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Exploring synergistic effects: Atorvastatin and electrical stimulation in spinal cord injury therapy

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ABSTRACT

Spinal cord trauma represents a significant clinical challenge, and improving patient outcomes is a main priority for many scientific teams globally. Despite advances in the understanding its pathogenesis, the overall mechanisms occurring in the spinal cord after traumatic injury remain unclear. This study explores the possible synergistic effects of a regenerative therapy that combines electrical stimulation with the anti-inflammatory drug Atorvastatin (ATR) after spinal cord injury (SCI). SCI was induced at the T9 segment under isoflurane anesthesia and applying a compression force of 40 g for 15 minutes. An oscillating field stimulator (OFS) was implanted subcutaneously, delivering a weak electric current (50 μA) that changed polarity every 15 minutes for six weeks to promote axonal growth at the injury site. Female Wistar albino rats were divided into four groups: SCI with non-functional stimulator (SCI + nOFS), SCI with functional stimulator (SCI+OFS), and two groups that received ATR together with stimulator for 7 days after injury (SCI+OFS+ATR, SCI+nOFS+ATR). Behavioral tests (hot-plate test and BBB scale) showed improvement in sensory and motor performance in animals treated with the combination therapy. The protein levels of astrocytes (GFAP), neurofilaments (NF-L), newly sprouting axons (GAP-43), and oligodendrocytes (PLP −1, CNPase) were analysed by Western blot. The results showed increased neurofilaments, newly sprouting axons and oligodendrocytes in groups receiving both individual and combination therapies, with a decrease in their concentrations in the following order: SCI+OFS+ATR, SCI+nOFS+ATR, SCI+OFS, SCI+nOFS. In addition, astrocyte protein levels were lower in the SCI+OFS+ATR group compared with others. Histological analysis showed a significant reduction in white and gray matter after SCI, but less white and gray matter volume loss was found in the groups receiving therapies (SCI+OFS+ATR, SCI+nOFS+ATR, SCI+OFS). These results suggest that the combination of Atorvastatin with OFS stimulation promotes neural recovery after SCI, highlighting the potential of combination therapies in enhancing regenerative outcomes.

Introduction

Acute traumatic spinal cord injury (SCI) has devastating effects. In addition to the deterioration or complete loss of motor, sensory and autonomic functions of the spinal cord, SCI also has a negative impact on the patient's mental health (Eli et al. 2021). Currently there is no effective treatment for SCI that can adequately restore lost tissue function.

SCI is a complex set of events and processes and cannot be cured by a single intervention. Therefore, the attention of the scientific community is focused on various therapeutic interventions to enhance the recovery of neuronal function.

In this study, we investigated the effects of a combination therapy, consisting of combined application of weak electrical field (Bacova et al. 2022) and the drug Atorvastatin (Bimbova et al. 2018) over a six-week study period.

Electrical stimulation is one of the current strategies to restore functional status after damage. Its goal is to restore nerve signal transmission by forming new neural pathways or promoting axon regeneration (Schuhfried et al. 2012).

A direct current electric field stimulates axonal outgrowth in only one direction (towards the cathode), and suppresses growth in the

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opposite direction (towards the anode). To restore both sensory and motor functions (ascending and descending nerve fibers), the principle of oscillating field stimulation (OFS) has been proposed to promote axonal overgrowth over the spinal cord lesion site in both directions (McGinnis and Murphy, 1992, McCaig, 1987, Borgens et al. 1993). The polarity of the OF stimulator's electric field changes periodically every 15 minutes. This time interval is sufficient to promote axon growth towards the cathode, but does not yet suppress growth in the direction away from the anode (Borgens et al. 1999, Shapiro et al. 2005, Li, 2019).

Atorvastatin (ATR) is a drug from the statins family, that is currently used to treat high cholesterol and coronary atherosclerosis. A number of benefits contribute to their use, including suppression of apoptosis, antioxidant and anti-inflammatory effects, immunomodulation and promotion of tissue regeneration. It is believed that they may exhibit neuroprotective properties, that contribute to reducing the severity of the pathophysiology (Blauw et al. 1997, Komukai et al. 2014, Pordal et al. 2015).

ATR has been studied in various ischemia-reperfusion and traumatic SCI models. Studies have shown its effectiveness in reducing inflammation, inhibiting apoptosis and demyelination after trauma (Bimbova et al. 2018). A significant decrease in the release of pro-inflammatory cytokines, inhibition of macrophage infiltration and microglia activation has also been reported (Pannu et al. 2005, Pannu et al. 2007, Gao et al. 2016).

ATR has been selected as a treatment for spinal cord injury therapy over anti-inflammatory drugs due to several compelling factors that highlight its unique benefits in promoting neuroregeneration. It was also selected due to its pleiotropic properties that extend beyond its primary role as a cholesterol-lowering agent. For example, ATR has been found to decrease important molecules involved in programmed cell death (apoptosis), such as caspase-3 cleavage and expression of Bcl-2 and Bax in neurons (Pan et al. 2010, Hasanvand et al. 2020, Lima et al. 2022).

The choice of ATR was also influenced by the promising data from our previous experiments published in the paper of Bimbova et al. (2018), where ATR had a positive effect.

We expect that the early application of electrical gradient (OFS) in combination with the administration of Atorvastatin in the acute phase of SCI will synergistically enhance regenerative the processes in the damaged tissue of the spinal cord.

Material and methods

Ethics statement

All animal experiments were performed in accordance with the European Communities Directive 2010/63/EU and ARRIVE guidelines. Studies were approved by the State Veterinary and Food Administration in Bratislava (Decision No. 4434/16–221/3) and by the Institutional Animal Care and Use Committee. Every effort was made to reduce the suffering of the rats used in this study and minimize the number of animals.

Experimental animals

A total of 32 adult female Wistar rats (250–350 g) were used in the experiment. Animals were randomly divided into 4 groups: rats with SCI induced by compression of the 9th thoracic segment (T9) and implantation of a non-functional stimulator (SCI+nOFS, n = 8), with SCI and implantation of a functional stimulator (SCI+OFS, n = 8) and two equivalent groups of SCI animals, that, in addition to implanted stimulators, received ATR (5 mg/kg, i.p.) daily for the first 7 days after compression (SCI+OFS+ATR, n = 8; SCI+nOFS+ATR, n = 8).

Surgery

Model of compression-induced spinal cord injury (SCI)

Surgical procedure was performed under isoflurane anesthesia (2 – 4 %; Vetpharma, Barcelona, Spain; in 1.5 – 2.0 L/min oxygen) delivered by facemask. The skin around the site of lesion was disinfected with betadine (EGIS Pharmaceuticals PLC, Budapest, Hungary) and shaved. A laminectomy was performed at the T9 level, which was the site of the injury. In addition to the main laminectomy, two small laminectomies were performed, two segments cranial and caudal to the site of injury. SCI was induced using a compression device with a plastic impactor weighing 40 g (base dimensions: 2,5×2,0; WxL) for 15 minutes (Fedorova and Pavel, 2019). Compression depth was not measured, as this is not a contusion injury model. In our model the impactor is gently placed on the surface of the spinal cord and ischemic-compressive injury is caused only by its weight (not by controlled impact).

Stimulator design

A custom-designed miniature oscillating field stimulator was developed specifically for this and our previous studies (For more details see Bacova et al. 2019, 2022). The device operates using two constant current sources, each configured to deliver an output current of ± 50 μA. The current polarity is automatically reversed every 15 minutes through a built-in timing unit. The stimulator, with compact dimensions of 22 × 12.5 × 3.5 mm and weight of 1.5 g, is powered by a 3 V lithium battery (48 mAh), providing sufficient energy for continuous operation over a minimum of 6 weeks without interruption.

Stimulator implantation

Prior to surgical implantation, the stimulator was encapsulated in a silicone-based material (Duosil Express, SHERA, Lemförde, Germany), selected and tested for biocompatibility to ensure safety in vivo. The insulated stimulator is sufficiently small to be implanted subcutaneously without causing harm or restricting the animal's movement.

Immediately after compression, two inert electrodes (Pt/Ir) attached to the OFS stimulator were inserted through small laminectomies into the epidural space cranially and caudally from the lesion area, and stimulator was implanted subcutaneously on the dorsal part of the rat's body (For more details, see Bacova et al. 2019, 2022).

Postoperative treatment

Atorvastatin administration

Selected groups of animals received the drug Atorvastatin (5 mg/kg i.p.; Fluka by Sigma Aldrich, St. Louis, MO, USA) for 7 days after compression. The powder of ATR (100 mg) was dissolved in sterile dimethyl sulfoxide (DMSO; 20 ml) solution and adjusted according to body weight.

Health status

To avoid post-operative infection, the animals received the antibiotic Amoksiklav (Sandoz Pharmaceuticals, Ljubljana, Slovenia; 30 mg/kg i.m.) and the analgetic Novasul (Richterpharma Wels, Austria; 2 ml/kg i.m.) for 3 days following surgery. For the purpose of preventing dehydratation following surgery, sterile saline solution (5 ml) (Bieffe Medital S.P.A., Grosotto, Italy) was subcutaneously injected for 5 days. The animals were housed individually with unlimited access to food and water. Every 7 days through survival period, animals were regularly weighed.

After SCI, urine was gently manually voided twice daily until bladder reflexes were restored. Recovery of bladder function in animals was assessed using a quantification method: 0 – manual bladder emptying; 1 – spontaneous bladder emptying.

After 6 weeks of survival, animals were decapitated and spinal tissue (+2, +1, LC, −1, −2) was collected for Western Blot analysis. For histological and immunohistochemical analyses, animals were

transcardially perfused using 300 ml of saline followed by 300 ml of 4 % paraformaldehyde.

Behavioral assessment

During the survival period, the functional improvement of neurological functions after SCI was tested by behavioral tests that focused on the recovery of sensory (hot-plate test) and locomotor functions. Locomotor functions were assessed using the Basso-Beattie-Bresnahan rating scale (BBB). Each animal involved in the study was individually assessed and graded.

Locomotor behavioral analysis

Recovery of hindlimb motor function was assessed every 7 days throughout the survival period. The BBB score focuces on the subjective assessment of hindlimb locomotor function using a 21-point rating scale. Each point on the scale represents a specific stage of joint movement, forelimb and hindlimb coordination, stability and tail position (0 – no detectable hindlimb movement, 21 – regular hindlimb movement) (Basso et al. 1995, Fehlings and Tator, 1995).

Sensory function analysis

To assess the improvement and recovery of sensory function, the hot-plate test was performed in all 4 experimental groups. The test was repeated every 2 weeks from the induction of SCI until the end of the survival period. The principle of this method consists in placing the animal on a heated plate with a temperature of 50 – 55°C, where the main monitored parameter is the latency time period – the time from placing the animal on the plate to the manifestation of pain (paw licking or jumping) (Giglio et al. 2006, Khandelwal and Khanna, 2020).

Spinal cord tissue processing

Western blot analysis

Segments (+2, +1, LC, −1, −2) of the spinal cord were homogenized in cold RIPA buffer containing protease inhibitor cocktail (Roche Diagnostic, GmbG, Manhelm, Germany, Sigma Aldrich). Protein content was measured using the Pierce TM BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). 20 micrograms per tube were separated on a NuPAGE™ 10–15 % Bis-Tris protein gel (Invitrogen™, MA, USA), and then transferred to a polyvinylidene difluoride membrane (Bio–Rad Lab., USA). The membranes were then blocked in 5 % nonfat milk for 90 minutes at room temperature and incubated overnight at 4°C with primary antibody diluted in 2,5 % TBS-T (milk/Tris-buffered saline and Tween®20). The following primary antibodies were used to analyse astrocytes: anti-mouse GFAP (1:1000; Merck, Millipore, Burlington, MA, USA), neurofilaments: anti-rabbit NF-1 (1:1000; Cell Signaling, Danvers, MA, USA), newly sprouting axons: anti-rabbit GAP-43 (1:500; Merck, Millipore, Burlington, MA, USA), oligodendrocytes: anti-rabbit PLP-1 (1:1000, Cell Signaling, Danvers, MA, USA), oligodendrocytes: anti-rabbit CNPase (1:1000; Cell Signaling Danvers, MA, USA). On the second day, the membranes were washed four times in TBS-T for 5 min and incubated with the following secondary antibodies: mouse anti-rabbit IgG HRP (1:10,000; Santa Cruz Biotechnology, TX, USA) and goat anti-mouse (1:10,000; Merck, Millipore, Burlington, MA, USA) diluted in 2.5 % milk/TBS-T for 90 min. Protein bands were visualized via chemiluminescence solution and exposed on a Fusion XT scanner (Vilber Collégien, France). After exposure, membranes were stripped through stripping buffer (Restore™ Plus Western blot Stripping Buffer, Thermo Fisher Scientific, MA, USA) and incubated with the monoclonal antibody against β-actin HRP (1:20,000; Abcam, UK) as a loading control for 1,5 hod. The relative expression of the target molecule was quantified by measuring the average optical density of the protein band using ImageJ software (version 2.1.0). This Western blot analysis was subsequently supported by an illustrative immunohistochemical method for complementary localization of the studied protein.

Immunohistochemical labeling

Isolated spinal cord tissue was frozen and cut into 25 μm slices using a cryostat (Leica CM1850, Wetzlar, Germany). Subsequently, sections were washed in PBS-T (0.1 M Phosphate-buffered saline + 0,3 % Triton X-100) three times for 10 min each. After washing, the spinal cord tissue was blocked in PBS-T with 5 % normal goat serum for 30 minutes at room temperature. To visualize reactive astrogliosis, outgrowing axons and oligodendrocytes, the primary antibodies were used: GFAP (1:600; mouse, Millipore, Burlington, MA, USA), GAP-43 (1:500, mouse, Abcam, Cambridge, UK), APC (1:200; mouse, Millipore, Burlington, MA, USA).

After overnight incubation at 4°C, the sections were washed again three times for 10 min each in PBS-T and incubated with secondary antibodies: goat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, Cat# 115–295–003) and goat anti-rabbit IgG (1:800; Jackson ImmunoResearch Laboratories, West Grove, PA, USA, Cat # 111–295–144) dissolved in PBS-T for 1,5 hod at room temperature. After that sections were washed a final time three times for 10 minutes each in PBS-T and placed on Superfrost Plus slides (Thermo Fisher Scientific, MA, USA). Mounting medium (Serva Serving Scientist, Germany) was used to adhere the cover-slips to the microscope Superfrost Plus slides. Selected areas were visualised and photographed using an Olympus BX51 fluorescent microscope (Tokyo, Japan) at 10x magnification. This method was not quantified (Fig. 3F).

Histological staining (Luxol fast blue/Cresyl violet)

The spinal cord tissue was isolated for histological analysis was cut using a cryostat (Leica CM1850, Wetzlar, Germany) into 25 μm sections and placed on Superfrost Plus slides (Thermo Fisher Scientific, MA, USA). Histological analysis was performed using standard Luxol fast blue/Cresyl violet (LFB/CV) staining. Spinal cord sections were incubated in 0.1 M PBS for 10 min, in 70 % ethanol for 120 minutes and in 0.1 % LFB solution for and overnight period. The following day, the spinal sections were washed in distilled water, decolored with 0.05 % lithium carbonate and 40 % ethanol in order to increase the visibility of the white and gray matter of the spinal cord, and then incubated for 10 min with 0,2 % CV. Consequently, the sections were washed with distilled water before being dried with a graded series of ethanol (80 %, 90 %, 99,8 %), cleaned with xylene and covered by cover-slips. Stained spinal cord sections were scanned using an automatic digital scanner Aperio AT2 (Leica Biosystems, Nussloch, Germany) with 20x magnification and analysed with ImageJ software (version 2.1.0).

Statistical analysis

All obtained data were statistically analyzed using GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA). Results are presented as mean ± SEM. Prior to conducting the variance analysis, a normality assessment was carried out using the Shapiro-Wilk and Kolmogorov-Smirnov tests, which verified that the data followed a normal distribution. This allowed for the application of a parametric test. To determine the level of significant statistic differences between experimental groups we used ordinary one-way and two-way analysis of variance (ANOVA) test with multiple comparisons using Sidak method and Friedman's Anova with Tukey's post-hoc test (P value < 0.05). Statistically significant differences were considered by P value ($P < 0.05$). Data for voiding score were evaluated by contingency table and then analysed by chi-square test followed by Yates correction. Statistically significant differences were considered by P value ($P < 0.05$).

Results

Physiological parameters

Weight changes

Weight loss is one of the most common consequences of SCI. The weight change was observed in all experimental groups during the first week after induction of spinal cord trauma. Subsequently in all

experimental groups, during the 4–6-week treatment period, the weight of the animals was gradually restored to the original weight (Fig. 1A).

Voiding score assessment

Animals with SCI lose the ability to urinate spontaneously. Thus, the bladder of the animals was emptied manually twice a day or as needed. Bladder function was restored within 7 days in the SCI+OFS+ATR group, but within 9 days in the SCI+OFS and SCI+nOFS+ATR groups. In the SCI+nOFS group, where no treatment was administered, bladder function was not restored until 17 days after spinal cord injury (Fig. 1B).

Behavioral testing

Motor function – BBB locomotor score

After induction of T9 compression, the animals were completely paraplegic with a neurological score of 0. The BBB score was assessed every 7 days, where we recorded a gradual increase in the neurological score (Fig. 2A).

During the first 2 weeks after SCI, there was no significant difference between the experimental groups (SCI+nOFS, SCI +nOFS+ATR, SCI-+OFS, SCI+OFS+ATR). A major improvement in motor functions was observed between 3 and 4 weeks after SCI in the SCI+OFS and SCI-+OFS+ATR groups compared to the SCI+nOFS and SCI+nOFS+ATR groups. In the SCI+OFS+ATR group, an increase in the BBB score was observed until the end of the survival period. After 6 weeks of survival, animals with combination therapy (OFS+SCI+ATR) were able to take regular steps with weight-bearing and more frequent coordination of forelimb and hindlimb movements (BBB score: 11.2 ± 0.85). In the SCI+OFS group, the animals were capable of regular weight-bearing steps and infrequent coordination of forelimb and hind-limb movement (BBB score: 10.3 ± 0.68). Animals in the SCI+nOFS+ATR and SCI+nOFS groups were able to stand independently when “sweeping” movements of hind-limb were observed (BBB scores: SCI+nOFS+ATR: 9.98 ± 0.82; SCI+nOFS: 8.53 ± 0.57).

Sensory function – hot-plate test

In the hot plate test, the rats with SCI injury (SCI+nOFS, SCI+nOFS+ATR, SCI+OFS and SCI+OFS+ATR) had particularly longer latency times than intact animals (value of the intact control – 4–5 s, unpublished data). Two weeks after SCI, the latency time of the experimental groups was prolonged: SCI+nOFS – 10.64 ± 0.97 seconds, SCI+nOFS+ATR – 11.98 ± 1.15 seconds, SCI+OFS – 16.35 ± 1.02 seconds, SCI+OFS+ATR – 8.46 ± 0.89 seconds. Gradually, after 4 weeks after induction of compression, we observed a decrease in the latency time, in groups of animals that received individual therapy (SCI+nOFS+ATR, SCI+OFS) and combination therapy (SCI-+OFS+ATR). The average latency times were: SCI+nOFS – 9.66 ± 1.05 seconds, SCI+nOFS+ATR – 9.28 ± 1.28 seconds, OFS+SCI – 8,55 ± 1.45 seconds, SCI+OFS+ATR – 7.66 ± 1.26 seconds. After 6 weeks of survival, animals with a functional electrical stimulator implanted in combination with ATR administration (SCI+OFS+ATR) had a shorter latency time compared with the other experimental groups. The average latency time was: SCI+nOFS – 8.90 ± 1.23 seconds, SCI+nOFS+ATR – 7.84 ± 1.43 seconds, SCI+OFS – 7.96 ± 1.31 seconds, SCI+OFS+ATR – 7.22 ± 1.23 seconds. After performing statistical analysis, no statistical significance was present (Fig. 2B).

Analysis of protein levels

Western blot analysis

To determine whether the combination of OFS stimulation and ATR administration affects neural tissue regeneration at the protein level, Western blot analysis was performed. To analyze spinal cord damage, level of GFAP protein was measured, which is one of the components of reactive astrocytes after SCI. The known role of GFAP is to form a physical barrier to isolate the epicenter of damage from the surrounding neuronal tissue. In SCI+nOFS rats, about 2-fold higher concentration of GFAP protein was observed in + 2 and −2 compared to the damage epicenter, i.e., in LC and adjacent + 1 and −1 segments. In other experimental groups, i.e. SCI+nOFS+ATR and SCI+OFS, there was a less obvious trend in the gradual increase of GFAP expression at the border of the damage epicenter. Interestingly, this protein increased at the border of the damaged epicenter was not observed in the SCI-+OFS+ATR experimental group. Moreover, the lowest GFAP expression in all spinal cord segments of this group was found (Fig. 3A). A significant decrease was observed in LC, −1 and −2 segments compared to + 2 and + 1 segments. A statistically significant difference occurred only in −2 segment between the SCI+OFS+ATR and SCI+nOFS groups *($P < 0.01$)*. Changes in neurofilament light chain (NF-L) concentration reflect the level of neuronal damage. Mostly higher levels of this protein were observed in SCI+OFS+ATR and SCI+nOFS+ATR compared to SCI+OFS and SCI+nOFS experimental groups (Fig. 3B). NF-L protein levels were highest in the SCI+OFS+ATR groups in segments + 2, LC and −2, in contrast to segments + 1 and −1, where there was a slight decrease in NF-L expression. Using one-way ANOVA, no statistical significance was found between groups. GAP-43 protein was selected to measure the levels of the newly sprouting nerve fibers in an effort to understand the molecular processes involved in axon growth,

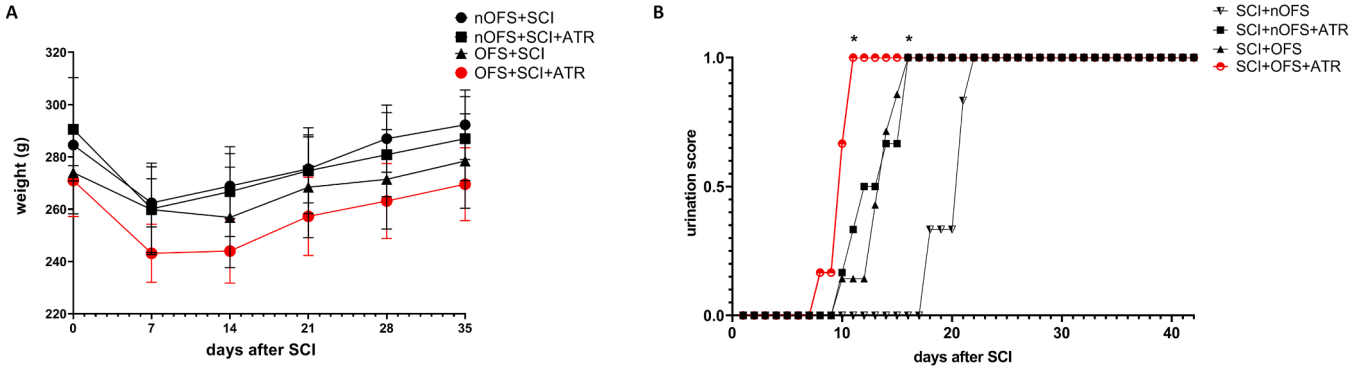

Fig. 1. Health status of animals after T9 spinal cord compression in the following groups: rats with non-functional stimulator (SCI+nOFS), with functional stimulator (SCI+OFS), with non-functional stimulator and administered ATR (SCI+nOFS+ATR), and with functional stimulator and administered ATR (SCI+OFS+ATR). (A) - Body weight changes in adult female Wistar rats after SCI. The animals were weighed every 7 days for 6 weeks. Data are presented as percentage values. (B) – Voiding score assessment in all experimental groups after SCI. Voiding score 0 – rats unable to urinate spontaneously; Voiding score 1 – rats with the ability to urinate spontaneously. SCI – T9 spinal compression injury; OFS – oscillating field stimulator; nOFS – non-functional oscillating field stimulator; ATR – Atorvastatin. Data are presented as MEAN ± SEM. Results for weight changes were statistically evaluated using a two-way Anova followed by a post-hoc Tukey's test. Results for urination were statistically evaluated using chi-square followed by Yates correction. The data were considered statistically significant at P < 0,05 (*).

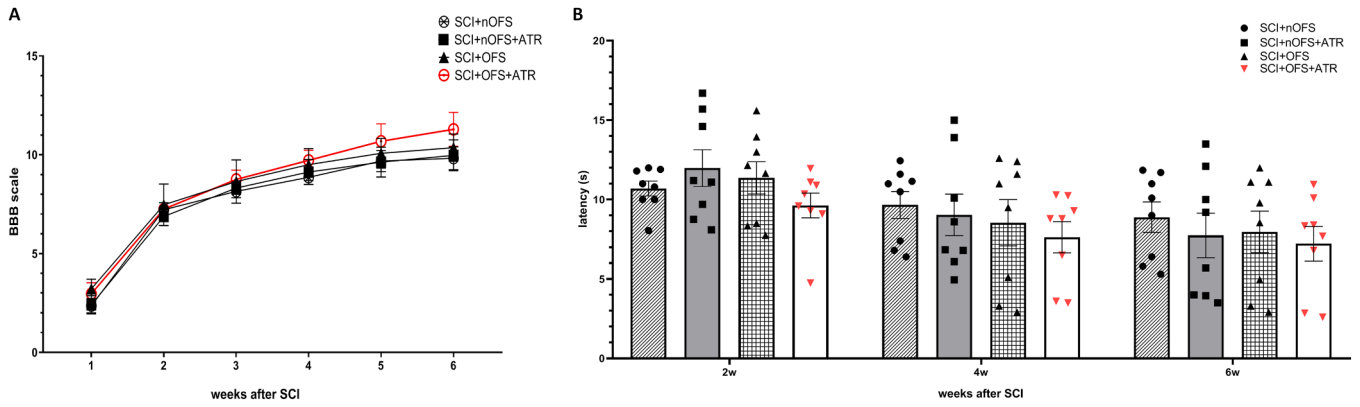

Fig. 2. Demonstration of functional improvement in motor and sensory functions after SCI in all experimental groups. (A) Assessment of hind-limb locomotion improvement in rats after SCI by BBB score. A visible improvement was observed after 3 weeks of evaluation in the combination therapy group (SCI+OFS+ATR). Slight improvement was in the individual therapy groups (SCI+OFS and SCI+nOFS+ATR) compared with the no – treatment group (SCI+nOFS). (B) Hot-plate test performed to observe recovery in sensory functions after SCI. The test was repeated every 2 weeks during the 6-week survival period. The most obvious changes were noticed in the SCI+OFS+ATR group compared to the other groups – SCI+OFS, SCI+nOFS+ATR and SCI+nOFS. SCI – T9 spinal cord compression injury; OFS – oscillating field stimulation; nOFS – non-functional electrical stimulation; ATR – Atorvastatin. Data are presented as MEAN ± SEM. Results were statistically evaluated using a two-way Anova followed by a post-hoc Tukey's test. No statistical significance was detected.

regeneration, and neuronal repair. The protein level was significantly increased in the group receiving combination therapy (SCI+OFS+ATR). The highest effect of the combination therapy was observed in the −1 segment. GAP-43 protein concentration was also high in SCI+OFS and SCI+nOFS+ATR rats compared to the SCI+nOFS group. A statistically significant difference was observed in the + 2 segment between SCI-+OFS and SCI+OFS+ATR; and between SCI+OFS+ATR and SCI+nOFS+ATR groups (+2: $P < 0.05$). Similarly, between SCI+OFS and SCI+OFS+ATR; SCI+nOFS and SCI+OFS+ATR; SCI+OFS+ATR and SCI+nOFS+ATR (-1: $P < 0,05$) in the −1 segment. Also, between SCI+nOFS and SCI+OFS+ATR groups (-2: $P < 0.01$) in the −2 (Fig. 3C). Another characteristic feature of traumatic spinal cord injury is demyelination of axons. Selected proteins (PLP-1 and CNPase) were used to determine whether the selected combination therapy promoted the regeneration of oligodendrocytes forming the myelin sheath. PLP-1 protein concentrations were significantly increased in the SCI + OFS group in all investigated segments. Compared to this group, there was a decrease in the level of this protein in the SCI+OFS+ATR, SCI+nOFS+ATR and SCI+nOFS experimental groups. Statistical significance was observed in the + 1 segment between SCI+OFS and SCI-+OFS+ATR, SCI+OFS+ATR and SCI+nOFS+ATR *(+1: $P < 0,01$; $P < 0,05$)*. Similarly, between the SCI+OFS group to all three other groups (SCI+nOFS, SCI+OFS+ATR, SCI+nOFS+ATR) *(-1: $P < 0,05$; $P < 0,01$)* in the −1 segment (Fig. 3D). In the case of CNPase, in the group with applied combination therapy (SCI+OFS+ATR) the highest increase in protein concentration was found in all spinal cord segments. High protein levels were also detected in the SCI+OFS and SCI+nOFS+ATR groups. The lowest protein level was in the SCI+nOFS group. Compared to the other segments, the lowest increase in CNPase protein concentration was observed in the −1 segment. In the LC, −1 and −2 segments, there was statistical significance between the SCI+nOFS and SCI+OFS+ATR groups *($P < 0.05$);* and in the −1 segment between the SCI+OFS and SCI+OFS+ATR groups *($P < 0.05$)* (Fig. 3E). Our results were also supported by the immunohistochemical method, representative images of the studied proteins are shown (Fig. 3F).

Analysis of spinal cord tissue preservation

Histopathological analysis of LFB/CV- stained spinal cord sections revealed a characteristic image of the lesion after SCI (Fig. 4A). A large loss of gray matter as well a valuable reduction of the surrouding white matter was observed in all experimental groups. The spinal cord tissue consisted of numerous cavities and cysts of various sizes. The greatest vacuolization was observed at the epicenter of injury, while both the cranial and caudal segments were affected to a lesser extent. SCI compression resulted in massive tissue loss mainly in the LC segment in the SCI+nOFS group (Fig. 4B). Quantitative histological examination showed a greater volume of spared tissue in all groups with applied therapies (SCI+nOFS+ATR, SCI+OFS, SCI+OFS+ATR) compared to the nOFS+SCI group. The increase in spared tissue occurred mainly in the epicenter of injury and segments adjacent to the LC segment (+1 segment: SCI+nOFS 76.82 ± 2,1 %; SCI+nOFS+ATR 95.43 ± 1.1 %; SCI+OFS 79.97 ± 6.2 %; SCI+OFS+ATR 87.15 ± 1.0 %; and −1 segment: SCI+nOFS 77.94 ± 5.1 %; SCI+nOFS+ATR 80.25 ± 3.2 %; SCI+OFS 84.32 ± 5.3 %; SCI+OFS+ATR 93.42 ± 0.2 %). However, an increase in the cumulative area of the total spared tissue in the combination therapy group was observed. It was identified that the most pronounced preservation of tissue volume with statistical significance (P value < 0,05) in the LC spinal cord segment of SCI+OFS+ATR rats (79.85 ± 3.5 %) than in the untreated SCI+nOFS group (42.05 ± 2.23 %).

Discussion

The present study describes the effect of a combination therapy consisting of immediate epidural implantation of an OF stimulator and administration of ATR 7 days after the induction of experimental T9 in the rat. The combined treatment was safe and successful. Our claims are in line with our previous studies which proved the safety and tolerance of epidural OF stimulator implantation in experimental animals (Bacova et al. 2019, 2022) and the effect of ATR on reducing the inflammatory response present in the spinal cord during the cascade of secondary processes (Bimbova et al. 2018).

The primary aims of this publication were as follows: 1. To evaluate the effectiveness stimulation in promoting axonal regeneration and restoration of functionality in neural pathways of the spinal cord following traumatic injury; 2. To assess the combined effect of electrical stimulation on the injured spinal cord together with validated individual treatments, specifically focusing on anti-inflammatory therapy using ATR; 3. To investigate the mechanisms underlying the protective effects of electrical stimulation on tissue regeneration by examining the involvement of cell populations crucial for the regeneration process, such as astrocytes, newly sprouting axons, neurofilaments and oligodendrocytes.

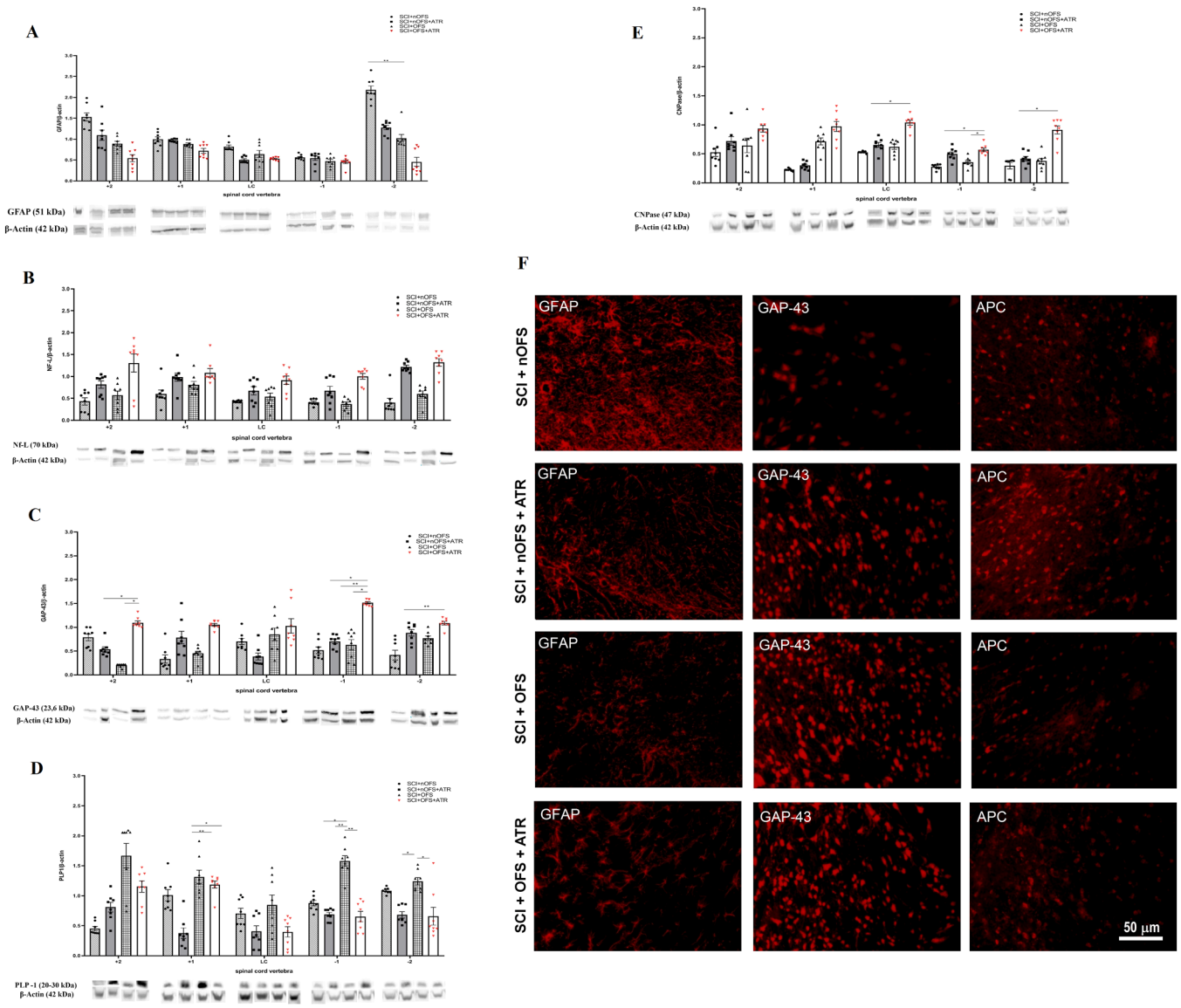

Fig. 3. Monitoring of changes in the levels of selected proteins after SCI and applied treatment by Western blot method. (A-E) – Graphs represent the protein level of accessed proteins: glial fibrillary acidic protein (GFAP), neurofilaments (NF-L), newly sprouting nerve fibers (GAP-43) and oligodendrocytes (PLP-1 and CNPase) relative to the β-actin 6 weeks after SCI. (A) GFAP protein level 6 weeks after SCI. Western blot analysis showed that the lowest GFAP protein level was in the group with applied combination therapy (SCI + OFS + ATR) compared to the group without treatment (SCI+nOFS) where the protein level was up to 2-fold higher. The GFAP protein level increased gradually in the following order: SCI+OFS+ATR, SCI+OFS, SCI+nOFS+ATR, SCI+nOFS. (B) NF-L protein level 6 weeks after SCI. The difference in NF-L protein levels was mainly observed in the spinal cord segments of ATR-treated animals. This was the case for both functional and non-functional electrical stimulator groups (SCI+OFS+ATR and SCI+nOFS+ATR). The optical density decreased in the order: SCI+OFS+ATR, SCI+nOFS+ATR, SCI+OFS and SCI+nOFS. (C) GAP-43 protein level 6 weeks after SCI. Data showed that the application of combination therapy had the highest effect – the high optical density level was in the SCI+OFS+ATR group, and then decreased in order: SCI+nOFS+ATR, SCI+OFS and SCI+nOFS. (D) PLP-1 protein level 6 weeks after SCI. For this protein, the highest levels were observed in the SCI+OFS group. In the groups where ATR was part of the treatment there was a decrease in the level of this protein (SCI+OFS+ATR and SCI+nOFS+ATR). (E) CNPase protein level 6 weeks after SCI. Western blot analysis revealed changes in CNPase protein in treated groups (SCI+OFS+ATR, SCI+OFS and SCI+nOFS+ATR) versus untreated (SCI+nOFS) group. Representative blots show protein levels for five distinct target proteins, with one band corresponding to each protein in each group. Band intensity reflects the abundance of the proteins, with dark bands representing higher exposure (and consequently higher protein concentration), and the lighter bands representing lower exposure (and lower protein concentration). All experiments were repeated independently with consistent results. (F) Representative images showing immunohistochemical staining of GFAP, GAP-43 and APC in spinal segments + 2 and −2. Scale bar – 50 µm. SCI – T9 spinal cord compression injury; OFS – oscillating field stimulation; nOFS – non-functional electrical stimulation; ATR – Atorvastatin; LC – lesion center (T9 compression). Data are presented as MEAN ± SEM. Results were statistically evaluated using a one-way Anova followed by a post-hoc Tukey's test (*P value < 0.05; **$P < 0.01$; ***$P < 0.001$).

Behavioral assessment

Restoration of sensory and motor abilities is one of the primary requirements for the effectiveness of SCI treatment; as such, behavioral testing is one of the most important components of experimental investigations (Fouad et al. 2020). They are used to record the degree of recovery after SCI and to evaluate the location and severity of the injury (Šedý et al. 2008, Ahmed et al. 2019). Sensitivity testing is useful to assess sensory pathways in experimental rats after SCI and therapeutic interventions (Lavrov et al. 2008, Ahmed et al. 2019). Sensory perception can be tested in the presence of heat (Hunskaar et al. 1986), cold (Yoon et al. 1994), or pain (Yu et al. 1998). According to Giglio et al.

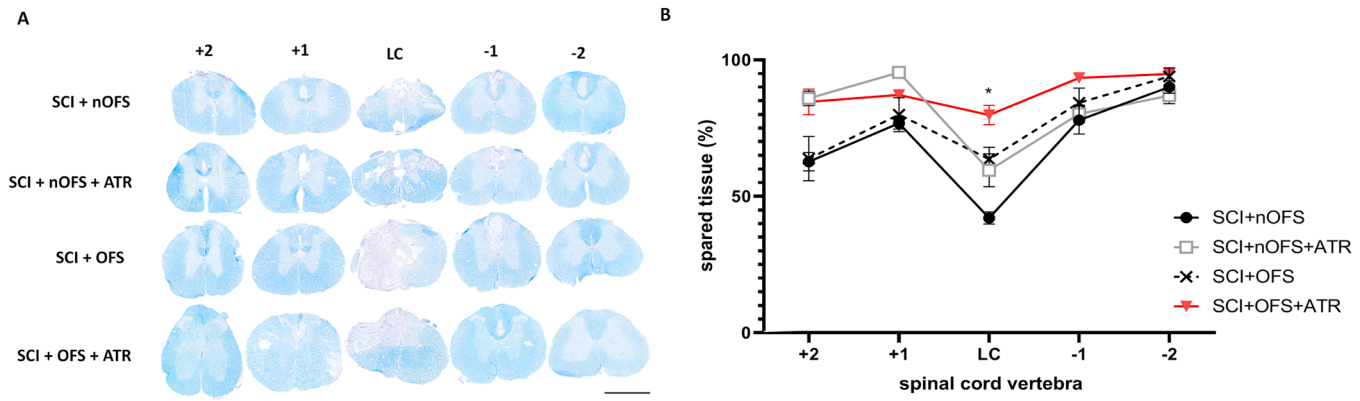

Fig. 4. Spinal cord tissue preservation. (A) – Representative transverse spinal cord sections stained with Luxol Fast Blue / Cresyl Violet at 6 weeks post-injury taken from 1.5 cm cranial (+2, +1) and caudal (-1,−2) segments at the lesion center (T9). Scale bar – 700 μm. (B) – Quantitative assessment of preserved spinal cord tissue revealed that amount of spared tissue was considerably larger in the treated groups (SCI+OFS+ATR, SCI+OFS and SCI+nOFS+ATR) in caudal segments ((-1,−2) SCI – T9 spinal cord compression injury; OFS – oscillating field stimulation; nOFS – non-functional electrical stimulation; ATR – Atorvastatin; LC – lesion center. Data are presented as MEAN ± SEM. Results were statistically evaluated using a one-way Anova followed by a post-hoc Tukey's test (*P value < 0.05).

(2006), the hot-plate test is the most accurate way to assess reflex function in order to identify sensorimotor deficiencies following an injury. Weekly hot-plate sensory recovery testing by Bacova et al. (2022) revealed significant differences in reaction latency between the treated (SCI+OFS) and untreated (SCI) groups over 8-week study period. It has been shown that ATR can affect latency in this sensory test. Kesim et al. (2012) demonstrated the anti-inflammatory and analgesic properties of ATR after acute and long-term application using hot-plate test. In our study, the hot-plate test showed, how the animal's sensory pathway function improved following the individual treatment (SCI+OFS, SCI+nOFS+ATR) and combination therapy (SCI+OFS+ATR) in compared to the group that received no therapy (SCI+nOFS). Our findings demonstrated behavioral testing implemented at 2,4 and 6 weeks following SCI resulted in the lowest latency. There was little difference in delay in the groups that received individual treatment suggesting to us that the use of combination therapy enhanced the recovery of sensory ascending pathways.

Common scoring systems such as the BBB scale, are used to for locomotor function and are a well-accepted technique for analyzing descending neural pathways following spinal cord injury (Basso et al. 1995, 1996a, 1996b). These grading systems are useful for comparing results between laboratories and cover a wide spectrum of motor impairments (Basso et al. 1996b). BBB test results from Bacova et al. 2022 study demonstrated a moderate and steady improvement in motor skills. This improvement reached statistical significance at week four in rats implanted with active OFS and continued for up to eight week. Bimbova et al. (2018) evaluated rats for 6 weeks using the BBB method after SCI and administration of single dose of ATR. The development of the neurological score showed no differences between the SCI and ATR-treated groups until day 24. Results varied significantly between days 30 and 42 (Bimbova et al. 2018). Differences in our results were observed 3–4 weeks after SCI induction, especially in the functional stimulator groups (SCI+OFS, SCI+OFS+ATR) in contrast to the other groups (SCI+nOFS+ATR, SCI+nOFS). This pattern persisted until the end of the survival period, when, after six weeks, motor function improved primarily in the SCI+OFS+ATR rats then in the SCI+OFS, SCI+nOFS+ATR and SCI+nOFS groups. Behavioral assessments verified enhancements in sensory and motor abilities in rats treated with ATR and with and implanted electrical stimulator, either separately or in combination.

GFAP – glial fibrillary acidic protein

The cytoskeleton of astrocytes contains GFAP, which has been proposed as a possible biomarker to assess the severity of injury (Ahadi et al. 2015, Wichman et al. 2023). After SCI, astrocytes become activated and exhibit aberrant proliferation, cell body enlargement, and upregulation of GFAP. During reactive gliosis, astrocytes are translocated and congregated around the epicenter of injury (Clifford et al. 2023). Astrocytes rapidly produce a range of inhibitory proteins, that ultimately lead to the formation of glial scar tissue in the SCI lesion (Okada et al. 2006, Sofroniew, 2009, Zhang et al. 2018). In the initial days following SCI, the level of GFAP increases, as the condition progresses from subacute to chronic. Interestingly, during the transition from acute to chronic SCI, the trajectory of GFAP overe time is highly correlated with the degree of neurological recovery, with a faster decline in those who recovered better (Leister et al. 2023). According to Moriarty and Borgens (2001), applied voltage reduced the amount of astrocytes, that accumulate in the area of injury and in the surrouding unaffected areas. Furthermore, the extension of astrocytic processes within the lesion was significantly suppressed by the applied voltage. Zhang et al. (2018) followed up on the above mentioned study and also confirmed that OFS can reduce astroglial scar formation after SCI in rats. Moreover, Pannu et al. (2005) showed a significant decrease in astrocyte activation when they injected ATR (5 mg/kg; orally) 7 days before SCI and once daily during the period of survival. Continuing with those studies, our choice was to use a combination therapy by applying OFS stimulation and administering ATR (5 mg/kg; i.p.) for 7 days after induction of compression. Our findings confirmed the previously observed effectiveness of individual therapies (Bacova et al. 2022 – SCI+OFS experimental group; Bimbova et al. 2018 – SCI+nOFS+ATR experimental group) to reduce GFAP in damaged segments. Moreover, we observed that the combination of OFS with ATR after SCI enhanced the effect of individual therapies and significantly reduced GFAP concentrations in the segments surrouding, the injury epicenter. This effect of the treatment caused that we did not observe GFAP barrier-like formations in the + 2 and −2 segments. This findings may indicate that the combined treatment significantly affected the injury epicenter.

Nfl – neurofilament light chain

Changes in the amount of structural protein of the neuronal cytoskeleton, such as Neurofilament – light chain (NF-L), indicate spinal cord damage (Kuhle et al. 2015, Gordon, 2020, Stukas et al. 2023). Bacova et al. (2022) observed that use of epidural stimulation improved axonal regeneration ability following SCI. According to their findings, rats with active OFS had significantly higher levels of Nf-L protein. Since pro-regenerative and significantly inflammatory processes begin already in the first week after the injury, our hypothesis was that the early administration of a weak OF current to the damaged spinal cord could

help as a trigger for the initiation of regeneration processes. After the application of a individual OF stimulation therapy, the results of Western blot analysis showed relatively low levels of Nf-L protein, especially in the caudal segments of the spinal cord. ATR has been shown to improve neurofilament regeneration following SCI, six weeks after injury, a single acute dose of ATR greatly enhanced the expression of neurofilaments in the dorsolateral spinal cord region (Bimbova et al., 2018). Based on these findings, the ATR was administered after induction of compression – either alone or as part of combination therapy. Therapy using OF stimulation was effective, but higher protein levels were observed in the experimental group treated with ATR only (SCI+nOFS+ATR). There was probably more suppression of inflammation and higher recovery. Moreover, the application of combined therapy (SCI+OFS+ATR) revealed a significant increase in the levels of neurofilament, especially in spinal segments + 2 and −2.

GAP-43 – growth-associated protein 43

The GAP-43 protein is crucial for the control of axonal growth and is widely distributed in the rat spinal cord. After compression injury, elevated levels of GAP-43 were observed in cell bodies and axons surrounding the injury site within four days (Curtis et al. 1993). This increase in GAP-43 suggests an effort to regenerate axons, which could greatly promote reinnervations and growth of nerves from the lesioned area (Aigner et al. 1995, Marufa et al. 2021). Consistent with previous studies (Bacova et al. 2022, Bimbova et al. 2018), individual treatments applied after SCI showed a significant increase in GAP-43 protein in animals receiving OFS with ATR compared to the nOFS group. These data demonstrated that OFS stimulates GAP-43 expression, which contributed to axonal regeneration following SCI. The mechanism of the effect of ATR on GAP-43 expression in the spinal cord after injury is not yet fully understood. Western blot findings were also confirmed by immunohistochemical analysis, which revealed an increase in GAP-43 positive fibers in the damaged segments of the spinal cord. The efficacy of individual therapies also varied, with ATR administration predominating in segments + 2 and + 1. By applying combination therapy (SCI+OFS+ATR), a beneficial effect of both individual therapies was observed, especially in the caudal segments of the spinal cord (-1 and −2).

PLP-1 – proteolipid protein −1

Oligodendrocytes provide trophic support, surround axons in myelin sheaths, and defend neurons and their axons (Baumann and Pham-Dinh, 2001, Li et al. 2020). SCI causes loss of tissue, including myelinated fiber tracts, which are essential for the transmission of sensory and motor impulses (Wrathall et al. 1998). Two of the main pathogenic processes that prevent functional recovery after SCI are oligodendrocytes loss and axonal demyelination (Almad et al. 2011). After experimental SCI, demyelinated axons have also been observed in the spinal cord (Gledhill et al. 1973, Griffiths and McCulloch, 1983, Bunge et al. 1993). One of the most common components (~ 50 %) of the total protein content of the myelin sheath is PLP-1 protein, along with myelin basic protein (30 % of CNS) (Eng et al. 1968, Hudson, 1990; Harlow et al. 2014). Findings by Wrathall et al. (1998) show that the concentration of myelinating cells in the chronically damaged spinal cord that express PLP mRNA is not significantly reduced. However, fewer PLP mRNA molecules are expressed in each cell and a higher proportion of cells than usual appear to have low protein levels (Wrathall et al. 1998). According to Becker et al. (2010) electrical stimulation encourages adult rat's endogenous neural progenitor cells to proliferate and differentiate into oligodendrocytes following spinal cord injury (Becker et al. 2010, Li et al. 2020). By activating sodium-dependent action potentials, electrical stimulation enhanced the development and myelination of oligodendrocytes (Ishibashi et al. 2006, Li and Li, 2017). So far, no specific link between ATR and PLP-1 after SCI has been described. In our results, electrical stimulation had a positive effect. The highest increase in PLP-1 protein concentration was observed in the SCI+OFS group. On the contrary, in the experimental groups where ATR was administered, the protein level was reduced. A possible explanation is that during its biosynthesis, the PLP-1 protein associates with myelin rafts in oligodendrocytes, which are membrane domains enriched in cholesterol and galactosylceramide (Simons et al. 2002). It also has a direct interaction with cholesterol (Simons and Gruenberg 2000). ATR is generally described as a medication used to lower cholesterol (Liao and Laufs, 2005, Aghazadeh et al. 2017). Thus, administration of ATR could suppress PLP-1 expression due to the connection between this protein and cholesterol described above.

CNPase – 2,3-cyclic nucleotide −3- phosphodiesterase

CNPase is an enzyme commonly used as a marker for oligodendrocytes (Bernardo et al. 2013, Wang, Almazan, 2016). Oligodendrocytes and dendritic cells have been reported to have very high concentrations of CNPase. Most research on CNPase expression has focused on its functions in myelinogenesis and glial cells that produce myelin, such as Schwann cells and oligodendrocytes (Yang et al. 2014). Based on the literature, we can conclude that the expression of CNPase in the white matter of the spinal cord serves as an indicator of the preservation of the white matter, plays a key role in the proper functioning of oligodendrocytes and myelination, and also in the response of the spinal cord tissue to injury (Kanno et al. 2010, 2019; Li et al. 2020). After injury, detection of CNPase immunoreactivity indicates that oligodendrocytes alterations occur rapidly and spread up to several millimeters from the site of injury. This precedes a significant extent of axonal damage, providing an early estimate to the final volume of intact white matter (Ek et al. 2012). Li et al. (2020) reported a positive effect of electrical stimulation applied after injury. After electrical stimulation therapy (ESCS), it was observed and increased differentation of oligodendrocytes (Li et al. 2020). Similarly, our results with the application of only electrical stimulation (SCI+OFS) showed higher concentrations of CNPase compared to the group where no therapy was applied (SCI+nOFS). Several studies demonstrated that the administration of ATR after SCI led to a reduction in inflammation, facilitation of axonal growth and increased remyelination process (McFarland et al. 2014, Bimbova et al. 2018, Lukacova et al. 2021). CNPase expression coincides with the presence of inflammatory mediators in microglia, which are increased upon activation. Our findings showed a positive correlation between ATR administration and CNPase concentrations, with a higher protein level observed in both groups where ATR was used either as a individual therapy (SCI+nOFS+ATR) or as part of a combination therapy (SCI+OFS+ATR).

Spinal cord tissue preservation

The cellular composition of the white matter of the spinal cord includes longitudinally running nerve fibers, neuroglia and blood vessels. In contrast, gray matter ir a more loosely structured tissue consisting of neurons, axon terminals, dendrites and neuroglial cells. This different cellular composition between white and gray matter is thought to influence the mechanical properties of these tissues (Nishida et al. 2020). Studies suggest that SCI results in damage to white and gray matter, leading to reduction of both types of tissue in the spinal cord. Gray matter degeneration occurs relatively rapidly, usually within 24 hours after injury, while white matter continues for up to 7 days after SCI (Ek et al. 2010, 2012). Ono et al. (1977) documented that severe compression of the spinal cord resulted in significant collapse and formation of cavities in the gray matter (Ono et al. 1977). The initial lack of visible damage in the surrouding white matter suggests that this tissue may have greater resistance to deformation compared to the deeper gray matter (Ek et al. 2010). Thus, previous studies suggest that the gray matter in the spinal cord is probably mechanically weaker, undergoing

almost immediate tissue loss, than the white matter (Ono et al. 1977, Baba et al. 1996, 1997, Nishida et al. 2020). Our results also confirmed greater gray matter tissue loss compared to white matter. Electrical stimulation plays an important role in enhancing the functional recovery of both white and gray matter after SCI. Bacova et al. (2019, 2022) explored the beneficial impact of oscillating field stimulation on tissue preservation in two experimental groups (SCI+OFS and SCI group). Their findings confirmed that OF stimulation treatment showed neuroprotective properties at the lesion site and in the surrounding tissue after SCI (Bacova et al. 2019, 2022). Our observations revealed a loss of significant gray matter with a decrease in adjacent white matter, especially at the site of lesion. Our findings are consistent with Bacova et al. (2019, 2022) results, indicating a protective influence on spinal cord tissue after the application of a individual therapy (SCI+OFS) compared to no therapy (SCI+nOFS). Changes in white and gray matter during electrostimulation therapy underscore its potential to induce structural and molecular changes that may aid neural repair and functional recovery in individuals with SCI. Furthermore, placing the cathode either cranial or caudal to the lesion site has been shown to promote specific direction-axon regeneration underscoring the importance of electrode placement in influencing regenerative processes in white and gray matter (Patel and Poo, 1982, Borgens et al. 1990, Hamid and Hayek, 2008). ATR studies have explored its effects on both white and gray matter in the spinal cord post-injury, finding that it contributes to tissue preservation and neural recovery. Pannu et al. (2007) administered ATR (5 mg/kg, oral) at different time points after injury (2, 4 and 6 hours) and observed its ability to reduce the expansion of necrotic lesion, suppress overall spinal cord damage, and prevent myelin loss. Post-injury neurodegeneration often manifests as white matter deterioration, including loss of tissue viability, axonal degeneration, and myelin damage, all of which were attenuated by ATR treatment following SCI. Based on this research, Bimbova et al. (2018) demonstrated the neuroprotective effects of a single dose of ATR (5 mg/kg, i.p.) immediately after a T9 compression injury, which reduce macrophage infiltration in both white and gray matter one day post-injury. Following to these findings, in our study ATR (5 mg/kg, i.p.) was administered seven days after compression injury. In the group receiving ATR therapy only (SCI+nOFS+ATR) the enhanced tissue preservation was observed compared to the SCI + nOFS group. In particular, the group with combined therapy (SCI+OFS+ATR) showed the highest levels of preserved white and gray matter in the spinal cord, which underline benefits of individual therapy approaches.

Conclusion

In our study, the effect of a specific individual treatment of SCI and a combined therapy was explored involving a weak oscillating field and the anti-inflammatory drug Atorvastatin. This investigation utilized behavioral, protein, histological and immunohistochemical analyses. We confirmed earlier research by Bacova et al. (2019, 2022), demonstrating that immediate epidural electrical stimulation activates regenerative processes in the acute phase of SCI and maintains efficacy in the chronic phase. In accordance with the findings of Bimbova et al. (2018) administration of ATR enhanced the regenerative potential of damaged tissue, promoted axon growth and increased the expression of neurofilaments. Our study demonstrated a beneficial effect of combined therapy (SCI+OFS+ATR) on tissue regeneration after 6 weeks of treatment. Notable results included reduced accumulation of GFAP – expressing astrocytes at the periphery of the primary lesion, enhanced axonal growth, increased concentrations of neurofilament and oligodendrocyte molecules, with the exception of suppressed PLP-1 expression after ATR application. Behavioral results showed only a tendency that did not reach significance during the survival period. It is reasonable to assume that over the longer recovery period this trend will continue and eventually become significant. Although these results are promising, further research is needed in this regard for fully optimized functional recovery.

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CRediT authorship contribution statement

Kuruc Tomas: Methodology, Investigation. **Kiss Bimbova Katarina:** Methodology, Investigation. **Magurova Martina:** Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Papcunova Stefania:** Software, Formal analysis, Data curation. **Bacova Maria:** Methodology, Investigation, Conceptualization. **Kucharova Karolina:** Supervision, Funding acquisition, Formal analysis. **Kisucka Alexandra:** Supervision, Methodology, Funding acquisition. **Galik Jan:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Lukacova Nadezda:** Resources, Funding acquisition, Formal analysis. **Ihnatova Lenka:** Visualization, Validation.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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