Effects of Seven Days of Aerobic Exercise Combined with Functional Electrical Stimulation on NT-3, NT-4, and GDNF Gene Expression in Wistar Rats with Spinal Cord Injury

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Abstract

Background: Spinal cord injury causes severe and often irreversible motor deficits, largely due to neuronal loss, impaired axonal regeneration, and dysregulation of neurotrophic signaling pathways. Neurotrophins such as neurotrophin-3/4 (NT-3/4), and glial cell line–derived neurotrophic factor (GDNF) play critical roles in neuronal survival, synaptic plasticity, and functional recovery following SCI. Additionally, rehabilitation through exercise-based and functional electrical stimulation (FES) have been shown to modulate neurotrophic factor expression and improve locomotor outcomes after SCI; however, the molecular effects of their combined application remain poorly understood.

Objective: Here we aimed to investigate the combined effects of aerobic exercise and functional electrical stimulation on NT-3, NT-4, and GDNF gene expression and locomotor recovery in a rat model of spinal cord injury.

Methods: Utilizing thirty-three adult male Wistar rats that were randomly assigned to five groups of healthy control, SCI control, SCI with exercise, SCI with FES, and SCI with combined exercise and FES, SCI induction was performed at the T11 vertebral level, where animals in the intervention groups underwent seven days of treadmill-based aerobic training and/or spinal electrical stimulation. The Basso, Beattie, and Bresnahan (BBB) scale was used to assess locomotor recovery. Gene expression levels of NT-3, NT-4, and GDNF were investigated using qRT-PCR. The results were then analyzed statistically using one-way ANOVA and Tukey's post-hoc test ($p<0.05$).

Results: The improvement in the locomotor function of FES group at week 2 post-injury was statistically significant, although aerobic exercise demonstrated effectiveness from week 4 onward. Notably, the combined exercise and FES treatment, resulted in the most substantial improvements. Significant upregulation of NT-3, NT-4, and GDNF was evident in the gene expression analysis of the combined intervention group compared to other groups ($p<0.01$), where FES alone showed significant increases in gene expression compared to the SCI control group ($p<0.05$).

Conclusion: The findings suggest that the combined administration of FES and aerobic exercises enhances the motor recovery results in upregulates the expression of neurotrophic factors in SCI rats, holding promises as a therapeutic strategy for SCI rehabilitation.

Keywords: Spinal cord injury, neurotrophic factors, functional electrical stimulation, exercise, gene expression

Introduction

Spinal cord injury (SCI) is a complex condition where cascade of pathological events occur followed by the primary traumatic insult, with the primary injury characterized by mechanical impairment of neurons, surrounding soft tissues, and the endothelial layers of blood vessels, culminating in cellular necrosis as a consequence of mechanical trauma or ischemic conditions (1), and the second phase unfolding over a period ranging from minutes to weeks' after the initial trauma and involves additional neuronal cell death driven by inflammatory processes, glutamate excitotoxicity, the release of excitatory amino acids, formation of glial scars, and ultimately, apoptosis, as various molecules and factors are secreted at the injury site that inhibit axonal regeneration (2). This chain of events and the increasing prevalence of this devitalizing condition demands an urgent need for innovative therapeutic strategies that can mitigate secondary injury and apoptosis while promoting axonal growth, where despite extensive efforts for SCI repairment, a definitive treatment remains Inaccessible, even though significant advances have been achieved in post-injury management, surgical interventions, and stem cell-based therapies.Under healthy conditions, neurons' ordinarily dormant regeneration capacity is activated by the activation of a number of repair-associated genes following injury (3-8). This reparative process is stimulated by growth factors; however, the specific effects of different growth factors on peripheral tissues—such as muscle—and various neuronal subtypes have not been fully elucidated. It is conceivable that a particular neurotrophin may exert substantial effects on one neuronal population while having minimal impact on another, and that individual neurons might respond differently to distinct neurotrophins (9).

Neurotrophins are vital for both the development and maintenance of the peripheral and central nervous systems (PNS and CNS), through upregulating neuronal survival, differentiation, growth arrest, and the apoptosis of sensory neurons (10-13). In response to the injuries of CNS, such as SCI, the expression levels of numerous neurotrophin genes are altered, further modulating axonal development and neuronal survival to enable neural

healing (13-15);Hence, targeted and sustained delivery of neurotrophins such as neurotrophin-3 and brain-derived neurotrophic factor to the injured spinal cord promotes axonal growth, enhances neuronal survival, and contributes to improvements in sensory and motor function in experimental models of spinal cord injury(16-19). Similarly, recent studies that focus on the recovery and rehabilitation of paralyzed limbs using functional electrical stimulation (FES), also report improvements in muscle readiness (13-15). Additional studies have also highlighted that exercise augmented by FES may prevent muscle atrophy, osteoporosis, and spasticity (20-23). FES has emerged as a powerful rehabilitative tool for restoring movement, offering not only physical exercise but also distinct therapeutic benefits, and has proved to contain great potentials as it provides patients with the dual benefit of receiving electrical stimulation while simultaneously engaging in active motor training (24). The positive impacts of physical activity and exercise on the brain have been well documented, including improvements in brain mass, neurotransmitter levels, synaptic plasticity, hippocampal neurogenesis, neuronal survival and differentiation, as well as elevated neurotrophin levels, and these neurobiological changes are closely linked to enhanced behavioral performance (25). Although the precise biological mechanisms underlying spinal cord repair remain largely unknown, substantial evidence indicates that secondary injury–phase molecular mediators (e.g., inflammatory cytokines/chemokines, oxidative stress products, and intracellular signaling cascades) critically shape the lesion microenvironment and thereby influence neurodegeneration versus reparative remodeling after SCI (26-30).

Given the growing interest in multimodal therapeutic strategies for SCI, increasing attention has been directed toward understanding how physical activity during the secondary phase of injury influences molecular mechanisms underlying neural repair. Previous studies have demonstrated that either exercise or FES alone can modulate neurotrophic signaling and improve functional outcomes after SCI; however, findings remain inconsistent, and most investigations have focused primarily on behavioral recovery rather than molecular adaptations. Moreover, the combined effects of exercise and FES on the expression of key neurotrophins—particularly neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and glial cell line–derived neurotrophic factor (GDNF)—have not been systematically investigated at the gene expression level.

To our knowledge, this study is among the first to comprehensively evaluate the effects of a combined exercise and spinal electrical stimulation protocol during the secondary phase of SCI on the spinal expression of multiple neurotrophin genes with distinct roles in neuronal survival, axonal regeneration, synaptogenesis, and maintenance of mature neurons and accordingly, we aimed assessing the effects of of selected aerobic exercise combined with FES on NT-3, NT-4, and GDNF gene expression in the spinal cord of rats with SCI.

Methods

Study Design and Experimental Overview

This randomized controlled experimental study examined the independent and combined effects of aerobic exercise and functional spinal electrical stimulation on locomotor recovery and neurotrophic gene expression following SCI in rats, where a total of 33 adult male Wistar rats (With each group consisting about 7 rats, as applied in prior similar studies (31), and feasibility considerations) were initially enrolled. After the SCI induction and a subsequent stabilization period, animals were randomly assigned to one of five experimental groups of healthy control, SCI control, SCI + aerobic exercise, SCI + functional electrical stimulation, and SCI + combined aerobic exercise and FES. Over the period of the experiment, four animals were excluded due to unsuccessful SCI induction, postoperative complications, and/or inability of completing the intervention protocol. Consequently, 29 rats completed the study and were included in the final behavioral and molecular analyses. It is notable that the SCI was induced prior to group allocation.

In order to allow stabilization and to minimize the influence of acute secondary injury processes of the animals following injury, all were monitored for two weeks, therefore the exercise and stimulation interventions were initiated 14 days post-injury and continued according to the predefined protocols. Animals in the combined intervention group received electrical stimulation concurrently with aerobic exercise, and single-intervention groups received only the assigned modality.

Utiling the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale, the recovery was assessed longitudinally, and molecular outcomes were evaluated through quantitative analysis of NT-3, NT-4, and GDNF gene expression in spinal cord tissue harvested after completion of the intervention period, alongside behavioral assessments conducted by observers that were blinded to group allocation to reduce assessment bias.

Animals and Housing Conditions

A total of 33 adult male Wistar rats (weighing approximately 225-275g, aging between 10 to 12 weeks, obtained from an accredited laboratory animal breeding facility) were initially included in the study. Rats were housed in standard plexiglass cages with a mesh door measuring 25 × 27 × 43 cm under controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 50–60%, and a 12-h light/dark cycle) with free access to standard laboratory chow and water throughout the study period, where all animals were allowed to acclimate to the housing environment prior to the initiation of experimental procedures. Health status was monitored daily, and all efforts were made to minimize stress and discomfort during handling, training, and experimental interventions.

Ethical Approval and Animal Welfare

Strict adherence to the Helsinki protocol's ethical guidelines for laboratory animal research was kept for all the animal housing and the experimentation procedures, and ethical approval was obtained from the Institutional Animal Care and Use Committee (IR.NKUMS.REC.1402.012), as the study was carried out at North Khorasan University of Medical Sciences' Animal Laboratory.

During the experimental period, animals were carefully monitored for signs of pain, distress, or postoperative complications, with appropriate anesthetic protocols were applied during all surgical and invasive procedures, and humane endpoints were established. Animals that exhibited severe complications, unsuccessful spinal cord injury induction, or inability to complete the experimental protocol were excluded from the study and handled according to ethical standards.

Induction of Spinal Cord Injury

Initially, through an intraperitoneal (i.p.) injection of ketamine (75-100 mg/kg) and xylazine (10 mg/kg)- using an insulin syringe-the rats were rendered unconscious, and the anesthesia was confirmed, the fur at the surgical site of the dorsal surface was shaved, and the entire surface was sterilized and disinfected sequentially with 70% ethanol followed by surgical betadine.

A midline incision was then made by cutting the skin to a length of 2.5±0.5 cm in the rostral-caudal direction along the vertebral column and after incising both the superficial and deep fascia and retracting the paraspinal muscles adjacent to the spinous processes of vertebrae T9 through T11, a dental drill connected to a small motor was used to perform a laminectomy at the T11 level.

Subsequently, the vertebrae were stabilized with a stereotaxic apparatus, and a 10-gram weight was dropped from a height of 25 mm onto the exposed spinal cord (or a hollow cylinder was used to deliver the impact) (32). Immediately after the injury, using 4-0 absorbable sutures, the fascia and muscles were sutured; however, in the sham-operated (control) group, only a laminectomy was performed and no SCI was induced.

After the surgery, each animal was individually housed in a different cage, in which the same temperature, humidity and light setup as before were maintained. Notably, the nutritional conditions provided to the SCI animals were identical to those that were from the healthy controls, in order to ensure unrestricted access to standard food and water.

Additionally, in order to verify of successful induction of the spinal cord lesion, the locomotor test of BBB was administered 24 hours' post-surgery, with the movement of the hindlimb joints serving as the evaluation criterion. Rats with scores above 3 on the BBB scale were excluded from the study as not properly induced. The same test was also utilized for motor recovery assessments, commenced two weeks after the lesion induction, and conducted in an open-field arena where each rat was individually placed and evaluate by two blinded observers for 4 minutes using the same BBB scoring system (32, 33).

Behavioral Assessment (BBB Locomotor Test)

Locomotor function was assessed using the BBB locomotor rating scale, a validated method for evaluating hindlimb motor recovery following spinal cord injury in rodents, with scores ranging from 0 (no observable hindlimb movement) to 21 (normal locomotion), evaluating joint movement, weight support, coordination, paw placement, and trunk stability.

Behavioral assessments were conducted in an open-field arena, where each rat was allowed to move freely for 4 minutes. Evaluations were performed weekly throughout the experimental period, beginning after SCI induction and continuing until the end of the intervention. Locomotor performance was independently scored by two trained observers who were blinded to group allocation to minimize assessment bias. When discrepancies between observers occurred, final scores were determined by consensus.

BBB assessments were used both to confirm successful induction of spinal cord injury during the early post-injury period and to monitor functional recovery over time. Animals that failed to demonstrate locomotor deficits consistent with SCI during early assessments were excluded from the study, as described in the Experimental Groups and Sample Size section.

Post-Injury Monitoring and Experimental Timeline

After the successful induction of the SCI, close monitoring of all the animals during the acute and subacute post-injury periods went through, where the rats were housed individually and observed daily for general health status, wound healing, signs of distress, and neurological recovery. Manual bladder expression was performed twice daily as required until spontaneous bladder function was restored. Additionally, to alleviate pain, enhance immune function, and control postoperative infections, the animals in all groups received 5 ml of acetaminophen syrup – which was diluted in 250 ml of water- for three days, intramuscular injections of vitamin B at a dose of 0.1 ml daily for one week, and gentamycin at 0.1 ml daily for up to three days. The stages of SCI induction and the experimental procedures are illustrated in Figures 1 and 2.

To allow stabilization of the injury and to reduce the influence of acute secondary injury mechanisms, animals were maintained without experimental intervention for a two-week post-injury period, during which time, locomotor function was assessed using the BBB locomotor rating scale to confirm the establishment and consistency of SCI prior to initiation of the interventions. Aerobic exercise and functional spinal electrical stimulation protocols were initiated 14 days after the induction of the SCI . Interventions were conducted according to the predefined experimental group assignments and continued for the duration of the intervention period. Animals that exhibited severe postoperative complications, inconsistent SCI presentation, or inability to safely participate in the intervention protocols during this monitoring period were excluded from further analysis.

The overall experimental timeline consisted of SCI induction, a two-week stabilization and monitoring phase, an intervention period involving aerobic exercise, functional electrical stimulation, or their combination, followed by behavioral and molecular outcome assessments.

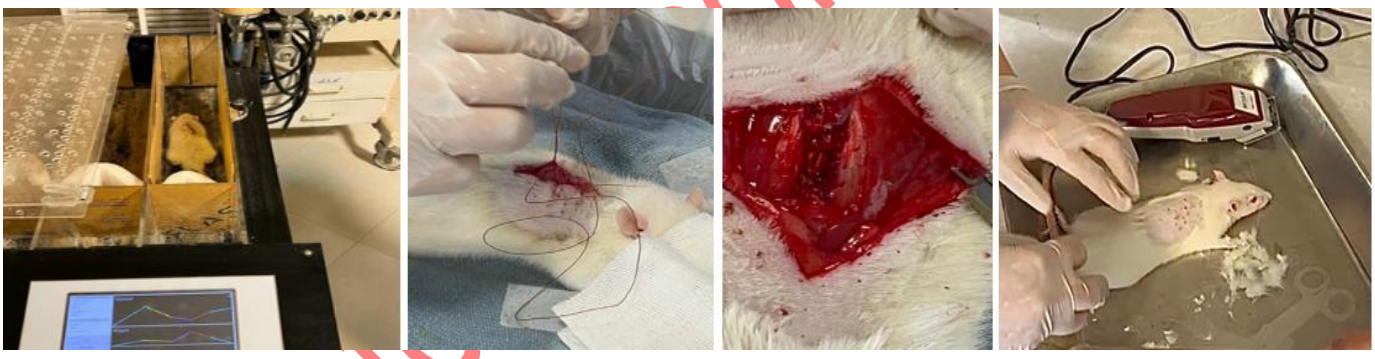

Figure 1. Stages for Inducing Spinal Cord Injury and Conducting Experiments; a) Shaving the animal's dorsal hair; b) Performing a laminectomy; c) Suturing the animal's back and post-surgical recovery; d) Exercise training implementing in spinal cord injury animals)

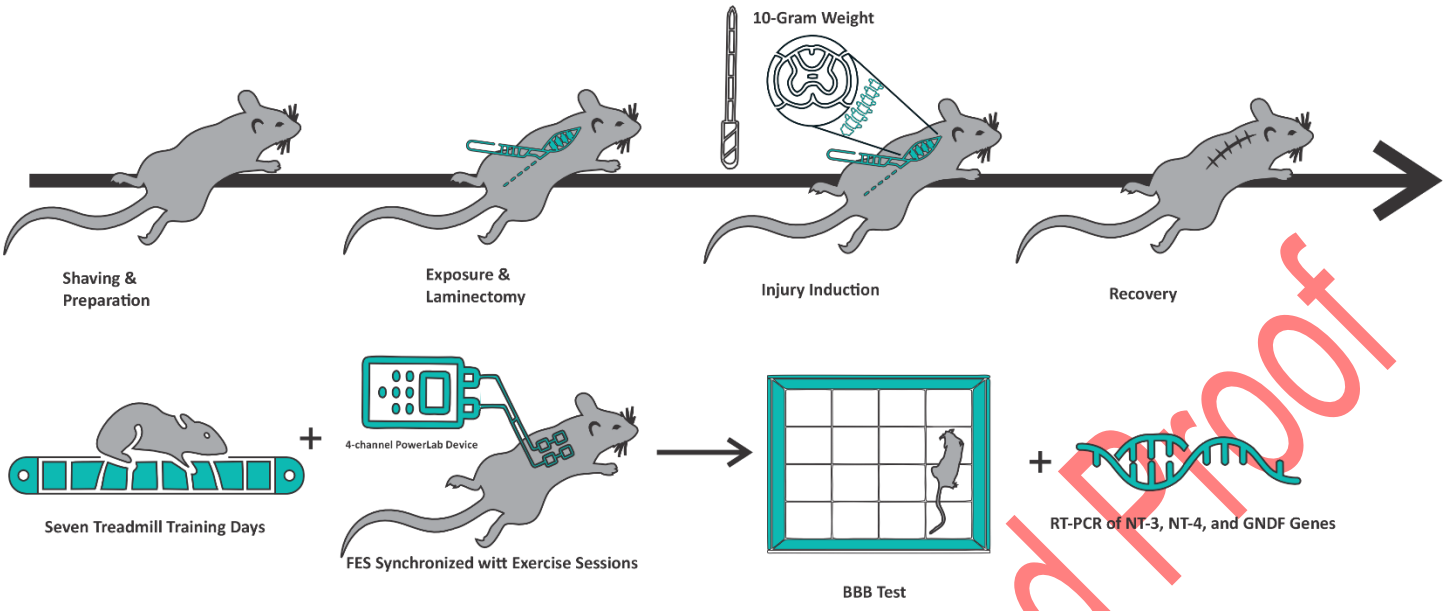

Figure 2. Stages for Inducing Spinal Cord Injury and Conducting Experiments

Aerobic Exercise Training Protocol

Aerobic exercise was performed on a motorized treadmill which was equipped with a body weight support system to facilitate safe locomotion beginning 14 days after spinal cord injury, where the subjected animals were acclimated to the treadmill for 3 days prior to training. The training protocol consisted of one session per day for seven consecutive days, with exercise duration progressively increasing from 20 minutes on day 1 to 58 minutes on day 7, and treadmill speed set at 8–12 m/min, corresponding to low-to-moderate exercise intensity as previously described for rodents with spinal cord injury. Short rest periods (1–2 minutes) were provided if signs of fatigue were observed, including frequent stopping or inability to maintain pace.

Functional Electrical Stimulation (FES) Protocol

Spinal electrical stimulation was delivered using a 4-channel PowerLab system (PowerLab®, ADInstruments), delivered through two custom-made 5-mm surface electrodes placed over the lesion level and secured for the stimulation period, with electrodes being securely fixed for the duration of each session to ensure consistent placement and stimulation delivery.

Electrical stimulation consisted of biphasic, charge-balanced rectangular pulses delivered at 50 Hz with a pulse width of 200 µs per phase (inter-phase interval 50 µs) in constant-current mode at an amplitude of 1.0 mA, with these parameters being selected to evoke effective neuromodulatory stimulation while minimizing the risk of tissue damage. Stimulation was delivered continuously during the exercise session, beginning at exercise onset and ending at exercise termination, resulting in a stimulation duration identical to the exercise bout. Electrode contact was confirmed before each session, and impedance was monitored to ensure stable delivery across sessions.

In animals assigned to the FES-only and combined intervention groups, stimulation was applied once daily during the intervention period, and for the combined intervention group, electrical stimulation was delivered continuously throughout the aerobic exercise session, beginning at exercise onset and terminating at the end of the exercise bout. Accordingly, the duration of electrical stimulation was identical to the corresponding exercise session and ranged from 20 to 58 minutes, depending on the training day. It is notable that, prior to each

stimulation session, electrode contact and positioning were verified, and impedance was monitored to ensure stable current delivery across sessions.

Tissue Collection and Euthanasia

Forty-eight hours after the completion of the final intervention session, animals were subjected to a 12-hour fasting period and subsequently re-anesthetized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), with adequate depth of anesthesia being confirmed prior to all procedures.

Under deep anesthesia, intact spinal cord tissue encompassing the lesion site was carefully harvested using sterile surgical techniques. Collected tissue samples were immediately frozen and stored at −70 °C until subsequent molecular analyses.

Following tissue collection, animals were humanely euthanized in accordance with institutional animal care guidelines and approved ethical protocols. All biological remains were disposed of following established biosafety and biohazard regulations.

Gene Expression Analysis

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was employed to evaluate the mRNA expression levels of GDNF, neurotrophin-3 and 4 in spinal cord tissue samples, where total RNA was extracted from frozen spinal cord tissue using a commercial RNA extraction kit (Addbio, South Korea) in accordance with the manufacturer's instructions. The concentration and purity of the isolated RNA were assessed spectrophotometrically by measuring absorbance at 260 and 280 nm (A260/A280). RNA samples were subsequently stored at −70 °C until further processing.

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a reverse transcription kit (Addbio, South Korea) following the manufacturer's protocol. Quantitative real-time PCR amplification was performed using SYBR Green Master Mix on a real-time PCR system.

Gene-specific primers for GDNF, NT-3, NT-4, and the reference gene β-actin were used. For GDNF, primers were designed to target the GFRα1 receptor in an exon–exon junction format using PRIMER3 and the online IDT primer design software and were synthesized by Gene Fanavaran Co. (Tehran, Iran). The primer sequences and specifications are provided in Table 1.

Reaction mixture volumes and component concentrations for each target gene were prepared according to the protocol detailed in Table 2, and real-time PCR amplification was carried out using a four-step thermal cycling program as described in Table 3. Lastly; relative gene expression levels were calculated using the 2^−ΔΔCt method, and all target gene expression values were normalized to the reference gene β-actin.

Table 1. **Gene Primers Characteristics**

Target	**PCR Product**	**TM (oC)**	**Primer**
NT-3	204	56.14	F: CTTTTGAGGGACCATTCGAG
		56.90	R: GCAGAAGTAACCATGGCATC
GDNF	207	57.58	F: AGACCGGATCCGAGGTG
		57.09	R: TCTTCGGGCATATTGGAGTC
Actin β	200	59.56	F: CGCGAGTACAACCTTCTTGC
		59.08	R: ATACCCACCATCACACCCTG

NT4	182	59.3	F: AGCTCAGTCTGTGTGGAATG
		60.07	R:CTCTTGCCAGGGTAAGTCAGG

Table 2. Required Quantities of Reagents for the Real-Time PCR Reaction

Materials	Volume used (µL)
Cybergene Master Mix	10
Primer Pair Mix	2
DNase free H2O	6
cDNA	2

Table 3. Thermal Cycling Program for the Real-Time PCR Reaction

Cycle	Time	Temperature	Step
١ Cycle	3 minutes	95 °C	Hold
45 cycles	30 Seconds	95 °C	Denaturation
	20 Seconds	58 °C	Primer Annealing
	30 Seconds	72 °C	Extension

Sample size calculation

An a priori sample size calculation was performed using G*Power software (Heinrich Heine University Düsseldorf, Germany) to ensure adequate power for detecting group differences in spinal cord NT-3, NT-4, and GDNF gene expression among the five experimental groups. The calculation was based on a one-way ANOVA (fixed effects, omnibus test) with an assumed moderate effect size (Cohen's f = 0.40), α = 0.05, power (1–β) = 0.80, and five groups, yielding a required total sample size of 35 animals (n = 7 per group), but due to availability constraints, 33 rats were enrolled, of which 29 completed the study (due to the mortality through the SCI induction process) and were included in the final analysis. This sample size also allowed for potential post-injury attrition commonly observed in experimental SCI models. All statistical analyses were conducted using the final number of animals available per group.

Data Analysis

After the extraction of data from the experiments, descriptive statistics were computed to determine the mean values and standard deviations, and to generate tables and graphs. Since the data were normally distributed (as evidenced by the result of the the test 'Shapiro-Wilk'), a one-way analysis of variance (ANOVA) was performed to assess intergroup variance differences and Post hoc tests were subsequently performed for pairwise group comparisons, with a p-value of < 0.05 indicating statistical significance for accepting or rejecting the null hypotheses. It is notable that BBB locomotor scores were compared between groups at each assessment week using one-way ANOVA followed by Tukey post hoc testing. This approach was selected a priori to allow direct between-group contrasts at clinically relevant timepoints and to accommodate occasional missing observations and unequal group sizes due to post-injury attrition. Classical repeated-measures ANOVA was not used because it is sensitive to incomplete longitudinal data and relies on sphericity assumptions in small samples. All statistical

analyses were performed using SPSS 20.0 (SPSS Inc.; Chicago, IL, USA), and graphs were generated using GraphPad Prism 5 (GraphPad Software Inc.; San Diego, CA, USA).

Results

Following exclusions due to unsuccessful SCI induction, of the 33 initially included rats, a total of 29 survived to the end of the experiment and were subjected to analysis, as 2 animals in the SCI group, 1 animal in the SCI with exercise group, 1 animal in the SCI with electrical stimulation group, and 2 animals in the SCI with combined exercise and electrical stimulation group were lost during the study period.

Evaluation of locomotor function using the BBB scale 24 hours after surgery and SCI induction yielded in scores below 3 for all animals and the mean locomotor scores for the groups were subsequently assessed at various time points, as illustrated in Figure 3. Significant differences in locomotor scores were observed among the groups at week 8 (W = 8, F = 72.58, P = 0.01), week 6 (W = 6, F = 93.74, P = 0.002), and week 4 (W = 4, F = 55.11, P = 0.02). Moreover, in the SCI group, the FES-treated subgroup exhibited a significantly higher locomotor score in week 2 post-injury compared to the SCI group (W = 2, P = 0.03), while in week 3, the exercise group showed a significant improvement relative to the SCI group (W = 3, P = 0.02). Across the subsequent weeks (weeks 4–8), the mean locomotor scores of the three intervention groups were significantly higher than those observed in the SCI group (P < 0.05).

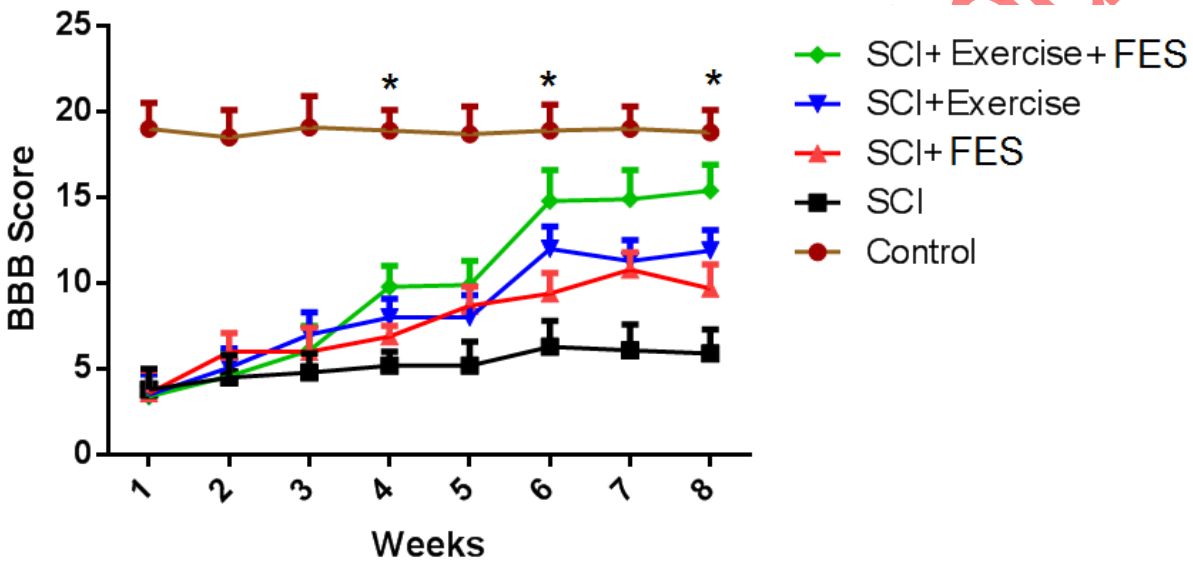

Figure 3. Motor test scores comparison between groups (*P < 0.05, **P < 0.01, **P < 0.001), illustrated as mean ± standard deviation (SD). The numbers of rats in each group were as follows: five experimental groups containg six healthy controls, five SCI controls, six SCI + aerobic exercise, six SCI + functional electrical stimulation, and six SCI + combined aerobic exercise and FES.

Similar with the previously described protocol, extraction of RNA was performed and following assessment of its concentration and purity, a sample of extracted RNA was subjected to electrophoresis on agarose gel. of 1.5%. The presence of the srRNA18 (approximately 9.1 Kb) and srRNA28 (approximately 5 Kb) bands confirmed the integrity of the RNA extraction. The data implied that expression of GDNF in the SCI+ FES model was significantly elevated compared to the groups of control, SCI, and exercise. Notably, a statistically significant increase was also observed in the group of combined exercise and FES. Furthermore, a statistically significant difference was noted between the exercise plus FES group and the FES–only group, with the most pronounced changes occurring in the combined intervention group. Figure4a presents the pairwise comparison of GDNF gene expression among the study groups.

The results also revealed that expression of NT-3 was enhanced in a significant manner in the SCI model receiving combined intervention of FES and exercise, when compared to other groups. Both the SCI with exercise and the SCI with FES groups demonstrated significantly higher NT-3 expression than the SCI and healthy control groups, with the combined intervention group showing the greatest effect. Figure4b depicts the pairwise comparison of NT-3 gene expression.

The findings show that NT-4 expression was increased significantly, in the model of SCI with FES relative to the SCI, SCI with exercise, and healthy control groups. This significant elevation was also observed in the combined intervention group. The difference found between the combined intervention group and the FES–only group, was not statistically significant. Figure4c illustrates the pairwise comparison of NT-4 gene expression.

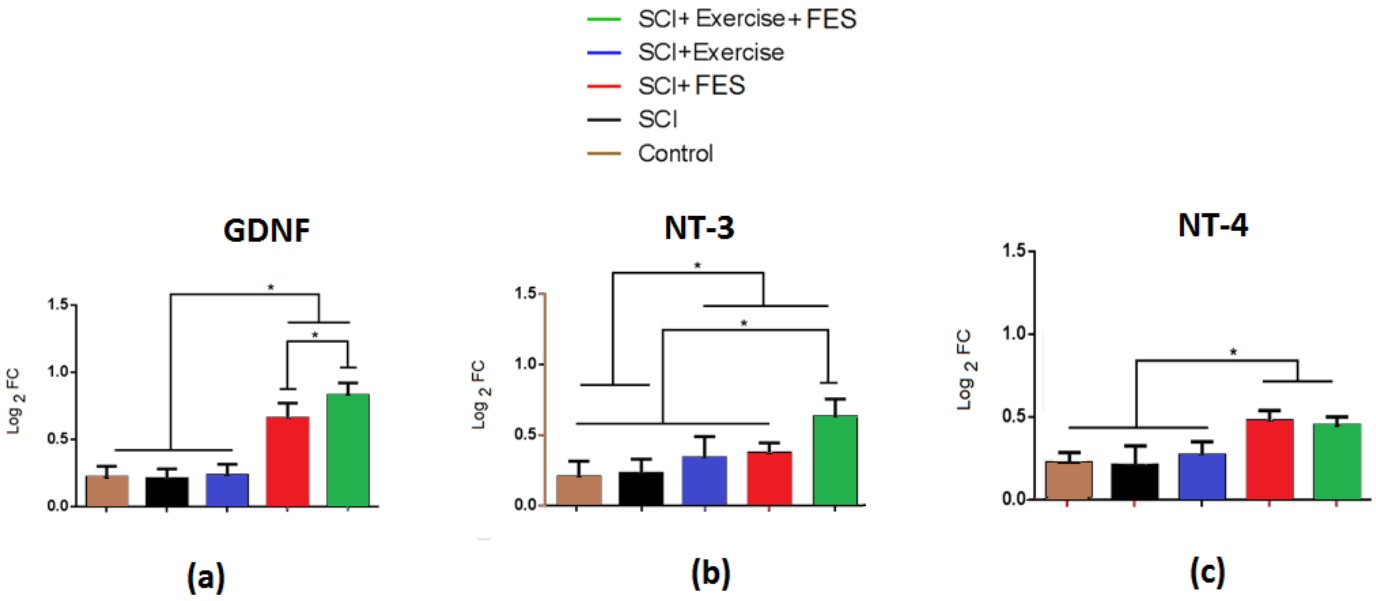

Figure 4. Relative mRNA expression levels of GDNF (a), NT-3 (b), and NT-4 (c) in spinal cord tissue across experimental groups: healthy control, spinal cord injury (SCI) control, SCI + aerobic exercise, SCI + functional electrical stimulation (FES), and SCI + combined aerobic exercise and FES. Data are presented as mean ± standard deviation. Statistical significance was determined using one-way ANOVA followed by post hoc testing.

Discussion

We conducted the study presented here, to evaluate the effects of aerobic exercises and FES on locomotor performance and the expression of the GDNF, NT-3, and NT-4 genes in rats with SCI and the results of locomotor assessments indicated that spinal electrical stimulation produced beneficial effects on motor function as early as two weeks' post-injury, however, the exercise protocols proved more effective than spinal electrical stimulation alone in facilitating locomotor recovery from week four onward. Simultaneous administration of exercise regimen and FES showed to cause the most significant improvements in motor function compared to each method alone. Further studies on Gene expression analyses revealed that electrical stimulation, both alone and on a greater degree in combination with exercise, elicited significant changes compared to the other groups, with the combined intervention yielding the most pronounced effects.

Regular, structured physical activity promote activity-dependent neural plasticity and skeletal muscle integrity, contributing to functional recovery following neurological injury (34, 35), and in the context of SCI, exercise-based rehabilitation has been reported to enhance reorganization of spinal and supraspinal motor circuits, facilitating adaptive plasticity within spared pathways (36, 37). Experimental and clinical studies further demonstrate that training-induced motor recovery is associated with changes in corticospinal excitability and improved motor cortex organization, even in neurologically intact individuals, highlighting the capacity of exercise to induce experience-dependent neuroplasticity (38-40).

At the molecular level, exercise has been shown to modulate dendritic remodeling, synaptic connectivity, neurotransmitter regulation, and ionic homeostasis within the central nervous system (41, 42). Accumulating evidence indicates that exercise-induced upregulation of neurotrophic factors, including NT-3, NT-4, BDNF, and GDNF, represents a key mechanism underlying these coordinated structural and functional adaptations (43-45). In SCI models, elevated neurotrophin expression following exercise has been linked to enhanced neuronal survival, axonal maintenance, and improved locomotor outcomes (46, 47). Consistent with this literature, the present study demonstrates that aerobic exercise modulates spinal neurotrophin gene expression and motor function in rats with SCI, supporting the role of activity-dependent neurotrophic signaling in spinal cord repair.

Spinal cord repair, through activity-dependent regulation of multiple biomolecular pathways, including neurotrophins, insulin-like growth factors, and inflammatory mediators, is influenced by exercise-based interventions, which collectively govern neuronal survival, axonal remodeling, glial scar formation, and cell fate decisions following SCI (36, 48, 49), among which molecular mediators, NT-3 and 4, and glial cell line-derived play critical and non-redundant roles in neurogenesis, axonal maintenance, and spinal circuit stability (50-52). Consistent with their essential biological functions, neurotrophins have been extensively investigated in both experimental models and clinical contexts as therapeutic targets for neurodegenerative and traumatic nervous system disorders (53, 54).

Genetic evidence further underscores the functional importance of these factors: NT-3 knockout mice exhibit severe proprioceptive deficits and impaired motor coordination due to loss of muscle spindle afferents, highlighting the requirement of NT-3 for sensorimotor integration (55, 56). Similarly, GDNF deficiency results in profound motor neuron loss and perinatal lethality, reflecting its indispensable role in spinal motor neuron development and survival (57, 58). In this context, the exercise-induced modulation of NT-3, NT-4, and GDNF expression observed in the present study provides a mechanistic framework linking physical activity to preservation of spinal circuitry and functional recovery after SCI.

As the NFTs are neuroprotective and implicated in neurogenesis and neuroplasticity, they are also upregulated in FES. There is a body of evidence that shows functional recovery in SCI post-injury (59). As FES and exercise

both can upregulate neurotrophins, this study presented data that show NT-3, NT-4, and GDNF are upregulated in both FES and Exercise groups, and the combination effect of these two interventions is neither competitive nor synergistic in both neurotrophin genes upregulation and functional outcomes. These effects are also repeated in a recent article that investigates the combination of treadmill running and electrical stimulation in peripheral nerve injury. There was a regulation of GDNF and NT-3 and optimal balanced effects elicited (60). In another study, the neuromuscular stimulation and aerobic exercise were shown to have a dose-dependent effect on neurotrophin regulation (61).

Skup et al. (2002) demonstrated that endurance training for eight weeks, can significantly increase the expression of gene related to the protein NT4/5 in the muscles of male Wistar rats (62). In a 2019 study, Yazdanian and colleagues reported the considerable association of cerebral ischemia with neuronal death in the CA1 region of the hippocampus, however, exercise markedly reduced ischemia-induced cell death (63). Detloff et al. demonstrated that acute exercise prevents the onset of neuropathic pain and the sprouting of non-peptidergic C-fibers following SCI and the findings further linked the development of neuropathic pain in SCI to significant reductions in the artemin and GDNF levels within the dorsal root ganglia (DRGs) and spinal cord, suggesting that GDNF, GFLs, or their downstream effectors may serve as key modulators of nociceptive fiber plasticity and thus represent promising targets for anti-allodynic therapies (64).

Peyman Ghasemi and colleagues (2020) investigated the impact of a four-week exercise regimen on hippocampal GDNF mRNA levels in a mouse model of Alzheimer's disease induced by Aβ1-42 injection. They found a significant difference in GDNF expression between groups ($p < 0.001$), with the exercise group exhibiting higher expression levels compared to the Aβ-42 group. Additionally, spatial learning and memory were significantly improved in the group receiving both exercise and Aβ-42 induction relative to the Aβ-42-only group. These results suggest that a four-week exercise protocol not only enhances spatial learning and memory but also upregulates neurotrophic factor expression, particularly GDNF, in the hippocampus (65).

Exercise rapidly and markedly influences dendritic sprouting, synaptic connectivity, neurotransmitter synthesis and regulation, as well as ionic homeostasis, with recent studies highlighting the exercise-induced elevation of neurotrophic factors is a fundamental mechanism underlying these interrelated effects (44, 66, 67).

Limitations

The relatively small sample size of our study in which 33 animals enrolled, with 29 completing the study, may limit the generalizability of the findings and statistical power and the as small cohorts can reduce the ability to detect subtle but clinically relevant differences in gene expression and functional recovery (68). Additionally, the variability in the operative and postoperative management of animals and complications such as infections, hindlimb injuries, and bladder rupture, may introduce additional confounding factors that may affect the interpretation of the outcomes, as previously recognized in preclinical SCI research (69). Notably, the duration of the study and follow-up period might not fully capture the long-term effects of the interventions on both motor recovery and neurotrophic gene expression (70), and nonetheless, inherent differences between rodent models and human SCI pathology pose challenges for direct clinical translation of these findings (71).

Future Directions

Based on the findings of this study, increasing the sample size and incorporating multi-center collaborations would enhance statistical power and may benefit the external validity of preclinical findings (72). longitudinal studies with extended follow-up periods would also help to better evaluate the durability of motor function improvements and sustained neurotrophic factor upregulations (73). Notably, studies focusing on the molecular mechanisms driving the observed synergistic effects through advanced proteomic and transcriptomic analyses (74), and translational studies involving larger animal models and,

eventually, controlled clinical trials will be essential to validate the efficacy and safety of these combined rehabilitative interventions and to bridge the gap between preclinical and clinical research (75).

Conclusion

This study demonstrated that a combination of aerobic exercises and FES significantly improves motor function and upregulates NT-3, NT-4, and GDNF expression in SCI rats, in a manner in which, FES-alone enhances early post-injury recovery, exercise-alone plays a more pronounced role in later stages, and the synergistic effects observed with combined exercise and FES highlight the potential of multimodal rehabilitation approaches for SCI. Based on these findings, future studies that elucidate the long-term benefits and underlying molecular mechanisms of these interventions, and their translational potential in clinical settings, optimizing exercise and stimulation parameters for enhanced neuroprotection and functional recovery are needed.

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