Investigation of the effects of electrical stimulation on BDNF and NGF levels in the sciatic nerve injury rat model

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ABSTRACT

The current study aimed to investigate the effects of electrical stimulation on brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels in rats with sciatic nerve injury. Twenty-eight rats were divided into four groups of sham (S, n=7), electrical stimulation (ES, n=7), sciatic nerve injury (SNI, n=7) and sciatic nerve injury+electrical stimulation (SNI+ES, n=7). An experimental nerve damage model was produced by applying a closing force to compress the sciatic nerve. Electrical stimulation was applied for twenty minutes at 200 µs, 2mA, and 20 Hz for fifteen days. Enzyme-linked immunosorbent assay analysis was used to evaluate the levels of NGF and BDNF. It was shown that the SNI group had higher brain BDNF levels than the other groups, while the S group had lower brain BDNF levels than the other groups (P<0.001). The ES and SNI groups had higher serum BDNF levels than the S and SNI+ES groups (P<0.01), while the SNI group had higher brain NGF levels than the S and SNI+ES groups (P<0.05). In comparison to the S and ES groups, the serum NGF levels in the SNI and SNI+ES groups were shown to be lower (P<0.01). Following the sciatic nerve damage, it was measured that the administration of electrical stimulation resulted in an increase in brain BDNF and a decrease in serum NGF. According to this research, electrical stimulation may have an impact on the release of NGF and BDNF after sciatic nerve damage.

Introduction

The rat sciatic nerve model is widely used to investigate functional, histological, and electrophysiological changes after different chirurgical repairs or pharmacological treatments, following peripheral nerve damage in experimental peripheral nerve studies (34). Many methods, including anastomosis, nerve conduits, non-neural tissue grafts, nerve grafts, combined grafts, and synthetic tubes, have been used in the treatments of peripheral nerve damages, and their possible effects on nerve healing have been revealed (8, 24, 28, 32). The importance of early physiotherapy applications following peripheral nerve injuries is emphasized, and it is known that methods that preserve or increase the muscle fiber diameter are effective in denervated muscle treatment. Electrical stimulation may also be useful to stimulate nerve regeneration in cases of nerve injuries (22). Electrical stimulation is used in orthopedic and neurological rehabilitation to create functional or therapeutic movements in upper motor neuron illnesses, including traumatic brain injury, spinal cord damage, cerebral palsy, stroke, or multiple sclerosis, by inducing muscle activation in paralyzed muscles to stand, walk, grip, and release (12, 17). Gordon et al. (7) reported in both human and animal studies that electrical stimulation may significantly accelerate axon growth and muscle reinnervation may occur significantly earlier. Electrical stimulation is not only a way to restore function; it is also used as a clinical efficacy motor function training tool (13).

The most well-characterized members of the neurotrophins are nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and both of them play specific roles in the regeneration, protection, and tropism of axotomized peripheral nerve fibers (15, 16, 18,

31). BDNF, in combination with other factors or alone, has been shown to have a positive effect following neurorhaphy (10, 20, 35). Frostick et al. (5) reported that BDNF promotes the survival and differentiation of motor neurons *ex vivo*, and it organizes the development of neuromuscular synapses and the function of preventing neuronal death following nerve damage *in vivo*. Vögelin et al. (35) presented that by surgically removing a twenty-millimeter portion of the sciatic nerve of rats *in vivo*, local continuous release of BDNF not only stimulates the regeneration of peripheral nerve but also provides rapid axonal growth and significantly reduces neuropathic pain. Shakhbazau et al. (31), in their study investigating neurotrophin synthesis in a two-week period in the transvertebral model of bilateral and/or unilateral transections of the sciatic nerves in rats, reported that increased NGF concentrations in the nerve segment of bilateral transection compared to the uniletral transection. After nerve axotomy, it is mentioned that neurotrophins (especially BDNF) have a direct role in enhancing the viability of the damaged neurons (6). In addition, BDNF mRNA expression in damaged sciatic nerves is up-regulated three days following nerve damage, and the up-regulation, which is deemed necessary for axonal regeneration, takes several weeks (23). In the literature, it has been indicated that BDNF released in the dorsal root ganglia is carried to the primary sensory neuron, and also BDNF levels change rapidly in the dorsal root ganglia following peripheral inflammation and nerve damage (27, 37). To further augment the functional outcome of peripheral nerve repair, neurotrophins have been used experimentally, and it has been suggested that neurotrophins increase neuronal cell survival and regeneration (4, 19). Huang et al. (11) indicated the potential of using electrical stimulation as a useful method to enhance functional recovery for delayed repair of peripheral nerve lesions in rats with chronic axotomized motoneurons. In addition, they presented that when brief electrical stimulation was applied to the proximal nerve stumps in the delayed nerve lesions, the BDNF gene expression was significantly up-regulated in the motoneurons in the anterior horn of the spinal cord. Park et al. (29) indicated that electrical stimulation may improve neural plasticity by boosting BDNF production in sciatic nerves such as soleus and medial gastrocnemius muscles. In a study on electrical stimulation application following femoral nerve cutting in rats, it was indicated that the gene expression of motoneuronal BDNF, which is organized by electrical activity, correlates with nerve regeneration (1).

The observed developments in functional behavior induced by electrical stimulation therapy can be partly mediated by molecular plasticity in the brain. It is suggested that electrical stimulation of peripheral nerves leads to the rise of genes related to regeneration, including BDNF. By enhancing the expression of structural protection proteins and neurotrophins, electrical stimulation may be used therapeutically to reduce muscle atrophy and improve muscle reinnervation (25). However, neural plasticity due to electrical stimulation and the general mechanisms mediating healing remain unclear (12). Moreover, research suggested that electrical stimulation may accelerate the healing of body function after peripheral nerve injury, but electrical stimulation still possesses the following problem to be solved: what changes in brain activity are induced by electrical stimulation (25). In the current study, the evaluation of BDNF and NGF release after sciatic nerve injury and whether electrical stimulation had any effects on the release of relevant factors were investigated.

Materials and Methods

In the present study, twenty-eight male, 8-10 weeks-old Wistar Albino rats (250–300 g) were provided from the Experimental Research Application and Research Center at Hatay Mustafa Kemal University (HMKU), Turkey. The rats were housed in a ventilated environment with 12-hour cycles of light and darkness, a humidity of 45 ± 5%, and a temperature of 21-1°C. Food and water were available *ad libitum*. The experiments on animals were approved by the Local Ethical Committee of Experimental Animal Ethics of HMKU, Turkey (approval no. 2019/10-5, 17/12/2019).

Animals and Surgical Procedures: To determine the minimum required sample size before the study, power analysis was performed using the criteria of a type 1 error rate of 0.05, power of 0.90, and effect size of 1 (36). The minimum required sample size was determined to be n=5 per group, totaling 20. However, to allow for potential losses during the study, 7 animals per group were used. Animals were equally divided into four groups (n=7 each): the sham (S) group, the sciatic nerve injury (SNI) group, the electrical stimulation (ES) group, and the sciatic nerve injury + electrical stimulation (SNI+ES) group. The animals were anesthetized for the operation with a xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (50 mg/kg) cocktail. Surgical procedures were carried out aseptically. The sciatic nerve was dissected (Figure 1) meticulously by forming a longitudinal skin incision on the right extremity at the level of the trochanter major and preserving the perineurium without traction injury in the sciatic nerve injury groups (SNI and SNI + ES). The sciatic nerve injury rat model was created by compressing the sciatic nerve with a special temporary aneurysm clip (Yasargil FE693K, Aesculap AG, Germany) with a closing force (50 g/cm) for two minutes in SNI and SNI + ES groups

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(Figure 2) (30), the wound was closed, and then the skin was sutured. In addition, skin and muscle were opened, respectively, and instantly closed in layers by the sciatic nerves left intact on the right extremities in the S and ES groups.

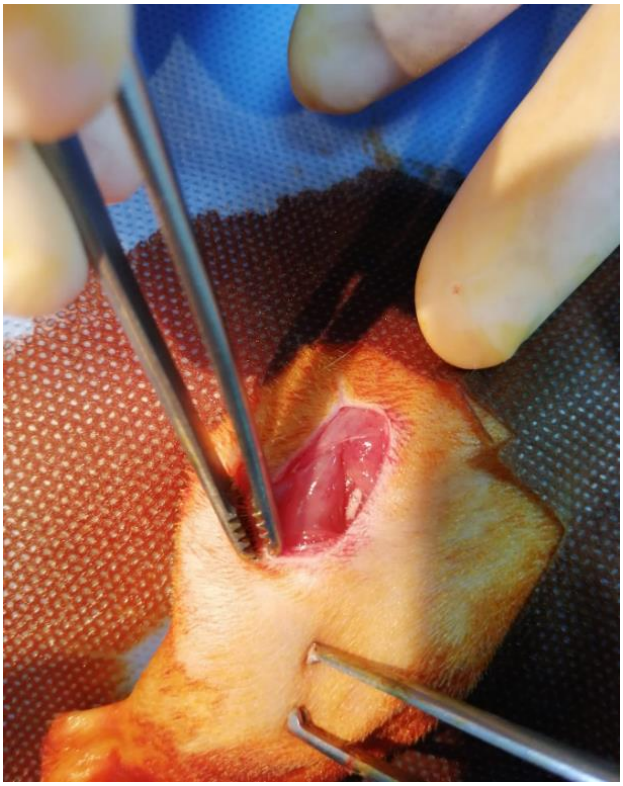

Figure 1. The exposure of the sciatic nerve.

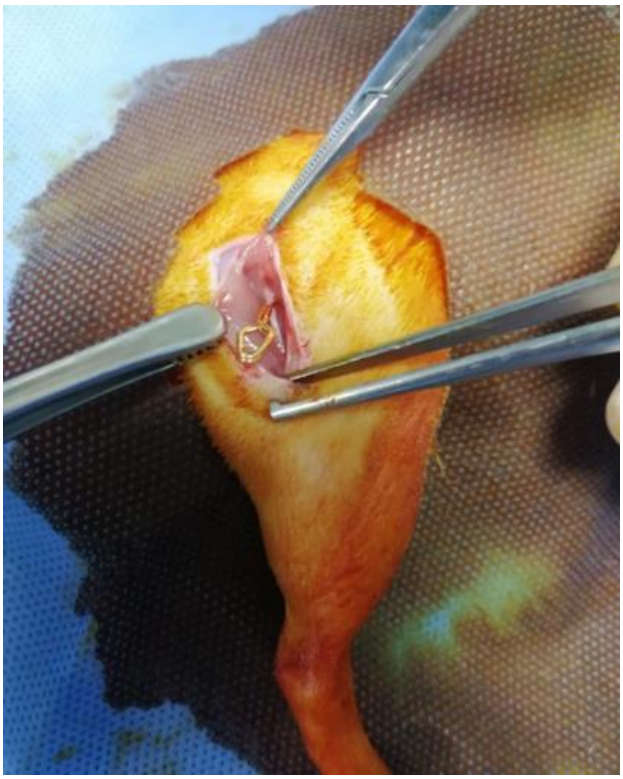

Figure 2. The sciatic nerve injury was made by compressing the nerve with a closing force.

Electrical Stimulation: The S and SNI groups received no treatment postoperatively. Electrical stimulation was commenced 3 hours following the operation. It was applied with two electrodes (Biomedical carbon film electrodes, Stimrodes); one of the electrodes was placed on quadriceps femoris muscle, especially 5 mm proximal to the injury site, and the other was placed on gastrocnemius muscle of the right extremity (Figure 3). Electrical stimulation was applied to rats in ES and SNI+ES groups at 20 Hz frequency, 200 µs current time, and 2 mA amplitude for 20 minutes parameters during 15 days with an electrical stimulation device (Chattanooga Intelect, Primera, England) (3, 22). In brief, the groups were formed as follows:

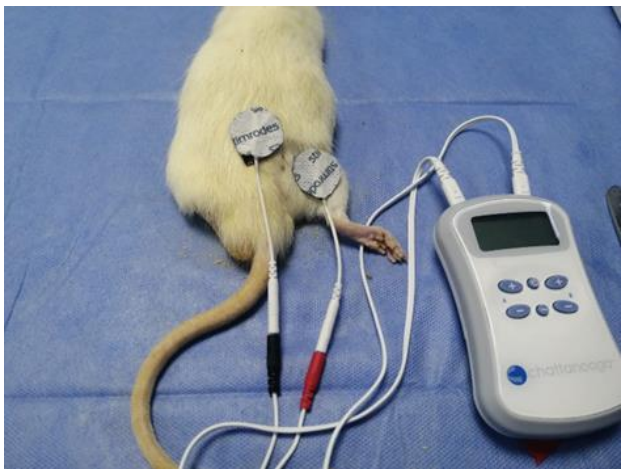

Figure 3. The application of electrical stimulation.

S Group: Only the tissues around the sciatic nerve were dissected from the dorsal surface of the right thigh of the rats, and the skin and subcutaneous tissue were closed properly without causing sciatic nerve injury.

ES Group: Only the tissues around the sciatic nerve were dissected from the dorsal surface of the right thigh of the rats, and the skin and subcutaneous tissue were closed properly without causing sciatic nerve injury. In addition, electrical stimulation was applied to the rats during fifteen days.

SNI Group: Right sciatic nerve injuries were experimentally induced in animals.

SNI + ES Group: Right sciatic nerve injuries were experimentally induced in animals, and then electrical stimulation was applied to the rats during fifteen days.

Sample Collection: On the 16th day of the study, the animals were anesthetized by using a mix comprising xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (100 mg/kg). Intracardiac blood was taken in serum clot activator tubes after the loss of righting, corneal, and withdrawal reflexes following anesthesia.

Rats were sacrificed with exsanguinations. Brain tissues were immediately harvested after the sacrification. The blood samples were centrifuged at 3000 rpm, +4 °C for 10 minutes, the serum was obtained, and then serum and brain tissues were kept at -80 °C until ELISA analyses.

Tissue Preparation: Brain tissues thawed, and the prefrontal cortex of the left hemisphere of the brain was used in the study. Approximately 100 mg of brain tissue sample was taken and homogenized by using an ultrasonic homogenizer (Bandelin Electronic UW 2070, Germany) in 1 ml phosphate buffer solution (pH: 7.4) in cooled tubes with ice (14). After centrifugation of the homogenates at 5,000 rpm, +4 °C for 30 minutes, the samples (supernatants) were obtained and kept at −20°C until enzyme-linked immunosorbent assay (ELISA) analyses.

Protein Analysis: Protein concentrations were measured spectrophotometrically (UV 2100 UV-VIS Recording Spectrophotometer, Shimadzu, Japan) according to Lowry's method (21). Brain protein levels were only determined to calculate neurotrophin levels. The method relies on the identification of cuprous ions, which are generated when proteins reduce cupric ions in an alkaline environment. Following a pre-treatment of the proteins with copper ions in an alkaline solution, the aromatic amino acids in the treated sample lowered the amount of phosphomolybdatephosphotungstic acid that was present in the Folin Reagent. This reaction resulted in a blue substance. By comparing the absorbance of the Folin reaction's final result at 700 nm to a standard curve of a standard (Bovine Serum Albumin) protein solution, the amount of protein in the sample was calculated.

BDNF and NGF Analysis: The levels of BDNF and NGF in the brain and serum samples were determined using the ELISA method. For this purpose, commercial ELISA test kits were used in the samples, and the procedure reported by the manufacturer was followed. Before beginning the ELISA analysis, the samples and solutions in the ELISA kits were brought to room temperature. The principles of the rat BDNF ELISA kit (201-11-0477, Sunred, China) and the rat NGF ELISA kit (201-11-0540, Sunred, China) are generally the same for each other. Briefly, the kits use a double-antibody sandwich ELISA to test the level of rat neurotrophin (NGF/BDNF) in samples. Neurotrophin was put in monoclonal antibodies. A rat neurotrophin monoclonal antibody has been used to pre-coat an enzyme well. The antibodies of neurotrophin were labeled with biotin and then combined with Streptavidin-HRP to constitute an immune complex. The choromogen solution A, the choromogen solution B, and the stop solutions were added, and the liquid color was ultimately changed to yellow. The color absorbance formed in the microplate was evaluated in the microplate reader (BioTek MQuant, USA), and the results were evaluated from the standard curve.

Statistical Analysis: The values obtained were evaluated by the Windows Statistical Package for the Social Sciences Program (IBM SPSS 22 version, Armonk, NY). Prior to the significance tests, all data were evaluated by the Shapiro–Wilk test for normality from the parametric test hypotheses and by the Levene test for the homogeneity of variances. The comparison of multiple groups was determined by analysis of variance (ANOVA) and post hoc Duncan's test. A difference of $P<0.05$ was considered significant. The values were expressed as mean ± standard error (SE).

Results

Brain-derived Neurotrophic Factor: Brain BDNF values were significantly ($P<0.001$) increased in the SNI group compared to other groups. Brain BDNF values in the S group were significantly ($P<0.001$) lower than in other groups. Brain BDNF values were found to be at almost the same values as the ES group (0.33 ± 0.008 ng/mg protein) and the SNI + ES (0.33 ± 0.006 ng/mg protein) (Table). However, serum BDNF levels in the ES group (1.62 ± 0.021 ng/ml) and in the SNI group (1.60 ± 0.024 ng/ml) were significantly ($P<0.01$) higher than the other groups. Serum BDNF values were determined to be at almost the same values as the S group (1.53 ± 0.007 ng/ml) and the SNI + ES (1.53 ± 0.024 ng/ml) (Table). Brain and serum BDNF values were significantly diminished in the SNI+ES group compared with the SNI group ($P<0.001$, $P<0.01$, respectively).

Table The BDNF and NGF levels of brain and serum (mean±SE).

Parameters	S (n=7)	ES (n=7)	SNI (n=7)	SNI+ES (n=7)	P
Brain BDNF (ng/mg protein)	0.29 ± 0.007^{c}	0.33 ± 0.008^{b}	0.36 ± 0.008^{a}	0.33 ± 0.006^{b}	<0.001
Serum BDNF (ng/ml)	1.53 ± 0.007^{b}	1.62 ± 0.021^{a}	1.60 ± 0.024^{a}	1.53 ± 0.024^{b}	<0.01
Brain NGF (ng/mg protein)	3.12 ± 0.13^{b}	3.57 ± 0.19^{ab}	3.77 ± 0.11^{a}	3.26 ± 0.14^{b}	<0.05
Serum NGF (ng/ml)	11.10 ± 0.30^{a}	11.23 ± 0.17^{a}	10.29 ± 0.24^{b}	10.26 ± 0.18^{b}	<0.05

[a-c]Different letters on the same column are statistically significant ($P<0.05$, $P<0.01$, $P<0.001$). NGF: Nerve growth factor, BDNF: Brain-derived neurotrophic factor, S:Sham, ES: Electrical stimulation, SN: Sciatic nerve injury, and SNI+ES: Sciatic nerve injury+electrical stimulation.

Nerve Growth Factor: Brain NGF values were elevated in the SNI group (3.77±0.11 ng/mg protein, P<0.05, Table) compared with the S group (3.12±0.13 ng/mg protein) and SNI + ES group (3.26±0.14 ng/mg protein). Meanwhile, brain NGF value increased in the ES group (3.57±0.19 ng/mg protein, Table) compared with the S group, but this increase was not significantly different (P>0.05). Brain NGF values were significantly diminished in the SNI+ES group compared to the SNI group (P<0.05). In addition, it was measured that the values of serum NGF in SNI and SNI + ES groups (10.29±0.24 ng/ml; 10.26±0.18 ng/ml, P<0.01, respectively) were lower than in S and ES groups (Table).

Discussion and Conclusion

It takes a long time for nerves to heal after injury. It is also known that neurotrophins such as NGF and BDNF possess a short half-life. Where the nerve is damaged, it is desirable to provide sustained secretion of these proteins *in vivo* for a long period (35). Various strategies have been developed to maintain controlled secretion of growth factors, including the implantation of mini-osmotic pumps, drug- or cell-containing polymers, viral vectors, agents conjugated with fibronectin receptors, gel foams, and films. However, although these systems have beneficial effects, it has been reported that each of them limits themselves significantly (10, 20, 35). Unlike the mentioned strategies, electrical stimulation was used in the present study to maintain an even longer-term release of neurotrophic factors. In the present study, the evaluation of BDNF and NGF release after sciatic nerve injury and whether electrical stimulation possessed any effects on the release of relevant factors were investigated.

It is stated that BDNF possesses a direct role in protecting the viability of damaged neurons after nerve axotomy (6). In addition, BDNF mRNA is up-regulated in damaged sciatic nerves three days following a nerve lesion, and upregulation takes several weeks, deemed necessary for axonal regeneration (23). It has been reported that BDNF synthesized in the dorsal root ganglia is transported to primary sensory neurons, and BDNF mRNA and protein rapidly change in the dorsal root ganglia following peripheral inflammation and nerve injury (27, 37). It is indicated that the differential regulation of BDNF in spinal motoneurons following sciatic nerve transection and ventral root avulsion increases in motoneurons to promote nerve regeneration following axotomy (9). In accordance with the literature, in the present study, it was determined that serum BDNF, brain BDNF, and brain NGF levels were significantly increased in the SNI group (1.05, 1.24, and 1.2-fold, respectively) compared to the S group. Thus, the present findings revealed that brain and serum BDNF and brain NGF levels increased in sciatic nerve damage.

Electrical stimulation application as a supporter of peripheral nerve regeneration was tested for the first time in 1982, and electrical stimulation of the regenerated nerve and its influence on motor recovery was found (26). Alrashdan et al. (2) indicated that the application of the electrical stimulation protocol at 100 μs, 20 Hz, and 2 mA for 30 minutes parameters instantly after the nerve crush injury accelerated axonal regeneration and increased BDNF mRNA levels in lumbal (L) 4, L5, and L6 dorsal root ganglion neurons at 5 days postoperation. It has been stated that when endogenous BDNF is inhibited by a functional blocking antibody applied within the first 3 days following damage, the enhancing influences of electrical stimulation on the regeneration of axons are eliminated and motor axonal regeneration is accelerated by BDNF with short low-frequency electrical stimulation (33). It has been reported that electrical stimulation exerts a supportive influence on the regeneration of nerves by elevating BDNF levels (1), while low-intensity electrical stimulation may enhance the regenerations of both sensory and motor neurons following injury (2). Park et al. (29) demonstrated that BDNF levels increase in the medial gastrocnemius and soleus muscles after the mentioned sciatic nerves are applied electrical stimulation with three distinct paradigms, including 1 ms / 40 Hz / 30 minutes, 1 ms / 40 Hz / 5 minutes, and 1 ms / 1 Hz / 30 minutes. Moreover, they suggested that electrical stimulation can enhance neuronal plasticity by boosting the production of BDNF, and all of the mentioned paradigms of the stimulus can modulate the amount of BDNF production. The findings from the present study are not far from previous reports in this area. In the present study, it was determined that serum BDNF and brain BDNF levels were significantly increased in the ES group (1.06 and 1.14-fold, respectively) compared to the S group. Similarly to the literature, it was revealed that electrical stimulation (at 200 μs, 20 Hz, 2 mA for 20 minutes during fifteen days) led to an increase in serum and brain BDNF values in the current study. Moreover, the mechanism by which electrical stimulation improves the nerve regeneration environment may be considered to be through the promotion of growth-related factors, especially BDNF.

When the denervated sciatic nerve was examined in terms of NGF with the intact sciatic nerve in the literature, it was reported that there was an increase in NGF levels in the denervated sciatic nerve (31). In the current study, it was revealed that while brain and serum BDNF levels and brain NGF levels increased, serum NGF levels decreased in sciatic nerve damage. Brain BDNF, serum BDNF, and brain NGF levels were significantly reduced in SNI+ES group compared with SNI group. It was observed that electrical stimulation led to an increase in brain and serum BDNF levels, but did not cause any changes in brain and serum NGF levels. Thus, it may not be accurate to say that

electrical stimulation enhances or deteriorates neurotrophin levels in the SNI+ES group when compared to the SNI group in the current study. Considering that it was revealed that electrical stimulation possesses an effect on the release of neurotrophins probably, the decrease in neurotrophin levels in the group that received electrical stimulation following sciatic nerve injury can be due to the fact that electrical stimulation accelerates the use of neurotrophin by metabolism to repair the damage. Shortly, in line with these data, it is thought that the release of BDNF and NGF and electrical stimulation have an influence on their release after sciatic nerve injury.

Since the period of nerve recovery after damage is lengthy and the half-life of NGF and BDNF proteins is short, a reliable prolonged sustained secret *in vivo*, precisely at the site of damage of the nerve, remains desirable (35). In this study, rat siatic nerve was injured and electrical stimulation was applied to provide a sustained secret of NGF and BDNF. In the present study, it was determined that serum BDNF, brain BDNF and brain NGF levels were significantly increased in the SNI group (1.05, 1.24, and 1.2- fold, respectively) compared to the S group, serum BDNF, brain BDNF and brain NGF levels were significantly decreased in the SNI+ES group (0.96, 1.09, and 0.87-fold, respectively) compared to the S group. It was thought that electrical stimulation reduced serum BDNF, brain BDNF and brain NGF levels in sciatic nerve damage. In addition, in the current study, it was found that the serum NGF levels of the SNI and SNI + ES groups decreased 0.93- fold compared to the serum NGF level of the S group. It is thought that this decrease is due to sciatic nerve damage. Because it was determined that the serum NGF levels of the SNI + ES group did not change compared to the serum NGF levels of the SNI group. Therefore, it was found that the electrical stimulation applied in this study had no effect on the serum NGF level in sciatic nerve damage.

The present study suggests that BDNF and NGF release after sciatic nerve injury and electrical stimulation possess an influence on the release. In the future, studies may be conducted to address the uncertainty regarding electrical stimulation, neural plasticity, and the mechanisms that mediate healing. It is predicted that the present study will contribute to scientific researches in the field of physiotherapy and neurochemistry.

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Ethical Statement

This study was performed after the animal experiment was approved by the Hatay Mustafa Kemal University Local Ethics Committee (Decision number: 2019/10-5, 17/12/2019).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

FK, EI, ZY and HA conceived, planned and carried out the experiments. FK and EI contributed to sample preparation. FK and EI contributed to the interpretation of the results. FK and EI took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting the findings this study are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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