Bio-Medical Materials and Engineering 24 (2014) 3629–3636
DOI 10.3233/BME-141190
IOS Press

Oscillating field stimulation promotes spinal cord remyelination by inducing differentiation of oligodendrocyte precursor cells after spinal cord injury

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Abstract. Demyelination is part of the cascading secondary injury after the primary insult and contributes to the loss of function after spinal cord injury (SCI). Oligodendrocyte precursor cells (OPCs) are the main remyelinating cells in the central nervous system (CNS). We explored whether oscillating field stimulation (OFS) could efficiently promote OPC differentiation and improve remyelination after SCI. SD rats with SCI induced by the Allen method were randomly divided into two groups, the SCI+OFS group and SCI group. The former group received active stimulator units and the latter group received sham (inoperative) stimulator units. Additionally, rats that only received laminectomy were referred as the sham group. The electric field intensity was 600μV/mm, and the polarity was alternated every 15 minutes. The results showed that the SCI+OFS rats had significantly less demyelination and better locomotor function recovery after 12-weeks treatment. The OFS treatment significantly increased the number of Gal C-positive OPCs after 2-weeks treatment. Furthermore, these rats had higher protein expression of oligodendroglial transcription factors Olig2 and NKx2.2. These findings suggest OFS can promote locomotor recovery and remyelination in SCI rats and this effect may be related to the improved differentiation of OPCs in the spinal cord.

Keywords: Oligodendrocyte precursor cells, remyelination, oscillating field stimulation, spinal cord injury, rat

1. Introduction

Neurites preferentially grow towards the cathode of an applied electric field and are resorbed from the anode [1]. This characteristic leads to the development of oscillating field stimulation (OFS), an

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extra-spinal treatment for spinal cord injury (SCI). OFS imposes a weak voltage gradient across the lesion site and reverses its polarity every 15 minutes which can promote the growth of nerve fibers in both directions [2]. Although the OFS treatment has been used in a phase 1 trial in human SCI patients, the underlying mechanism is not well illustrated. In the past twenty years, research mainly focused on the effect of OFS on enhancing neural regeneration and reducing scar formation [3]. Recently, the emphasis has been on enhancing remyelination after SCI [4,5]. The myelin sheath is important to neuronal function, providing trophic support to the axon and enabling rapid nerve impulses. Due to primary damage (i.e. mechanical impact) and secondary damage (i.e. complex biochemical changes) post-SCI, axons undergo demyelination at the site of injury [6]. Oligodendrocytes are myelin-forming cells in the CNS. Oligodendrocyte precursor cells (OPCs) are immature oligodendrocytes which can differentiate into oligodendrocytes under certain conditions [7]. Our preliminary animal experiments have showed that OFS can stimulate remyelination, so it is proposed that promoting differentiation of OPCs may be beneficial for remylination after SCI. Thus, in the present study, by establishing an SCI animal model treated with OFS, our team investigated whether the OFS treatment could promote the differentiation of OPCs, improve remyelination and ultimately benefit functional improvement after SCI in rats.

2. Materials and methods

2.1. Stimulator fabrication

The proposed electrical schematic of the OFS was shown in Figure 1. The power of stimulator units was supplied by a 3.3 V battery (CR2032) with a capacity of 210 mAh. A binary ripple counter with a 47 nF capacitor and two resistors (910 kΩ and 6.2 MΩ) was used to oscillate the current with an interval of about 15 minutes. Three operational amplifiers (OA) with two 1 MΩ resistors set the reference voltage level at 1.5 V. The OAs were connected to the ripple counter each with a 130 kΩ resistor. Three pairs of

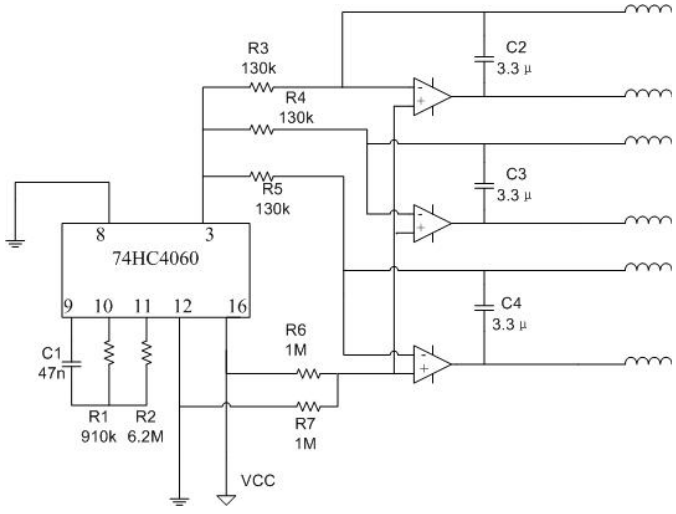

Fig. 1. The proposed electrical schematic of the OFS.

electrode leads were connected with the OAs. Electrodes with helix profile were fashioned from 0.2 mm diameter Pt-Ir (90/10) wire. Thus, when the OFS was fully implanted into the experiment animals, a direct current of about 12 μA was delivered into the biological tissues between each pair of electrodes. When the stimulator was assembled, the battery and PCB were put into a cylinder made of polycarbonate, which is a kind of biocompatible waterproof material. Epoxy was then poured into the cylinder to avoid water penetration. After the epoxy was dry, the stimulators including the leads were all sealed with silicon. Sham stimulators were fashioned with the same units but rendered inoperative by attaching the battery with nonconductive materials and fabricating the circuit with other non-functional components.

2.2. Experimental animals and groups

30 adult female Sprague-Dawley (SD) rats weighted 200-230 g were divided into 3 groups, the SCI (n=10), SCI+OFS (n=10) and Sham (n=10) groups.

2.3. Surgical procedures

Rat models of SCI were built according to the spinal cord contusion method of Allen [8] at T10. The insulated tips of the stimulating electrodes were inserted into the two laminectomies rostral and caudal to the injury site. The electrodes were stitched to the paravertebral musculature. The SCI+OFS rats received active stimulator units (electric field intensity, 500 μV/mm; polarity was alternated every 15 minutes), while the SCI rats received inoperative stimulator units. The Sham rats only received laminectomy.

2.4. 2Behavioral assessment

The Basso-Beattie Bresnehan (BBB) score was used to evaluate the locomotor of rats [9]. A score of 0 represents complete paralysis, and a score of 21 means complete mobility. Scoring was performed once weekly for the first two weeks and then bi-weekly thereafter until 12 weeks after SCI.

2.5. Luxol fast blue staining

12 weeks after the injury, the rats of three groups were killed and perfused with 4% cold paraformaldehyde transcardially. 2-cm length spinal cords including the injury site were removed and cut into 10 μm sections. The Luxol fast blue stained sections were photographed and analyzed by using Image-pro Plus software. The spared white matter area was represented as the percentage of the LFB-positive area in total area of the spinal cord.

2.6. Immunofluorescence staining

The rats were sacrificed at 14 d post-injury, the spinal cord tissue experienced slicing (5 μm) and was double immunofluorescence stained. The mouse anti-Gal C (1: 200; Chemicon) primary antibodies, the goat anti-mouse IgG (1:200; Beyotime) second antibodies and DAPI (1 μg/mL; Invitrogen) were used.

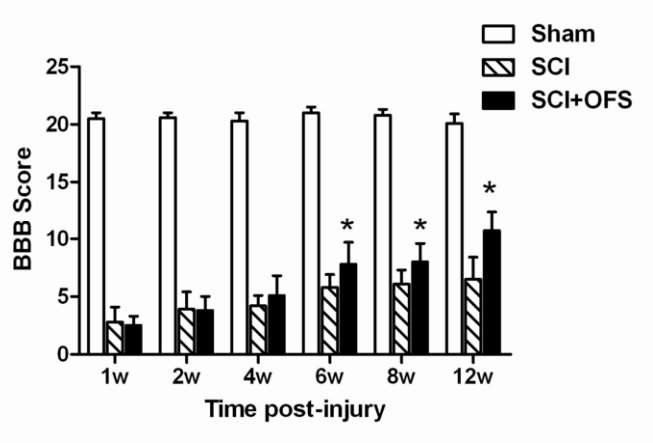

Fig. 2. Locomotor recovery was assessed by the BBB Locomotor test. The hindlimb function of rats in each group was scored as from flaccid paralysis (score 0) to normal gait (score 21) at 1 w, 2 w, 4 w, 6 w, 8 w and 12 w after the SCI. Statistical results showed that the mean score of rats in the SCI+OFS group was higher than that of the SCI rats at 6 weeks post-injury, and remained higher thereafter ($p<0.05$). * $p<0.05$ compared to the SCI group, n=10.

2.7. Western blotting assay

At four weeks post-injury, the spinal cord samples were dissected and centrifuged. The supernatants of spinal cord were collected and protein concentrations were examined. After electrophoresis and transferring, the PVDF membranes were incubated at 4°C overnight with anti-Olig2 antibody (1:1000, Abcam), anti-NKx2.2 antibody (1:1000, Abcam) or anti-β-actin antibody (1:5000, Abcam), followed by detection with the secondary antibodies (1:100, Beyotime).

2.8. Statistical analysis

All data were expressed as "mean ± SD". SPSS Software 12.0 was used for statistical analysis. For comparison of BBB behavioral test, the two-way analysis of variance (ANOVA) was used followed by the post hoc Bonferroni test. For comparison of simple effects, the one-way ANOVA was used followed by the Tukey's post hoc test. The differences were considered significant, if $p < 0.05$.

3. Results

3.1. Behavioral assessment

The motor function was assessed at 1 w, 2 w, 4 w, 6 w, 8 w and 12 w after SCI. The hindlimbs of the SCI and SCI+OFS rats were completely paralyzed and showed few hindlimb movements one week after the injury. A gradual improvement for rats in the SCI and SCI+OFS groups appeared and the score reached about 7 at week 4. No significant differences were observed between the SCI and SCI+OFS rats within 4 weeks ($p>0.05$). At six weeks and later, the mean score of rats in the SCI+OFS group was significantly higher than that of the SCI rats ($p<0.05$), and remained much higher thereafter ($p<0.05$). The scores remained to be 21 in the Sham rats as shown in Figure 2.

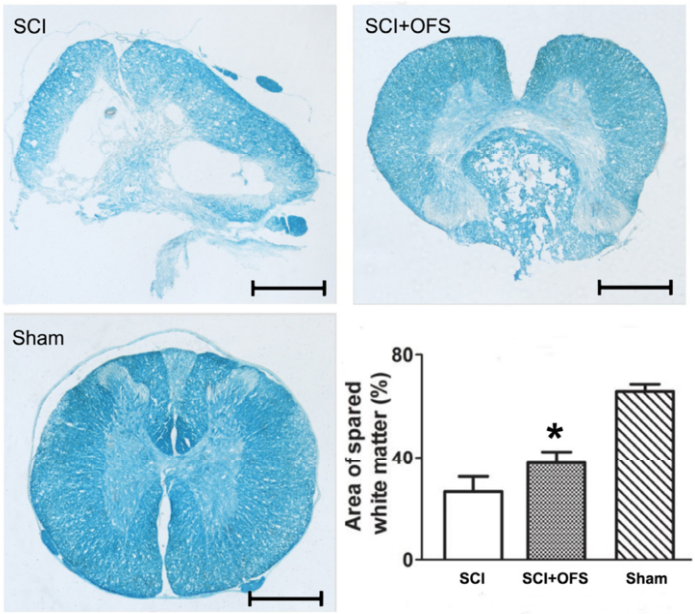

Fig. 3. OFS promotes remyelination after SCI. Quantitative results of spared white matter of each group are shown. The area of LFB-positive white matter in the SCI+OFS group was significantly larger than that in the SCI group. * $p<0.05$ compared to the SCI group. Scale bar =500 μm, n=5.

3.2. Luxol fast blue staining

The myelin area of spinal cord sections was detected by LFB staining, myelin appeared to be blue after staining. LFB staining of myelin was mostly restricted to the white matter in the sham group (Figure 3). The content of LFB-positive staining of the myelin in the SCI+OFS group was significantly larger than that in the SCI group ($p<0.05$). This observation suggests that OFS promotes remyelination after SCI.

3.3. Immunofluorescence staining

To investigate the phenotypic fate of OPCs post-injury (Figure 4), the spinal cord sections were double-immunostained with DAPI and different specific markers. In the first week after surgery, no significant difference was observed between the SCI and SCI+OFS groups. Two weeks after injury, the number of NG2 positive cells in the SCI+OFS group was significantly lower than that in the SCI group, but the number of Gal C positive cells was significantly more than that in the SCI group ($p<0.05$), implicating that the majority of OPCs differentiated into pre-mature oligodendrocytes.

3.4. Western Blotting Assay

Western blot assay revealed the Olig2 and NKx2.2 protein levels for different treatment groups (Figure 5). The relative expression of Olig2 protein in the SCI group and SCI+OFS group were 35.17 ± 3.26, and 62.39 ± 3.17 , NKx2.2 protein in the SCI group and SCI+OFS group were 43.26 ± 3.58 and 71.32 ± 4.88. OFS treatment increased the expression of Olig2 and NKx2.2 significantly ($p<0.05$).

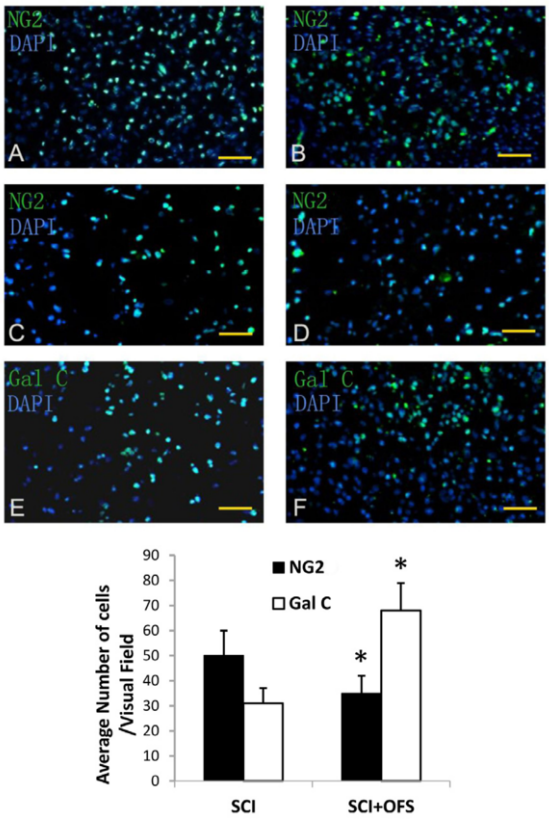

Fig. 4. Double immunolabeled DAPI+ cells and different markers of OPCs in the spinal cord (A–D) NG2+/DAPI+ OPCs. (E–F) Gal C+/ DAPI+ pre-matured OPCs. There was no significant difference in NG2+/DAPI+ cells between the SCI group (A) and the SCI+OFS group (B) 1 week after the injury. Two weeks after the injury, the number of NG2+/DAPI+ cells in the SCI group (C) was significantly higher than that in the SCI+OFS group (D), but the number of Gal C+/DAPI+ cells in the SCI group (E) was significantly lower than that in the SCI+OFS group (F). The results indicated the majority of OPCs differentiated into pre-mature oligodendrocytes. Bar=100μm * p <0.05 compared to the SCI group, n=5.

These observations showed that after SCI, the OFS could significantly promote the expression of neurotrophic factors.

4. Discussion

SCI is a common injury to the CNS. Although complete recovery of the injured spinal cord is still impossible, lots of efforts are being applied to promote nerve regeneration and functional restoration [4]. OFS is one of the current research approaches, which facilitates nerve regeneration in both directions. To date, possible mechanisms of OFS include reduction of astrocyte number and changes in blood flow within the injury site post-traumatic spinal cord [3].

SCI is known to result in demyelination with relative sparing of axons and therefore cause remarkable nerve dysfunction [10]. Enhancing remyelination helps to preserve axons and restore conduction velocity [11]. In the present study, we revealed that the LFB-positive area was significantly greater

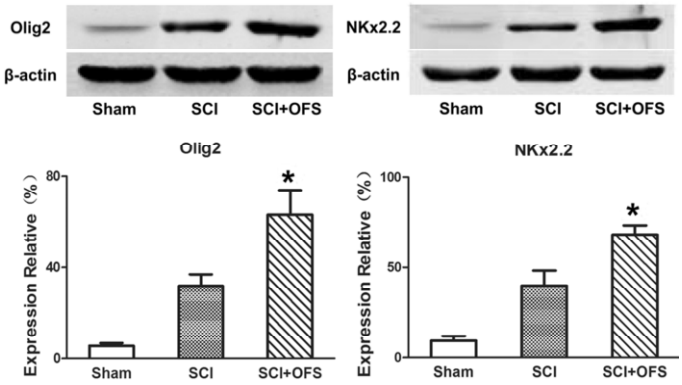

Fig. 5. OFS enhances the expression of oligodendrocyte transcription factors. The expression of Olig2 and NKx2.2 proteins after SCI was detected by Western blotting. β-actin was used as a protein loading control. The protein levels of Olig2 and NKx2.2 in the SCI+OFS rats were significantly higher than that in the SCI rats. * $p<0.05$ compared to the SCI group, n=5.

inwhite matter of the spinal cord in the SCI+OFS rats. It suggests that the OFS treatment efficiently promotes remyelination of the spinal cord after SCI. In this study, the results of the BBB score test indicate that the OFS treatment improves locomotors recovery.

OPCs are extremely motile and can differentiate into oligodendrocytes, which is a critical step for remyelination after SCI [12]. Therefore, the effects of the OFS treatment on differentiation of OPCs based upon the outcome of phenotypical staining were evaluated. After treated for 14 days, the OFS rats showed enhanced Gal C-positive cells and markers for pre-mature oligodendrocytes. Previous studies have reported that Nkx2.2 and Olig2 regulate OPC differentiation in the spinal cord [13–15]. Hence, the Nkx2.2 and Olig2 protein levels were further investigated, and the results confirmed that the protein levels of Nkx2.2 and Olig2 were increased in the SCI+OFS rats. These findings suggest that OFS can improve differentiation of OPCs.

5. Conclusion

The OFS treatment can promote functional improvement and remyelination after SCI in SD rats, and the neuro protective effect may be associated with the improved differentiation of OPCs. This study elucidates the effects of OFS on remyelination and provides new theoretical basis for treating SCI with OFS.

Acknowledgement

We are grateful to our technical staff for assistance with the electric stimulation equipment. This work was supported by National Natural Science Foundation of China (No. 51177162 & No. 51307166).

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