Epidural oscillating field stimulation increases axonal regenerative capacity and myelination after spinal cord trauma

Maria Bacova, Katarina Bimbova, Alexandra Kisucka, Nadezda Lukacova, Jan Galik*  

Abstract  
Oscillating field stimulation (OFS) with regular alterations in the polarity of electric current is a unique, experimental approach to stimulate, support, and potentially guide the outgrowth of both sensory and motor nerve fibers after spinal cord injury (SCI). In previous experiments, we demonstrated the beneficial effects of OFS in a 4-week survival period after SCI. In this study, we observed the major behavioral, morphological, and protein changes in rats after 15 minutes of T9 spinal compression with a 40 g force, followed by long-lasting OFS (50 µA), over a 8-week survival period. Three groups of rats were analyzed: rats after T9 spinal compression (SCI group); SCI rats subjected to implantation of active oscillating field stimulator (OFS + SCI group); and SCI rats subjected to nonfunctional OFS (nOFS + SCI group). Histopathological analysis of spinal tissue indicated a strong impact of epidural OFS on the reduction of tissue and myelin loss after SCI in the segments adjacent to the lesion site. Quantitative fluorescent analysis of the most affected areas of spinal cord tissue revealed a higher number of spared axons and oligodendrocytes of rats in the OFS + SCI group, compared with rats in the SCI and nOFS + SCI groups. The protein levels of neurofilaments (NF-l), growth-associated protein-43 (marker for newly sprouted axons), and myelin basic protein in rats were significantly increased in the OFS + SCI group than in the nOFS + SCI and SCI groups. This suggests a supporting role of the OFS in axonal and myelination after SCI. Moreover, rats in the OFS + SCI group showed great improvements in sensory and motor functions than did rats in the nOFS + SCI and SCI groups. All these findings suggest that long-lasting OFS applied immediately after SCI can provide a good microenvironment for recovery of damaged spinal tissue by triggering regenerative processes in the acute phase of injury.  

Key Words: axonal regenerative capacity; behavioral assessment; epidural stimulation; motor recovery; myelin regeneration; oscillating field stimulation; spinal cord injury  

Introduction  
Spinal cord injury (SCI) results in permanent or partial motor, sensory, and autonomic impairment. There is a need to seek or participate interventions during the acute phase of injury to reduce secondary injury (Ahuja et al., 2017), but no clinically relevant therapies are available to manage the neurological loss. Electrical stimulation (ES) has been shown to effectively reduce neuropathic pain and muscle atrophy (Huang et al., 2019; Thomaz et al., 2019) and promote regeneration of motor functions. Therefore, ES has been considered a promising treatment for SCI (Levison and Moritz 2017; Darrow et al., 2019). Various types of stimulation treatment have been used with promising results: epidural ES (Wagner et al., 2018), functional ES (Marquez-Chin and Popovich, 2020), intraspinal ES (Saigal et al., 2004; Mondello et al., 2014), and transcutaneous ES (Hofstetter et al., 2015). Although ES promotes the anatomical plasticity of the central nervous system after SCI (Hassannejad et al., 2019) and activates residual neuronal pathways (Smith and Knikou, 2016), the underlying mechanisms are not fully understood. The main reasons for nonfunctional signal transduction after SCI are limited growth capacity of axons, failed axonal sprouting, and loss of myelination of surviving and newly sprouting axons due to the formation of scar tissue around the site of injury, lack of trophic support and limited regenerative capacity of axons caused by demyelination, followed by axonal degeneration (Alizadeh et al., 2015). ES has been shown to be beneficial in these posttraumatic events (Carmel et al., 2010; Jack et al., 2020). Thus, enhancing the functional connectivity of spared circuitry using ES may be a viable means of promoting functional recovery after SCI.

Myelin sheaths formed by oligodendrocytes are responsible for the fast action potential transmission of the CNS. Following SCI, some axons and oligodendrocytes are initially lost via necrosis and mechanical injury (Almad...
et al., 2011). As injury progresses, a massive loss of oligodendrocytes occurs through apoptosis and autophagy that results in demyelination of the injured and spared axons (Casha et al., 2001; Pielem et al., 2014). Although spared axons reinnervate naturally in the CNS after a traumatic event (Salgado-Ceballos et al., 1998), these remyelination attempts are often insufficient and limited due to post-traumatic environment changes (Xing et al., 2014; Hesp et al., 2015).

Oscillating field stimulation (OFS) reverses the polarity of the electric field every 15 minutes to stimulate the regeneration of both ascending and descending neural pathways, which makes it a promising strategy in various models of SCI (Borgens et al., 1999; Li et al., 2019). In our previous experiments, we demonstrated that epidural OFS had neuroprotective effects on spinal tissue during the 4-week study period (Bacova et al., 2019). In this study, we further revealed the effects of epidural OFS applied immediately after spinal compression injury on myelin regeneration, axonal and oligodendrocyte survival, and functional recovery during a 8-week study period. We also observed several major behavioral changes in rats after epidural OFS.

Materials and Methods

Ethics statement

All experiments were approved by the Animal Use Committee at the Institute of Neurobiology, Slovak Academy of Sciences, as well as by the State Veterinary and Food Administration in Bratislava (approval No. 715/19-233/2019, approval No. 4426/16-211/2019, May 3, 2021) and conducted in accordance with the EC Council Directive (2010/63/EU) regarding the use of animals in research. All efforts were made to minimize the number of rats and their suffering during the experiments.

Experimental animals

Thirty-six 3–4 month-old female Wistar rats, weighing 250–300 g, were included in this study. They were randomly assigned to four experimental groups: Intact control (no surgical intervention; n = 6), SCI (T9 compression only; n = 10), OFS + SCI (SCI followed by OFS; n = 10), and nOFS + SCI (SCI followed by implantation of non-functional OF stimulator; n = 10) groups. Animals were housed individually with food and water provided ad libitum. The study design is shown in Figure 1.

Figure 1  Experimental timeline.

Spinal cord compression and OF stimulator implantation

Animals underwent spinal cord compression at the ninth thoracic vertebral region (T9). They were anaesthetized using 250 mg/kg xylazine (25 µl/kg) and 200 mg/kg ketamine (20 µl/kg), and placed in a custom-made miniature stimulator with an oscillating electric field (OF; 50 µA) (Figure 2A) were implanted into the epidural space cranially and caudally to the lesion site (for more details see Bacova et al., 2019). The OFS was applied for 30 minutes at room temperature in PBS with 5% normal goat/rabbit serum and 0.3% Triton-X100. To visualize oligodendrocytes, neurofilaments, and outgrowing axons, the following primary antibodies were used: APC (1:200; monospecific, Leica; Cambridge, MA, USA, Cat# C2826), AFC (1:500, rabbit; Abcam, Cambridge, UK, Cat# ab129990). To visualize myelin (MBP) (1:200; rabbit; Sigma, Cat# M8321), the slices were pre-incubated with 9.9% ethanol for 15 minutes. After overnight incubation at 4°C, the sections were washed in PBS with 0.3% Triton X-100 and incubated with secondary antibodies (FITC goat anti-mouse IgG, 1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-145; FITC goat anti-rabbit IgG, 1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-003; Rhodamine, 1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-006; 1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-004) to stain cell nuclei for 30 minutes. After incubation, the slices were finally washed with PBS-T, rinsed with distilled water, and placed on the slides. Selected regions (dorsal and lateral funiculi of the spinal cord, T9) were captured by fluorescent microscope (Olympus BX51, Tokyo, Japan) with 40x magnification and then analyzed using image software (Version 1.52).

Western blot analysis

Spinal cord segments (T7–T11) were homogenized and the protein concentration was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The samples (20 µg per lane) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%, 500 V) and then transferred into a polyvinylidene difluoride membrane (Bio-Rad Lab, Hercules, CA, USA). After membranes were blocked in 5% skimmed milk (Blotting-Grade Blocker, Bio-Rad Lab) for 2 hours at room temperature, labeled spinal slices were washed again with PBS with 0.3% Triton X-100 and incubated with DAPI (1:10,000, Sigma, Cat# D9542) to stain cell nuclei for 30 minutes. After incubation, the slices were finally washed with PBS-T, rinsed with distilled water, and placed on the slides. The sections were incubated with 250 µl PBS with 0.3% Triton X-100 and incubated with secondary antibodies (FITC goat anti-mouse IgG, 1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-145; FITC goat anti-rabbit IgG, 1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-003; Rhodamine, 1:200; Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-006; 1:200, Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat# 115-095-004) to stain cell nuclei for 30 minutes. After incubation, the slices were finally washed with PBS-T, rinsed with distilled water, and placed on the slides. Selected regions (dorsal and lateral funiculi of the spinal cord, T9) were captured by fluorescent microscope (Olympus BX51, Tokyo, Japan) with 40x magnification and then analyzed using image software (Version 1.52).

Behavioral assessment

The recovery of neurological functions after experimental SCI was analyzed using multiple behavioral tests. Rat sensory and motor functions were evaluated using the hot plate test, open field test and Basso, Bresiner, Bresnahan (BBB) locomotor rating scale during the survival period. Each rat in the experiment was assessed and evaluated individually. All behavioral tests were performed by the same investigator blinded to study parameters to minimize rat stress level and achieve objective results.

Exploration and anxiety tests

The spontaneous activity of rats was assessed using the modified open field test (Martinez et al., 2009), which started 4 weeks post-SCI and was conducted once weekly until the end of study period. The rats were tested in the open field, which was a 60 cm × 45 cm transparent plexiglass box with a non-slippery floor. During a 5-minute interval, basic motor activity, exploration, anxiety, and depressive behavior were observed and evaluated. All monitored parameters were obtained by the descriptive method of direct observation and using the automated system for data processing and analysis (EthoVision XT, Noldus, Wageningen, The Netherlands). The rats were acclimated to the testing room for 20 minutes before testing. The test consisted of placing a single rat in the center of the open field, and when the rat was stabilized, the Open Field program was automatically registered using a video-tracking system and reported as the distance traveled (in centimeters). The duration of exploratory behavior and time spent grooming or freezing were manually scored from video recordings. The arena was cleaned with 70% ethanol between each trial.

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It is well known that after spinal cord trauma, there is massive tissue damage. To prevent tissue damage, the rats were immediately removed after the pain manifestation, or if no response occurred within 40 seconds.

Neurological assessment

The restoration of hind limb motor functions was analyzed using the BBB locomotor scale once every 3 days over the entire study period. The BBB locomotor scale was a 0–21 rating scale that classifies and evaluates hind limb movement, torso stability, gait coordination, paw placement, and tail positioning (Basso et al., 1995).

Cell and tissue analysis

Spinal tissue integrity and the extent of spinal cord injury were analyzed using Image software 1.52 (NIH). After calibrating the scale, the RGB image was converted to 8-bit and then into black-white (BW) format using the Huean threshold (Fedorova and Pavel, 2019) which separates the colored part of the spinal cord and the background. Adjusted images were then used to assess total area of preserved spinal tissue and white matter area. For quantitative analysis of the spinal tissue, selected spinal cord sections were captured by fluorescent microscope (Olympus BX51) with 40x magnification. The same exposure time was used for all compared microphotographs. Fluorescently stained oligodendrocytes and neurofilaments were quantitatively analyzed using ImageJ software in predetermined regions of white matter – dorsal and lateral spinal cord funiculi (Figure 3). The size of analyzed area was the same in all evaluated images (1.1 mm × 1 mm). Selected images were converted into 8-bit and then into black-white (BW) format using the Huean threshold. For quantitative analysis of oligodendrocytes and neurofilaments, the density of fluorescent cells was measured as a mean fluorescence intensity relative to the total sample area. For each spinal segment, five spinal sections were selected for analysis.

Statistical analysis

Power Analysis was performed to verify that the number of animals in groups was sufficient (GPower 3.1 software, University of Kiel, Kiel Germany) based on our statistical tests (one-way analysis of variance). The actual power of the analyzed tests was more than 80%. The experimental data were analyzed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). All data are expressed as means ± standard deviation. One-way analysis of variance followed by the post hoc Tukey’ Honest Significant Difference (HSD) test was used to determine statistical significance between all experimental groups. Correlation analyses were performed with Pearson correlation test and linear regression analysis. Correlation analyses were performed with Pearson correlation test. A level of P < 0.05 was considered statistically significant.

Results

Changes in spinal tissue integrity and myelin preservation after epidural OFS

It is well known that after spinal cord trauma, there is massive tissue damage of spinal parenchyma, leading to great white and gray matter loss. To examine spinal tissue preservation after SCI and OFS, LFB/CV-stained spinal sections were analyzed. Quantitative histological evaluation revealed remarkable degree of sparing of white and gray matter in all examined spinal segments. There was obvious visible difference in spinal tissue integrity between whole spinal cord slices (Figure 4A) and white matter area (Figure 4B) in rats from the OFS + SCI groups. Spinal tissue loss mainly occurred within the epicenter of injury in all experimental groups. Histopathological analysis, however, showed that the percentage of spared tissue at the epicenter of injury (T9) was significantly (P < 0.05) increased in the OFS + SCI group (54.46 ± 8.60%) than in the nOFS + SCI (42.92 ± 6.69%) and SCI group (37.92 ± 2.85%) groups. To determine the extent of spared tissue after SCI and OFS, we also analyzed white matter areas in selected spinal segments (Figure 4C). Epidural OFS strongly promoted myelin preservation, with the most evident changes evaluated in the cranial (T8) spinal segment (78.75 ± 6.5%), compared to the non-stimulated (nOFS + SCI, 54.33 ± 11.6%) and SCI groups were preserved. As shown in Figure 4C, the maximal difference in myelin preservation in the caudal area to the lesion site (T10 and T11) in the OFS + SCI group than in the intact control groups (P < 0.05). Severe tissue loss and damage after spinal cord injury mainly occurred in the dorsal and lateral white matter areas. These regions in the spinal cord consist of nerve fibers (corticospinal tracts) that primarily regulate motor functions and movement control. Therefore, we will focus on these areas using fluorescent and densitometric analyses in future researches.

Effect of epidural stimulation on oligodendrocyte survival and regeneration after spinal trauma

To test the assumption whether OFS applied early modulates the process of remyelination by oligodendrocyte regeneration after trauma was to determine the number and density of oligodendrocytes (APC cells) in the most affected regions. We noticed that the number of oligodendrocytes in both the dorsal cord lateral region was significantly higher in the OFS + SCI group compared to the nOFS + SCI and SCI groups (Figure 5A). The greatest increase in the number of APC cells in the dorsal funiculi was observed in the segments adjacent to the injury site (Figure 5B), compared to the dorsal and lateral white matter areas analyzed in the T8, T9, and T10 spinal segments (T8: OFS + SCI group, 70.6 ± 2.9; nOFS + SCI group, 52.2 ± 2.5; SCI group, 50.6 ± 3.9; T9: segment: OFS + SCI group, 72.4 ± 5.3; nOFS + SCI group, 49.1 ± 2.9; SCI group, 45.9 ± 2.1). Similarly, the most significant change in the number of oligodenrocytes in the lateral region was observed in T10 spinal segment (OFS + SCI group, 81.4 ± 4.1; nOFS + SCI group, 55.2 ± 3.6; SCI group, 43.6 ± 2.4, P < 0.01). As shown in Figure 5C, the number and density of APC cells were increased in the OFS + SCI group than in the nOFS + SCI and SCI groups. The greatest difference in myelin preservation in the OFS + SCI group compared to the nOFS + SCI groups (P < 0.01). The density of APC cells in the dorsal and lateral white matter areas was significantly higher in the OFS + SCI group than intact control groups (P < 0.05). The greatest differences in the number of APC cells in the dorsal funiculi were observed in the OFS + SCI group compared to the SCI group (P < 0.01). The number of APC+ nerve fibers was higher in the dorsal T11 spinal segment (OFS + SCI group, 15.4 ± 0.7%; nOFS + SCI group, 11.5 ± 0.6%; SCI group, 10.6 ± 0.6% and T10 (OFS + SCI group, 14.7 ± 0.4%; nOFS + SCI group, 10.7 ± 0.5%; SCI group, 10.1 ± 0.6%).

Regenerative capacity of axons after spinal trauma and OFS stimulation

Damage to the spinal cord causes irreversible disruption of nerve fiber integrity, mainly at the epicenter of injury, which is one of the main issues for restoring spinal cord functions after SCI. To evaluate the effect of OFS applied early on preservation of spared axons after spinal trauma, we quantified neurofilaments (NF-1) in the most affected regions of white matter – dorsal and lateral spinal cord funiculi. As shown in Figure 6A, the number of newly sprouted GAP-43+ axons was significantly elevated in the OFS + SCI group (5.9 ± 0.2%) and SCI group (6.2 ± 0.6%). Interestingly, the number of newly sprouted GAP-43+ axons was slightly elevated (7.3 ± 0.4%) in the OFS + SCI group, compared with the nOFS + SCI group (5.4 ± 0.2%). There was also a significant difference in the number of newly sprouted GAP-43+ axons at the lesion site (T8) in the OFS + SCI group than in the intact control groups (P < 0.05). The density of newly sprouted GAP-43+ axons was significantly higher in the OFS + SCI group (3.0 ± 0.5%) than with the nOFS + SCI group (1.7 ± 0.6%) and SCI group (1.4 ± 0.4%) (all P < 0.05).

Changes in NF and GAP-43 protein levels after spinal trauma followed by epidural OFS treatment

To determine whether OFS affected axonal regeneration at the protein level, we performed western blot analyses of NF-1 and GAP-43 proteins. As shown in Figure 7, at 8 weeks after spinal compression, there continued to exist a considerable decline in NF-1 and GAP-43 protein levels in all experimental groups compared to the intact control group. NF-1 protein levels were reduced in all studied spinal segments in the OFS + SCI group compared to the nOFS + SCI and SCI groups. Statistically significant differences were observed in only the segments (T8, T10) adjacent to the epicenter of injury (P < 0.05). Figure 1.A Figure 2 | Principle of stimulation using oscillating electric field. It is known that the axons respond immediately to the voltage gradient and tend to orient themselves parallel to the long axis of such a gradient. Nerve fibers grow faster and preferentially towards the cathode (negative pole of applied electric field), and are distracted from the opposite pole. (A) Schematic picture of oscillating field stimulator being prepared for stimulation. (B) Subcutaneous stimulator with epidural implantation of stimulating Pt wires. (C) Caudally placed cathode stimulates the regeneration and growth of motor nerve fibers. Cathode placed cranially to the lesion promotes the recovery of sensory fibers. Oscillating polarity of electric field every 15 minutes allows stimulation of the outgrowth of sensory and motor axons simultaneously.
Spinal tissue integrity assessment after NF-100 | Regenerative capacity of damage axons after spinal trauma and OFS treatment. Analysis of changes in the number of oligodendrocytes after spinal trauma and epidural OFS treatment.

Figure 4 | Spinal tissue integrity assessment after spinal cord compression and OFS. (A) Histological staining of the spinal injury epicenter in all experimental groups (scale bar: 600 µm). (B) Spinal cord tissue integrity at the site of injury and in adjacent cranio-caudal regions of the spinal cord. (C) Preservation of white matter in all experimental groups. Data are presented as the mean ± SD (n = 5) and were analyzed using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; *P < 0.05, ***P < 0.001. nOFS: Implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

Figure 5 | Analysis of changes in the number of oligodendrocytes after spinal trauma and epidural OFS treatment. (A) Immunohistological representative microphotographs of spinal cord dorsal funiculi within the T11 spinal segment 8 weeks post-spinal compression injury (scale bar: 500 µm). (B) Quantitative analysis of oligodendrocytes (APC+ cells) in dorsal and lateral funiculi. (C) Results of oligodendrocyte densitometry in dorsal and lateral white matter areas. Data are presented as the mean ± SD (n = 5). Results were statistically analyzed using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; *P < 0.05, **P < 0.01. nOFS: Implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

Figure 6 | Regenerative capacity of damage axons after spinal trauma and OFS treatment. (A) Fluorescent microphotographs of longitudinal spinal cord sections at the site of injury after 8 weeks of survival (scale bar: 1000 µm). The fluorescent intensity of spared axons (neurofilaments [NF-1]; green) and newly sprouted axons (growth-associated proteins [GAP-43]; red) after spinal compression and OFS (scale bar: 100 µm). (B) Quantitative analysis of spared axons in the dorsal and lateral funiculus (transverse spinal cord sections). (C) Densitometric analysis of axons in the longitudinal sections of the spinal cord lesion. Epidural OFS greatly supported axonal sprouting in the spinal cord lesion after 8 weeks of animal survival. Data are presented as the mean ± SD (n = 5). Results were statistically analyzed using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; *P < 0.05, **P < 0.01. nOFS: Implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

7A). There was a more significant difference in GAP-43 protein level in the outgrowing nerve fibers between groups. Spinal segment T8 was the most affected spinal cord region by OFS. A significant increase in GAP-43 protein level was observed in the OFS + SCI group compared to the nOFS + SCI and SCI groups. A neuroregenerative effect of OFS treatment was also observed in the caudal spinal segments, in which GAP-43 protein level was significantly elevated (T10: P < 0.05; T11: P < 0.01) in the OFS + SCI group compared to the nOFS + SCI and SCI groups (Figure 7B).

Differences in myelin immunoreactivity and protein levels after spinal cord compression and OFS treatment.

MBP is the most abundant protein component in the myelin membrane in the CNS. By interacting with lipids in the myelin membrane, MBPs maintain the correct structure of myelin and are therefore considered important for the myelination process (Deber and Reynolds, 1991). To evaluate the changes after epidural OFS treatment, we analyzed the immunoreactivity of MBP and NF-1 co-localization in each experimental group. MBP fluorescent staining showed moderate fluorescent signals in all groups, with a slight loss of signal in the dorsal and lateral white matter areas. Co-localization with markers specific for NF-1 shows visible differences between the stimulated and unstimulated animals. Figure 8A shows reduced MBP/NF-1 signals in the SCI and nOFS + SCI groups, compared to the OFS + SCI group.

To confirm the results, we performed protein analysis of MBP. Western blot results revealed major differences in MBP level (Figure 8C) between experimental groups at 8 weeks after surgery. MBP protein expression was
markedly decreased in the nOFS + SCI and SCI groups than in the intact control group in all studied segments (Figure 8B). Interestingly, an elevated level of MBP protein relative to that in the intact control group was observed only in the OFS + SCI group at the site of injury and in cranio-caudal segments. While MBP protein levels decreased in unstimulated groups, the OFS + SCI group showed significantly higher values at the caudal and cranial segments ($P < 0.001$, $P < 0.05$).

Functional recovery of spinal cord after early epidural OFS
Motor function restoration
After T9 spinal compression, rats were completely paraplegic with a neurological score of 0. BBB scores increase in each group over time (Figure 9A). In the first 3 weeks after surgery, no statistically significant differences in BBB score were observed between OFS + SCI and nOFS + SCI and SCI groups. As shown in Figure 9B, the first prominent motor improvement ($P < 0.05$) in BBB score was observed in the OFS + SCI group 4 weeks after SCI, compared with the nOFS + SCI and SCI groups. An increasing trend was observed in the OFS + SCI group until the end of study period. At 8 weeks post-surgery (Figure 9C), rats subjected to implantation of OF stimulators were able to move extensively in all three joints with an independent posture and slight frequent “sweeping” movements of the hind limbs (nOFS + SCI group, $9.7 ± 0.6$; SCI group, $9.8 ± 1.3$).

Behavioral analysis data showed significant changes ($P < 0.01$) in locomotor activity between the experimental groups during the 8-week study period (Figure 10A and E). Rats not subjected to OFS treatment showed lower spontaneous locomotor activity and velocity compared to rats subjected to an active OFS. Considering that reduced locomotor functions in SCI groups could interfere with exploratory behavior, which is the rodent’s fundamental type of behavior, we evaluated exploration along with locomotor activity. An increased exploratory activity was observed in the OFS + SCI group than in the nOFS + SCI and SCI groups (both $P < 0.05$; Figure 10D). On the contrary, significantly more manifestations of depressive behavior (freezing frequency, $P < 0.01$; freezing duration, $P < 0.05$) were observed in the nOFS + SCI and SCI groups compared to the nOFS + SCI group (Figure 10C). Grooming and self-cleaning licking, as a sign of comfort behavior, was also evaluated. Rats subjected to active OFS showed more grooming than those not subjected to stimulation ($P < 0.01$; Figure 10B). There were no significant differences in the frequencies of urination and defecation during the OFS between groups.

Sensory function
During the hot plate test, a radical prolongation of response latency (time when the rat started to show nociceptive signals as forepaw/hindpaw withdrawal or licking) was observed in rats with SCI. At 4 weeks post-SCI, the mean response latency was 7.9 ± 0.2 seconds in the intact control group and it was significantly prolonged in all experimental groups (17.9 ± 2.1 seconds). In following weeks, we observed gradual shortening of response latency in all tested rats, with more pronounced differences in the OFS + SCI group (Figure 11B). Our results showed that rats subjected to OF had profoundly shorter latency 8 weeks post-SCI (10.9 ± 0.6 seconds) compared to rats subjected to non-functional stimulation (14.6 ± 0.8 seconds), and rats only subjected to SCI (14.4 ± 0.7 seconds). At 8 weeks post-SCI, a strong reduction in hot plate response latency was observed in rats with high BBB score, as confirmed by correlation analysis (Figure 12A). Similarly, hind limb regeneration as evaluated by neurological BBB score was strongly correlated with the percentage of spared spinal cord tissue after injury (Figure 12B).

Discussion
Application of OFS is known to facilitate the bidirectional regeneration of axons after SCI (Borgens et al., 1999; Hamid and Hajek 2008; Walters, 2010; Zhang et al., 2014; 2015). Results of our previous study confirmed the beneficial properties of short-term epidural OFS via the implantation of the miniature, originally designed OF stimulator (Bacova et al., 2019). The main objective of the present study was to investigate the further impact of constant epidural OFS on functional recovery after T9 spinal compression during long-term survival.

![Image](https://www.nrronline.org/ResearchArticle/2022/12/2734 figure7.jpg)

**Figure 7 | Assessment of neurofilament and newly formed axon protein levels after SCI and OFS treatment.** (A, B) Graphs depict the protein levels of neurofilaments (NF-I; A) and newly sprouted axons (growth-associated proteins [GAP-43], B) relative to the β-actin level, compared to the intact control group. Data are presented as the mean ± SD ($n = 5$). The results were statistically analyzed using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; $P < 0.05$, ***$P < 0.01$, ****$P < 0.001$. Con: Intact control; nOFS: implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

![Image](https://www.nrronline.org/ResearchArticle/2022/12/2734 figure8.jpg)

**Figure 8 | Immunoreactivity and protein level of myelin basic protein in spinal tissue 8 weeks after spinal compression injury and OFS treatment.** (A) Fluorescent illustrations of co-localization of myelin basic protein (MBP; green) and neurofilament (NF-I; red) within the T11 spinal segment in all experimental groups (right, scale bar, 100 μm; left, scale bar, 1000 μm). (B) Western blot analysis revealed major changes in MBP protein level in treated (OFS + SCI) and untreated (SCI and nOFS + SCI) groups of animals compared to the intact control animals. (C) Immunoblotting picture of MBP. Data presented as means ± standard deviation ($n = 5$). The results were statistically analyzed using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; $P < 0.05$, ***$P < 0.01$, ****$P < 0.001$. Con: Intact control; nOFS: implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

![Image](https://www.nrronline.org/ResearchArticle/2022/12/2734 figure9.jpg)

**Figure 9 | Neurological evaluation of hind limb locomotor activity after spinal injury and OFS using rat BBB scale.** (A) Rats with OFS showed better recovery of hind limb functions during the 8-week study period. (B) Four weeks post-SCI, significant differences in functional recovery started to appear. (C) At the end of the study period, the differences between the experimental groups were even more pronounced. Data are presented as the mean ± SD ($n = 10$). The results were statistically analyzed using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; $P < 0.05$, ***$P < 0.01$. BBB: Basso, Bresnahan locomotor rating scale; nOFS: Implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.
The effect of epidural OFS on spontaneous locomotor activity and stress/comfort-related behavior in rats after spinal compression injury.

(A) Locomotor activity (distance, velocity) in animals after spinal trauma and followed OFS were evaluated during 5-minute testing interval. (B, C) Stress/comfort-related behavior, frequency and time spent in freezing and self-grooming were evaluated by exploratory behavior analysis of animals during the open field test. (D) Locomotor activity in experimental animals. (E) The data were obtained at the end of experiment (8 weeks post-SCI). Data are presented as the mean ± SD (n = 5). The results were statistically evaluated using one-way analysis of variance followed by the post hoc Tukey’s honest significant difference test; **P < 0.01; ††*P < 0.001; †††*P < 0.0001; nOFS: Implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

Figure 12 | Correlation analysis of response latency in hot plate test and spared tissue analysis versus BBB neurological function scores (Pearson’s correlation test). Significant negative correlation was found between neurological function evaluation versus hot plate response latency (A), while BBB score was significantly positively correlated with histological analysis of tissue integrity (B) at 8 weeks post-SCI and OFS. Scatterplots of individual values (n = 5) with regression line correlation coefficient (r) and coefficients of determination (R²) calculated by linear regression analysis. BBB: Basso, Baetge, Bresnahan locomotor rating scale; nOFS: Implanted electrical stimulator without stimulation; OFS: oscillating field stimulation; SCI: spinal compression injury.

One of the most evident changes after SCI is the massive loss of spinal cord tissue (Smith and Jeffrey, 2006). In this study, early epidural stimulation using an oscillating electric field was shown to be protective on spinal cord tissue and myelin preservation. LFB-positive staining of the myelin showed that epidural OFS markedly increased the myelin area in the segments adjacent to the lesion site. Previous studies have proposed that ES can promote spinal tissue integrity and contribute to remyelination after SCI via improving differentiation of oligodendrocyte precursor cells (Zhang et al., 2014; Jing et al., 2015). Oligodendrocytes, as specialized glial cells, are known to play a key role in myelin formation, integrity, and maintenance (Simons and Nave, 2015). To investigate the association between myelin recovery and oligodendrocyte regeneration after OFS, a quantitative and densitometric analysis of immunolabeled oligodendrocytes was performed. Our results confirmed that epidural OFS not only reduces oligodendrocyte loss, but also promotes their density directly in areas of greatest tissue damage (T8, T10 spinal segments). Mature oligodendrocytes are also the main cells that express myelin protein, such as MBP (Bernardo et al., 2013). Therefore, we used a western blot analysis of MBP to verify the impact of epidural OFS on the promotion of remyelination after SCI. We found that OFS strongly increased MBP levels in cranial and caudal spinal segments of the lesion. A similar beneficial outcome was reported in a study of epidural stimulation after SCI, in which the stimulation upregulated MBP and mRNA levels and reduced oligodendrocyte loss by promoting their differentiation and inhibiting apoptosis (Li et al., 2020). Our findings in this study indicate that long-term epidural OFS may contribute to the recovery of myelination after T9 spinal injury by inhibiting oligodendrocyte loss and promoting tissue and myelin regeneration.

Axonal regeneration after SCI is significantly limited. The insufficient activation of regenerative processes in neuronal cells is one of the key factors contributing to the lack of axonal post-traumatic regeneration. Moreover, growing axons need to be oriented correctly to re-establish functional reconnections across the lesion site. Several experimental studies have reported that ES applied early after SCI might be crucial for the promotion of axonal overgrowth (Haan and Song 2014; Zhang et al., 2015). Our hypothesis is that OFS-induced sparing of spinal tissue should correlate with enhanced axonal regenerative capacity. For that reason, we performed quantitative and densitometric analyses of spared NF-1 and GAP-43 nerve fibers, which corresponded to newly outgrowing fibers with the ability to rebuild neural connections after CNS damage. Our data showed more pronounced regeneration, increased number of NF-1 fibers and density of new GAP-43 fibers in selected regions in the animals with active OFS. Our findings were also confirmed by western blot analysis, which indicated a profoundly increased protein level of NF-1 and GAP-43 in rats with active OFS. We assume that the early application of weak OF current to the damaged spinal cord could be a trigger for the initiation of the regenerative processes since the pro-regenerative and major inflammatory processes are initiated within the first week after SCI (Bimbova et al., 2018).

In the context of these findings, behavioral tests confirmed a progressive improvement of the motor function in rats with active OFS. We noticed the most obvious changes 7 weeks after the traumatic event, which indicates the gradual effect of the initial OFS to restore motor function. Tian et al. (2016) reported functional improvement after OFS (40 µA) within 4 weeks after SCI; however, the effect of stimulation remarkably decreased after 10 weeks. The BBB test performed in this study showed slow and gradual improvement in motor functions, which became statistically significant in rats subjected to active OFS at 4 weeks and continued further for up to 8 weeks.

The early neurological improvement might be due to the modulation of the inflammatory response caused by the reduction of reactive astrocytes involved in glial scar formation, as a response to OFS application (Bacova et al., 2019). This beneficial outcome correlates with the spared white matter area, which was approximately 20% higher in the stimulated group of animals. Besides
locomotory impairment, the inflammatory response after SCI often affects the development of neuropsychiatric disorders, including anxiety and depression (do Espirito Santo et al., 2019). To exclude the negative impact of OFS applied early after injury on spontaneous activity, mental health and well-being of rats, we performed open field testing in rats. In our recent study, we demonstrated significantly increased motility, exploration, and self-cleaning (comforting type of behavior – grooming), indicating that the behavior of rats in the OFS + SCI group was not altered by OFS induced by one application. An impact of OFS on behavioral recovery after SCI is the regeneration of sensory functions. In a recent study, we used the hot plate test to examine the integrity of supraspinal pathways in animals with or without active OFS. The hot plate test is often used to detect sensory system disorders, such as hypoaesthesia, allodynia, or loss of sensory functions caused by SCI (Fischer and Peduzzi 2007; Sedy et al., 2008; Jalan et al., 2017). Our results showed that the recovery of sensory functions did not differ between the untreated animals and the animals that underwent OFS until 7 weeks post-SCI. Thus, our findings were consistent with the results of the neurological assessment, in which the most visible changes between groups were also observed 7 weeks after SCI. Despite the fact that we noticed differences between the groups within the first week of testing, a statistical significance was demonstrated up to 7 weeks after SCI. We expect that, for optimal assessment of sensory and motor function recovery, long-term experiments in the future are necessary.

In our recent study, we used histological, immunohistochemical, molecular and behavioral methods to investigate the potential of weak, long-term OFS on tissue regeneration and functional recovery after severe spinal cord damage. Our results confirm that immediate, epidural OFS can act as a suitable therapeutic strategy that can contribute to the activation of regenerative processes in the acute phase of SCI, with a long-term effect on the chronic stage of disease. Despite many beneficial properties of OFS, further research is needed due to the limited number of studies focused on OFS after SCI. Their outcomes are very difficult to compare because of wide divergence of models and methods of stimulation. OFS should not be considered as a sole treatment for SCI, it should potentiate recovery that can be achieved by other strategies. Combining several therapeutic strategies to achieve maximal functional recovery, areas for our further study.

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Conflicts of Interest: None declared.

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