22 %) and traffic accidents (38 %). The degree of injury in SCI patients is depends on the

precise location and extent as well as the spinal level of the injury[4]. Besides the direct consequences of failed sensory, motor and autonomic nervous system function, later problems including chronic pain, muscle wasting, pressure sores and urinary infections would aggravate the injury in SCI patients [5].

Initial spinal cord injury induces a series of complex molecular events called 'secondary injury'[6], including free radical-induced apoptosis, inflammation, phospholipase A2 activation, glutamate excitotoxicity, and induction of intrinsic and extrinsic apoptotic pathways. Nuclear factor kappa B (NF- κ B) is one of the major pro-inflammatory transcription factors and plays a critical role in inflammation activation[7]. Moreover, excessive cytokines, neurons apoptosis and glia apoptosis are closely related to the secondary injury [8]. Dual specific phosphatase-14 (DUSP14, also known as MKP6) dephosphorylates MAPK family members p38 MAPK, JNK MAPK and ERK MAPK *in vitro*[9]. Down regulation of DUSP14 elevates hypoxia/reoxygenation-induced activation of NF- κ B and MAPKs signaling, and further aggravates the apoptosis and cardiac dysfunction [10].

Electroacupuncture (EA) is a modified traditional Chinese medicine approach and it could improve sensory and motor function in spinal cord transection[12]. Moreover, electroacupuncture has been widely used in the therapy of peripheral nerve injury for a long time in China [14, 15], but the molecular mechanism remains enigmatic. Therefore, the objective of this study was to explore the effect of EA on nerve regeneration via analyzing the behavior, cell death and inflammation in spinal cord injury rat model.

EXPERIMENTAL

Animals

Six week-old male Sprague-Dawley rats (170 - 210 g) were obtained from Shanghai Lab Animal Research Center (Shanghai, China) and kept in cages 12 h light/dark cycle, 23-25 °C) with food and water *ad libitum*. This study was approved by the Animal Care and Use Committee of The Sixth People's Hospital Affiliated to Shanghai Jiaotong University (approval no. 2017-0014) and in accordance with the Guidelines for the Care and Use of Laboratory Animals [17].

Spinal cord injury (SCI) model

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg), and then fixed in the prone position. A longitudinal incision was sectioned in the middle skin in the rat back after shaved. Then, the vertebral laminae from T₉ to T₁₁ were resected. The SCI model was established according to Allen's falling strike method [18]. The skin and muscles were closed in layers after the injury. Rats in negative control group underwent the same operation except the strike-injury. Manual

emptying of bladder was performed 3 times daily until intrinsic function was recovered.

EA treatment

In the EA treatment group, acupuncture needles (0.25 mm × 13.00 mm, Suzhou Medical Appliance Factory, Suzhou, China) were inserted into the *Jiaji* (EX-B2) / *Mingmen* (GV4) acupoints at 3 days after operation. A low frequency electronic pulse therapeutic apparatus (Model G-6805-2, Shanghai Medical Electronic Apparatus, Shanghai, China) was connected. The acupoints were stimulated by a 1 mA current intermittent wave at the frequency of 2Hz for 20 min daily. Muscles at the acupoints were characterized by shaking slightly during EA execution. Stimulating at the non-acupoint on the right forelimb was served as electrical stimulation control.

Intraspinal injection of DUSP14 over-expression viral supernatant

DUSP14 (NM_001270836.1) over-expression lentivirus vectors were constructed by JRDun Biotech (Shanghai, China). Viral supernatants were diluted in rat serum to the required concentration and intraspinal injection into the injury side at 6 h after SCI operation.

Experiment design and sample collection

This study was divided into 3 parts. In the 1st part, a total of 21 SD rats were used (3 normal rats, 3 negative control rats and 15 SCI rats). Three rats in SCI group were sacrificed at 0, 6, 12, 24 and 72 h after operation respectively. Rats in normal control group and negative control were sacrificed at 72 h after operation. The injured / normal spinal cord tissue sample was collected. The mRNA expression and protein level of DUSP14 at each time point were measured.

In the 2nd part, 36 SD rats were divided into 6 groups (6 rats per group): negative control group, SCI group, EA *Jiaji* (EX-B2) acupoint treatment group, EA *Mingmen* (GV4) acupoint treatment group, EA *Jiaji* (EX-B2) + *Mingmen*(GV4) acupoints treatment group, and EA non-acupoint treatment group. The EA treatment was performed at 6 h after operation. Combined Behavioral Score (CBS) protocol[19]was employed to analyze the behavior of rats at 24 h after operation. Then the rats were sacrificed and the spinal cord tissue sample was collected. The mRNA expression and protein level of DUSP14 were tested at the end.

In the 3rd part, 30 SD rats were divided into 5 groups (6 rats per group): negative control group, SCI group, EA *Jiaji* (EX-B2) + *Mingmen* (GV4) acupoints treatment group, DUSP14 over-expression viral supernatants intraspinal injection group and empty plasmid control group. The EA treatment and intraspinal injection were performed at 6 h after operation. Rats were sacrificed at 24 h after operation and the spinal cord tissue sample and regeneration fluid were collected. The contents of TNF- α and IL-6 were tested using ELISA method. Cell apoptosis of spinal cord tissues were tested by TUNEL assay. The protein levels of DUSP14, NF- κ B, cleaved Caspase-3, cleaved poly ADP-ribose polymerase (PARP), TGF-activated kinase 1 (TAK1) and phosphorylated TAK1 (p-TAK1) were measured by western blot.

ELISA assay

The spinal cord regeneration fluid was extracted. The TNF- α and IL-6 content were measured using the rat TNF- α (ab100785, Abcam, USA) and IL-6 (ab100772, Abcam, USA) ELISA kits according to the instructions.

TUNEL assay

Apoptosis-induced DNA strand breaks were measured using a TUNEL assay kit (11684817910, Roche, USA) in accordance with a previous report[20]. Spinal cord tissue samples were rapidly fixed in 10% formaldehyde. After standard operation of dehydration, clearing in xylene, and paraffin embedding, 3–5 μ m sections were cut and mounted. Then the samples added with 50 μ L TUNEL reaction mixture were incubated in dark at 37 °C for 1 h. Finally, the 3' ends of DNA are labeled with dUPT (fluorescein conjugated) and show deep brown. Finally, the sample sections were observed using a fluorescence microscope (TE2000U, Nikon, Japan). Moreover, the apoptotic index of each sample was calculated by counting the positive and negative stained cells in fields of vision.

Polymerase chain reaction

Total RNA of spinal cord tissues was isolated using TRIzol reagent (15596018, Thermo Fisher, USA) and then reverse transcribed into cDNA using the first strand cDNA synthesis kit (K1622, Thermo Fisher, USA). Q-RT PCR was performed on an ABI 7500 system (ABI-7500, USA) using SYBR Green qPCR Master Mixes (4385612, Thermo Fisher, USA) with the primers (Table 1). Relative mRNA expression was calculated using the 2^{- Δ Ct} method normalized to GAPDH.

Table 1: Primers used in real-time fluorogenic PCR assay

Gene name	Sequence (5'-3')	Description
DUSP14	ATGGCTCCTCGGATGA TTTC	Forward
DUSP14	CATTGACGACGCAGGT GATG	Reverse
GAPDH	GGAGTCTACTGGCGTC TTCAC	Forward
GAPDH	ATGAGCCCTTCCACGA TGC	Reverse

Western blot

The total protein was isolated and then determined using a BCA protein assay kit (23227, Thermo Fisher, USA). Besides, nucleoprotein was extracted using a commercial kit (P0027-3, Beyotime Bio, China) according to the instruction. The proteins (35 μ g each sample) were separated using 10 % SDS-PAGE. Then target protein was transferred onto a PVDF membrane and blocked using 5% nonfat milk at 25 °C for 1 h. Then the membranes were incubated with primary antibodies DUSP14 (1:2000, NBP1-81046, Novus Bio, USA), Cleaved caspase3 (1:1000, #9661, CST, USA), Cleaved PARP (1:1000, #9545, CST, USA), NF- κ B p65 (1:1000, Ab16502, Abcam, USA), TAK1 (1:1000, #5206, CST, USA), p-TAK1 (1:1000, #9339, CST, USA), Histone H3 (1:2000, #4499, CST, USA) and GAPDH (1:1000, #5174, CST, USA) respectively. Followed by incubation with HRP (Horseradish Peroxidase)-conjugated goat anti-rabbit IgG secondary antibody (1:1000, A0208, Beyotime Bio, China) for 1 h. Finally, protein level was measured using a chemiluminescent imaging system (Tanon 5200, Tanon, China) and normalized to GAPDH/H3.

Statistical analysis

Data are presented as mean \pm SD. Statistical analysis was processed by SPSS 20.0 software (SPSS, IBM, Chicago, USA). Differences between two samples were analyzed by paired Student's t-test. One-way analysis of variance followed by Dunnett's test was performed for three or more groups. $p < 0.05$ was considered as significant.

RESULTS

Effects of spinal cord injury on DUSP14 expression in rats

SCI model was established according to Allen's falling strike method. Then relative mRNA and protein expression of DUSP14 at the injury site

were tested at 0, 6, 12, 24 and 72 h after operation. As shown in Figure 1, relative mRNA and protein expression of DUSP14 were decreased with time in SCI rats.

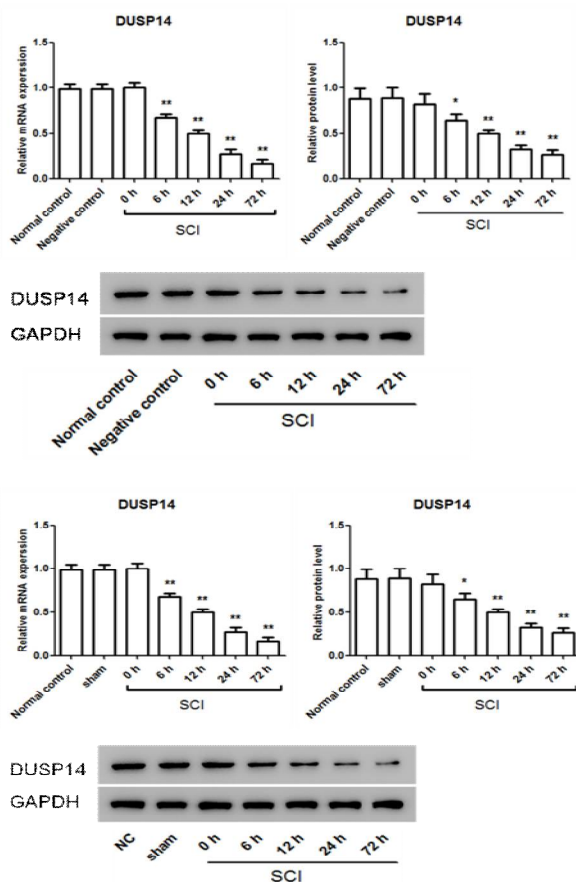


Figure 1: Effects of spinal cord injury on DUSP14 expression in rats. GAPDH was regarded as the loading control; * $p < 0.05$, ** $p < 0.01$, compared with negative control group

EA treatment ameliorated the sensory and motor function of SCI rats and up-regulated the DUSP14 expression

The Combined Behavioral Score (CBS) protocol [19] was employed to analyze the behavior of rats at 24 h after operation. Moreover, relative mRNA and protein expressions of DUSP14 were determined by q-RT PCR and western blot respectively. The present results indicated that EA (acupoints) treatment could ameliorate the sensory and motor function of SCI rats (Figure 2 a) and up-regulated the DUSP14 expression in SCI rats (Figure 2 b and c). Besides, EA double acupoints treatment showed better treatment effects. Moreover, there was no difference between EA non-acupoints treatment group and SCI model group (Figure 2).

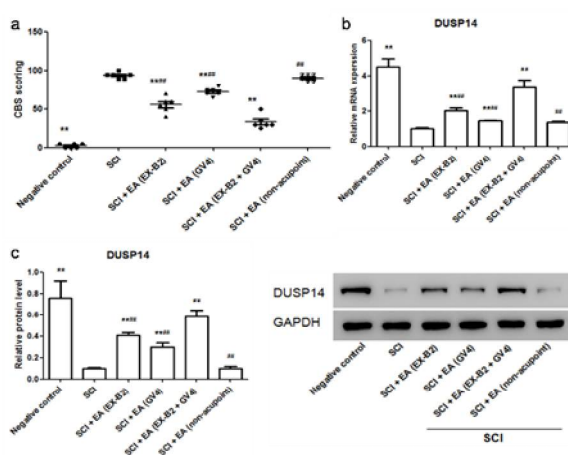


Figure 2: EA treatment ameliorated the behavior of rats and up-regulated the DUSP14 expression in SCI rats. The CBS scoring of SCI rats was decreased by EA acupoints treatment (a). Relative mRNA and protein expression of DUSP14 were increased by EA acupoints treatment (b and c); * $p < 0.05$, ** $p < 0.01$, compared with the SCI group; ### $p < 0.01$ compared with SCI + EA (EX-B2 + GV4) treatment group

EA treatment ameliorated the inflammation and apoptosis at the spinal cord injury site and up-regulated DUSP14

As shown in Figure 3, the concentration of TNF- α and IL-6 in spinal cord regeneration fluid were increased in SCI rats but decreased by intraspinal injection of DUSP14 over-expression viral supernatants at the spinal cord injury site (Figure 3 a). Further, SCI-induced increase TNF- α and IL-6 were ameliorated by EA (double acupoints) treatment (Figure 3 a). The apoptosis-induced DNA strand breaks were increased in SCI rats but decreased by EA (double acupoints) and intraspinal injection of DUSP14 over-expression viral supernatants (Figure 3 b). Furthermore, the protein levels of DUSP14 and NF- κ B p65 (cytoplasm) decreased in SCI rats but increased by EA (double acupoints) and DUSP14 over-expression (Figure 3 c). Moreover, the protein levels of cleaved caspase3, cleaved PARP, NF- κ B p65 (nucleus) and p-TAK1 showed an opposite trend. Besides, the protein levels of TAK1 showed no change (Figure 3 c).

DISCUSSION

SCI is a serious problem with high prevalence and causing heavy burden to family and social. Initial spinal cord injury induced secondary damages such as inflammation and apoptosis are the major target of most current neuroprotective strategies [6]. The present results indicated that EA acupoints treatment could be an effective treatment method against spinal cord injury.

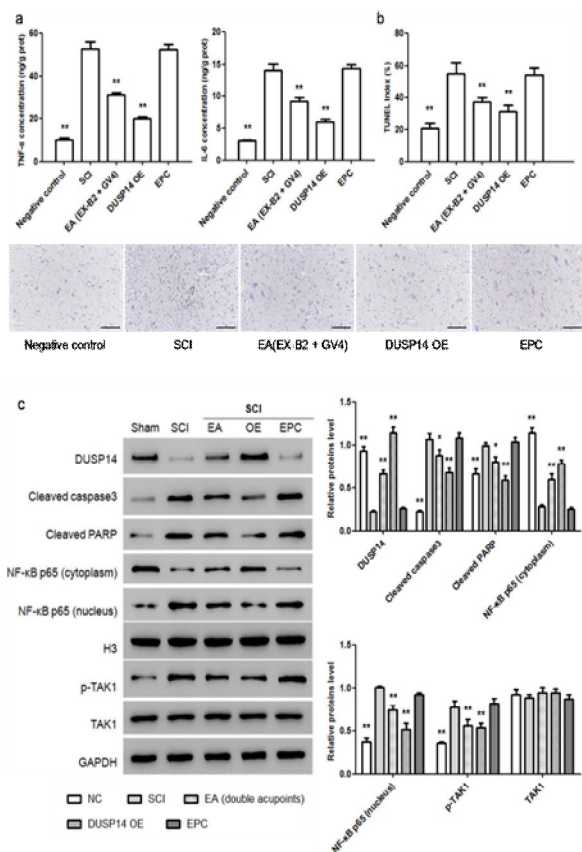


Figure 3: EA treatment ameliorated the inflammation and apoptosis at the spinal cord injury site and up-regulated DUSP14. The concentration of TNF- α , IL-6 and apoptosis-induced DNA strand breaks (TUNEL index) were increased in SCI rats but decreased by DUSP14 OE and EA treatment (a, b). Effects of EA in combine with DUSP14 OE on proteins expressions in SCI rats (c). Scale bar: 10 μ m; * p < 0.05, ** p < 0.01, compared with the SCI group/EPC group

Electro-acupuncture (acupoints) treatment ameliorated the behavior of SCI rats. Moreover, the inflammation and apoptosis induced by SCI was improved by EA (acupoints) treatment via the up-regulation of DUSP14.

Dual specific phosphatase-14 is a phosphatase and dephosphorylates MAPK family members [9]. It plays a key role in protecting cardiac from hypoxia/reoxygenation injury via suppressing activation of MAPKs and NF- κ B signaling [10]. TGF-activated kinase 1 is closely linked with the MAPK and NF- κ B signaling. Activated TAK1 can accelerate the MAPK and NF- κ B signaling pathways and resveratrol attenuates the inflammation by the inhibition of TAK1 activity [21,22].

A previous study indicated that the post-traumatic inflammation is mediated by the TNF- α -NF- κ B cascade [23], and the post-traumatic

inflammation was ameliorated by methylprednisolone (MP)- the effective treatment to improve neurological function in patients with acute SCI [23]. Another report showed that IL-6 mediated inflammatory via inducing aquaporin 4 (AQP4) expression in rat spinal cord astrocytic [24]. In the present study, the increased concentrations of TNF- α and IL-6 as well as protein level of NF- κ B p65 (nucleus) and p-TAK1 were decreased by EA (acupoints) treatment and DUSP14 over-expression in SCI rats. The expression of DUSP14 was up-regulated by EA (acupoints) treatment.

In recent years, the regain of lost nerve function after spinal cord injury has been widely studied [25]. A previous study has also reported that electrical stimulation enhances nerve regeneration with no effects on neuropathic pain [26]. Electro-acupuncture is a modified traditional Chinese medicine approach. It has been reported to induce BDNF, NT-3 and NGF expressions in spared L6 dorsal root ganglion in cats subjected to removal of adjacent ganglia [27]. Moreover, EA has been confirmed to improve chronic neuropathic pain after spinal cord injury [28,29].

In this study, the behavior of SCI rats (CBS scoring) were improved by EA *Jiaji* (EX-B2) / *Mingmen* (GV4) acupoints treatment. Moreover, the TUNEL index of SCI rats was decreased by EA *Jiaji* (EX-B2) + *Mingmen* (GV4). The apoptosis-related cleaved caspase 3 and cleaved PARP were decreased by EA *Jiaji* (EX-B2) + *Mingmen* (GV4) and intraspinal injection of DUSP14 over-expression viral supernatants in SCI rats.

CONCLUSION

Electro-acupuncture acupoint treatment affords neuroprotective effects in SCI rats. It would be an effective treatment on SCI in patient with minimal side effect.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. This study was conceived and designed by Yaochi Wu. Data were collected by Jingjie Xu, Shenghong Zhang and Shisheng Li and analysed by Junfeng Zhang and Yaochi Wu. The manuscript was written by Junfeng Zhang and Yaochi Wu and submitted by Yaochi Wu. All authors read and approved the manuscript for publication.

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