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Investigating the Effects of Brief Electrical Stimulation Duration on Sciatic Nerve Regeneration and Functional Recovery in a Rat Transection Model

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ABSTRACT

Introduction: Periodic and brief electrical stimulations (ES) are used as therapeutic protocols to improve nerve regeneration and functional recovery in various nervous system disorders. Periodic ES is applied transcutaneously for several sessions post-surgery, but brief ES is applied directly to the nerve during the surgery. Brief ES has no negative effects on functional recovery but applying periodic ES may delay the recovery. In most research studies, brief ES has been applied for 1-hour, although in some studies shorter durations were used. In this research, to reduce the risk of infection and cost, brief ESs with different durations (1-hour and shorter durations) were studied in a comparative study.

Material and Methods: The right sciatic nerve of 24 adult male Wistar rats was transected and sutured to a silicone tube. Experimental groups were stimulated by 10, 30, and 60 minutes ES (20Hz, 3V, 100µs). The hot plate test was done biweekly**.** At the end of the experimental period (12 weeks), the histomorphometric assessments were performed on the intra silicon tube segment of the regenerated nerve and its tibial branch.

Results: Hot plate test results showed an increase in the regeneration speed in experimental groups; furthermore, the 60-min ES group had better outcomes in histomorphometric assessment than other groups that may be due to the ES effect on the neuronal cell bodies.

Conclusion. As the results indicate, the 60-min ES had a better outcome compared to other groups. Other specifics of a brief ES such as frequency, pulse width, and waveform (monophasic or biphasic) may be studied in future research.

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Introduction

Peripheral nerve trauma is common in clinics, and despite advanced surgical methods and other interventions such as pharmacological treatments and electrical stimulation (ES), the functional recovery is not still satisfactory [1-8]. ES as a therapeutic approach can be used in two ways, brief ES and periodic ES. Brief ES is a low-intensity ES that is applied directly to the proximal stump of the nerve for a short duration (usually 1 hour) after surgery [9-17]. Periodic ES is known as TENS (Transcutaneous Electrical Nerve Stimulation) which is applied transcutaneously and repeated for several days after a repaired surgery [18-21].

Numerous studies have been performed to evaluate the effects of both types of ES on peripheral nerve functional recovery. For example, in an experiment was done by Lu et al [21] the daily application of a 15 minutes-periodic ES with 1 mA, 2 mA, and 4 mA amplitudes for 5 weeks showed that 1 mA ES has improved the functional recovery more effectively than higher amplitudes. In another study [20], a similar ES was applied using different frequencies; 1, 2, 20, and 200 Hz. The results revealed that the outcome of 2 Hz frequency is much better than others. Moreover, some data also confirms that applying periodic ES results in a delay of regeneration after nerve injury [18, 19]. Therefore, there are some concerns about the parameters of periodic ES which are needed to be optimized.

Brief ES has been shown to enhance remyelination of the regenerating nerve by increasing the expression of brain-derived neurotrophic factor (BDNF), trkB receptor, protein zero mRNA, and protein level [9,17, 22]. Al-Majed [10] suggested that brief ES may increase the speed and accuracy of regenerating fibers in the regenerating nerve. Moreover, Wan [15] reported that the number of axons and the diameter of the myelin sheath were increased in brief ES. Furthermore, Zhang [17] mentioned that brief ES speeds up remyelination after sciatic nerve crush injury in rats.

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The duration of brief ES is a parameter that influences the quality of nerve regeneration and functional recovery [10, 14, 22, and 23]. In this respect, it was mentioned that more than 1-hour duration of brief ES has no beneficial effects on the number of motoneurons [10] but even reduces the number of sensory axons due to downregulation of trkB receptor expression [22]. On the other hand, it has been shown that less than 1-hour duration of brief ES improves nerve regeneration [14, 23-25].

In this research, we aim to investigate the effects of brief ES with durations of less than 1 hour on nerve regeneration after injuries. The rat sciatic nerve transection model was used and after suturing the transected nerve, brief ES pulses were applied directly to the repaired nerve for 10 min, 30 min, and 60 min (3V, 100µs, 20Hz). The different aspects of functional recovery were assessed using the hot plate test and histomorphometric analysis of regenerated sciatic nerve.

Materials and Methods

Animals

In this research, 24 adult male Wistar rats, weighted 250-350 g, were used. All animals were kept under the standard laboratory conditions and allowed tap water as well as standard rat chew pellets ad libitum. All animal protocols were consistent with the guidelines issued by the Ferdowsi University of Mashhad Ethics Committee (IR.UM.REC.1397.043).

Experimental Groups

Adult male Wistar rats underwent right sciatic nerve transection surgery and then randomly divided into 4 groups with 6 rats per group: experimental groups 1, 2, and 3 respectively received 10-minutes, 30-minutes, and 60-minutes ES immediately after surgery, and a negative control group that received no ES.

Surgical Procedure

The right gluteal region of deeply anesthetized rats (100 mg/kg of ketamine HCL+ 20 mg/kg of xylazine, both were purchased from Alfasan, Woerden, Holland) were shaved and then the skin and muscles were opened by a 3 to 4 cm long incision located below the great trochanter. After exposure, the sciatic nerve was transected using a sharp blade and the created 6 mm long gap between the proximal and distal stumps was repaired by a 10 mm long silicone tube (1.47 mm ID, 1.96 mm OD). As shown in Figure 1, to fix the silicon tube, the epineurium of the transected nerve stumps was sutured to the silicon tube using two 8.0 nylon sutures (SUPASIL; Supa, Tehran, Iran). The inside space of the silicon tube was filled with 9% sodium chloride solution. The ES was applied before closing the muscles and skin using 4.0 silk sutures (SUPASIL; Supa, Tehran, Iran).

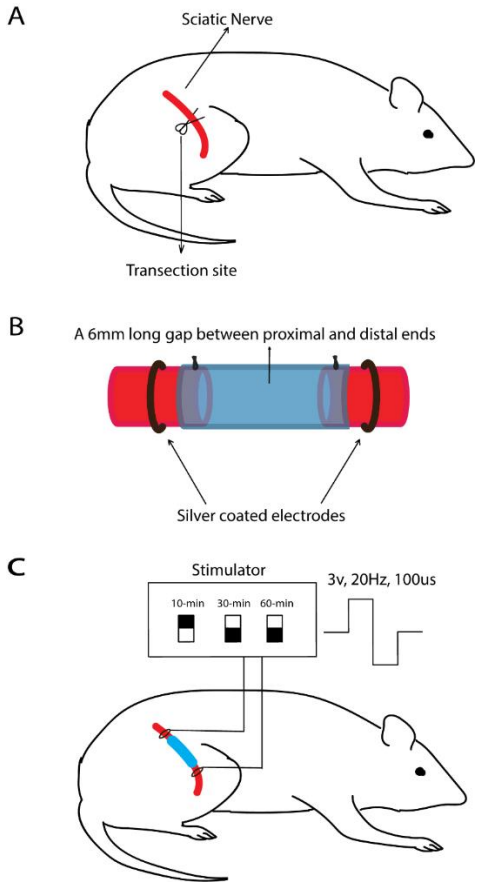

Figure 1. A schematic of the experimental procedure. (A) A paradigm for the right leg sciatic nerve injury in a Wistar rat. (B) The proximal and distal ends of the transected nerve are connected by a silicon tube and positive and negative silver-coated electrodes are placed on the proximal and distal ends, respectively. (C) The biphasic ES is delivered to the repaired nerve for 10, 30, or 60 minutes (3V, 100µs, 20Hz).

ES Procedure

After suturing the silicon tube to the transected nerve stumps, each rat in the experimental groups received ES for a specified duration. The ES was a series of voltage-controlled square pulses characterized by a 100µs width, 20 Hz frequency, and 3V amplitude produced by a custom-built stimulator. The custom-built electrical stimulator is consisted of a rechargeable lithium-battery as the power source and a microcontroller based control and stimulation system for handling the duration and specifics of the pulses produced by the device. The pulses were delivered through two silver-coated copper wires wrapped around the repaired sciatic nerve stumps so that, the positive electrode was located on the proximal part and the negative electrode was located on the distal part (Figure 1).

Evaluation of Sensory Nerves Regeneration

We used a hot plate test to evaluate the regeneration rate of sensory nerves. Briefly, the sole of the right foot of each rat was placed on a 56°c hot plate (model M13, PARS AZMA Company, Tehran, Iran) and the time-lapse of withdrawal was recorded.

The trial was stopped when the reaction time exceeded 12 seconds to prevent injury [26]. This process was repeated 3 times with 5 minutes intervals. This test was done every two weeks starting at 4th-week post-surgery up to week 12.

Histomorphometric Evaluation

At the end of the experiment (week 12), rats were sacrificed, using controlled atmosphere killing method (CO_2 exposure) and the intra silicon tube segment of the regenerated nerve as well as the tibial branch of the sciatic nerve was sampled and fixed in 2.5% glutaraldehyde solution (Merck, Germany) for 2 hours. For post-fixation, the specimens were transferred to 1% tetroxide osmium for another 2 hours. Then, they were dehydrated, blocked in resin, prepared in 1-µm thick sections using a rotary microtome (Leica, Germany), stained by toluidine blue 1% (TAAB CO., UK), and examined by a light microscope (Olympus BH2, Japan) equipped by a digital camera (OlympusDP71, Japan). Morphological analysis was done using images taken at 40x and 200x. For quantification, all counting was made with NIH ImageJ software. In this software, sampling schemes are selected based on a system of intervals in a numbered population within the nerve cross-section profile is named systematic random sampling. For nerve fiber stereology, there are single sampling boxes as units on a given cross-section of a nerve. Once a starting box is identified, the following boxes are selected at a distance away from the former box, systematically. For counting the nerve fibers, half of the circumference is shown as solid lines and the other is shown as dotted lines. The fibers on the dotted lines are counted and those on the solid lines are ignored.

Statistical Analysis

Statistical analysis was performed using Minitab 17.0 software (Minitab Inc., USA) and the data for hot plate test are presented as mean ± standard error of mean (SEM) while histomorphometric data are represented as Dot Plots. To detect any significant statistical difference between groups, one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test, as post-hoc were conducted and statistical significance was accepted at a confidence level of 95% ($p<0.05$).

Results

Hot Plate Test

Figure 2 shows the results of the hot plate test. The withdrawal reflex latencies at the 8th and 10th weeks post-surgery were significantly decreased in experimental groups compared to the negative control group ($p<0.05$); which is a sign of accelerated recovery of sensory fibers. There was no significant difference in the withdrawal reflex latency between all groups at the 12th week which means if all the animals have enough time, they will recover equally [27].

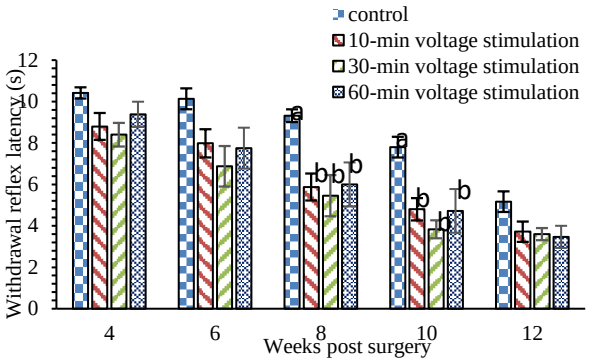

Figure 2. Comparison of the withdrawal reflex latency at 4 to 12 post-surgery weeks. Different letters represent the significant difference (n=6, $p<0.05$).

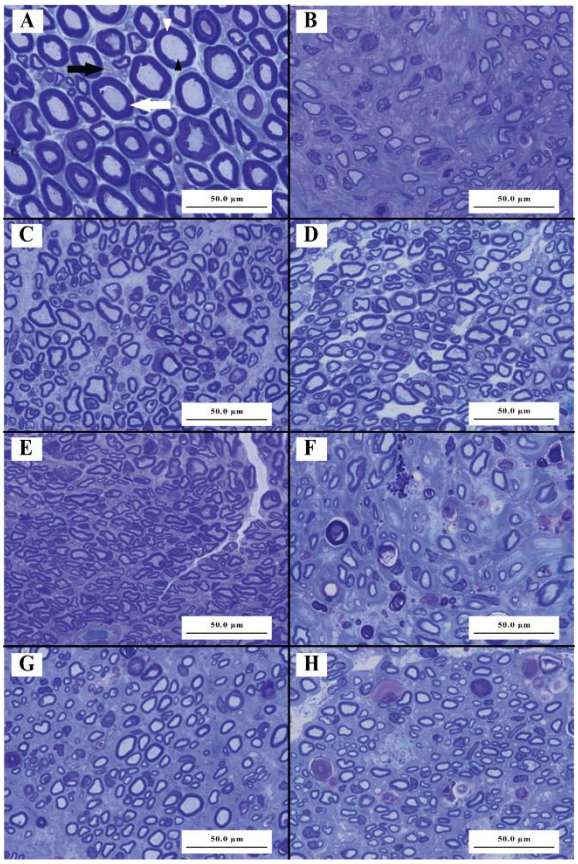

Figure 3. The semi-thin toluidine blue-stained sections of the intact nerve (A), the silicon tube segment of the regenerated nerve (B-E), and the tibial branch of the regenerated nerve (F-H). In (A), the white arrow shows a myelinated fiber; the black arrow indicates a non-myelinated fiber; the white arrowhead points to a myelin sheath; the black arrowhead shows an axon. B-E belongs to the control; 10 min ES; 30 min ES; 60 min ES of the silicon tube segment; respectively. F-H belongs to control, 10 min ES and 60 min ES of the tibial branch of the sciatic nerve; respectively. The bar size=50µm.

Morphometric Analysis of the Regenerated Sciatic Nerve in the Silicon Tube

In Figure 3 the semi-thin toluidine blue-stained sections of the intact nerve (A), the silicon tube segment (B-E), and the tibial branch of different groups (F-H) are presented. The density of myelinated fibers in the experimental

groups is increased compared to the control group, in both silicon tube segment and tibial branch of the regenerated sciatic nerve.

The statistical results of the morphometric analysis are illustrated in Figures 4 and 5. These data show that the mean number of regenerated fibers in the silicon tube is significantly higher in the 60 min ES group compared to the control, 10 min ES, and 30 min ES groups (P<0.05) (Figure 4A). The myelin sheath thickness, axonal diameter, and fiber diameter data are presented in Figures 4B, 4C, and 4D, respectively. Except for the 30 min ES group, in comparison with the negative control group, myelin thickness is significantly increased in 10 min and 60 min ES treated groups (P<0.05). There is no significant difference between experimental groups in myelin sheath thickness (Figure 4B). The axonal and fiber diameters show no significant difference between the control and ES groups (Figure 4C, D).

Morphometric Analysis of Tibial Branch of the Regenerated Sciatic Nerve

The results of statistical analysis of regenerated tibial branch segment of sciatic nerve show no significant difference in the mean number of axons and myelin sheath thickness between experimental groups and the control group (Figure 5A, B) while in comparison with the negative control group, the axonal and fiber diameters are significantly increased in 60-min ES group (p<0.05) (Figure 5C, D).

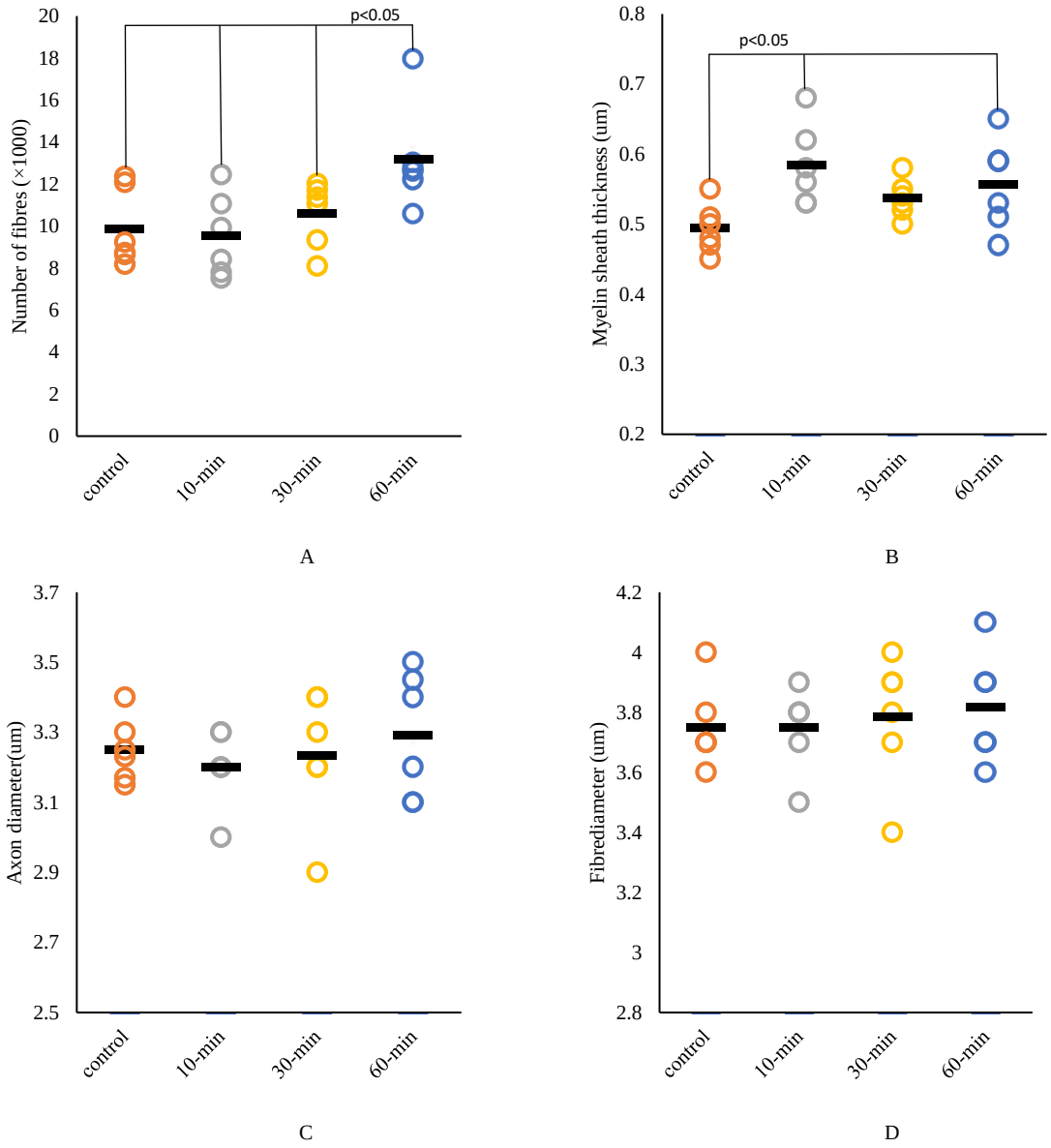

Figure 4. Comparison between morphometric parameters of the silicone tube segment at week 12 post-surgery (n=6). (A) The number of fibers; (B) myelin sheath thickness; (C) axonal diameter; (D) fiber diameter.

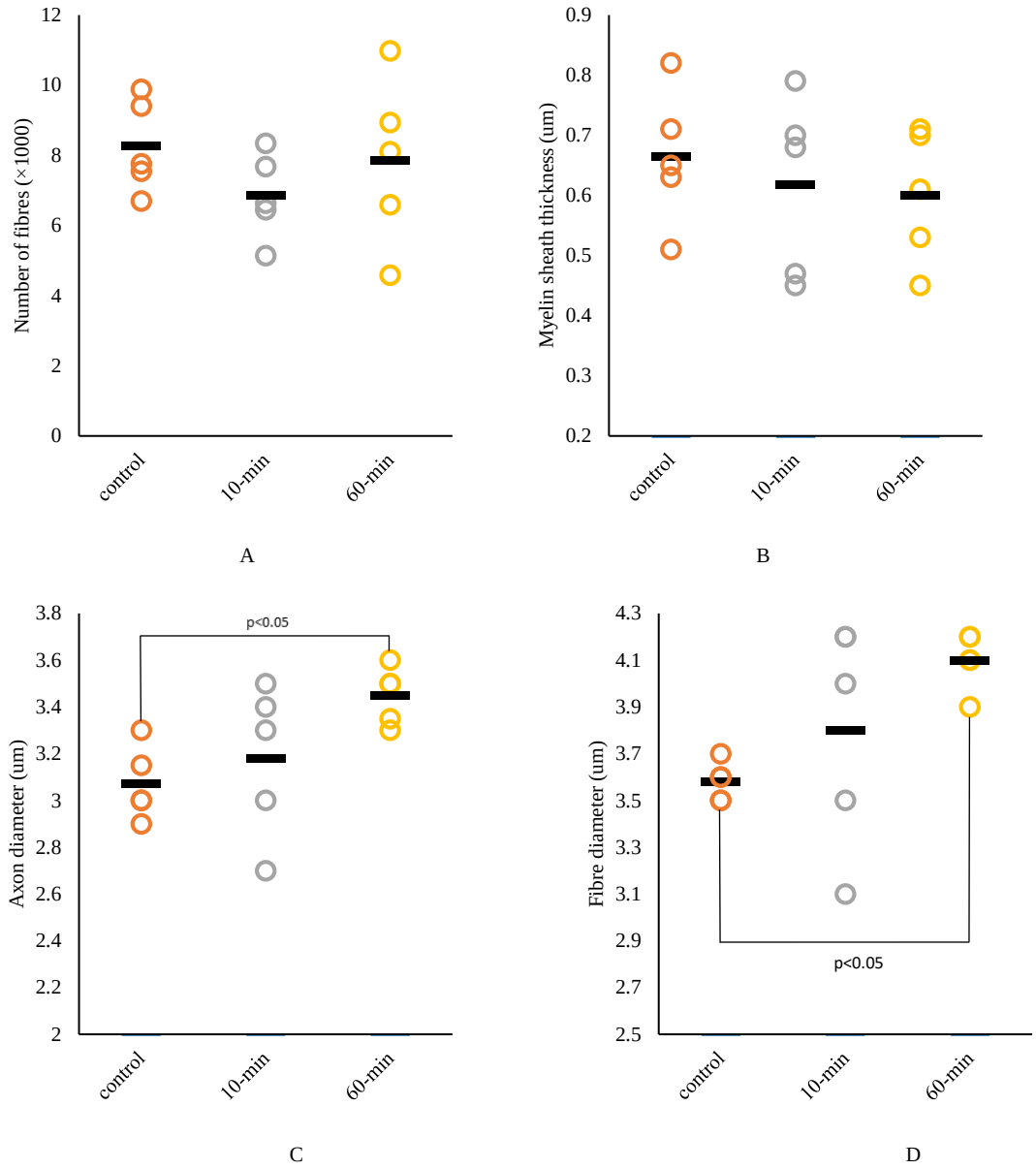

Figure 5. Comparison between morphometric parameters of the tibial branch of the regenerated sciatic nerve at week 12 post-surgery (n=5). (A) The number of fibers; (B) myelin sheath thickness; (C) axonal diameter; (D) fiber diameter.

Discussion

Depending on the severity of injuries, peripheral nerve injury is accompanied by some degree of degeneration in the perikaryon of neurons and their nerve fibers. In the case of nerve transection, the Wallerian degeneration occurs throughout the distal part of the injured nerve as well as up to the first Ranvier node of the proximal part [28]. If the neuronal cell body survives, it starts to regenerate the neural process via the growth cones [28]. Depending on the severity of the lesion and the distance between the two nerve endings, it takes about one month that the regenerating nerve fibers to pass the injured site and enter the distal stump [10]. It has been shown that some medications such as nerve growth factors administration [5] and the application of ES accelerates this process and improves functional recovery [11].

It has been suggested that the mechanism of the beneficial effects of ES application on regeneration is due to its influence on the metabolism of the cell body [10, 29] and increased production of matrix metalloproteinase (MMPs) enzymes in dorsal root ganglion neurons [30] and spinal motoneurons [31]. Moreover, through the up-regulation of P0, Par-3, and BDNF genes expression, ES application probably leads to earlier initiation of myelin synthesis by Schwann cells [15].

The main limiting factor in the application of ES is the duration of ES in which the patient/animal must be unconscious and the site of injury open. This may increase the risk of wound infection. Previous research showed that applying more than one-hour ES not only

affects the survival of motoneurons but also reduces trkB receptor gene expression and leads to poor recovery of sensory axons [10, 22]. However, no comprehensive research has been done on the effect of times less than an hour. To overcome these challenges, this study aimed to examine the effect of brief ES application on nerve repair so that, the one-hour ES (as a gold standard) is reduced to 10-min.

Although in earlier studies both ES electrodes were placed on the proximal stump of the injured nerve [9-17] in the present study the positive and negative electrodes were placed on proximal and distal stumps of the injured nerve, respectively [32, 33]. The mean number of regenerated axons in the silicon tube in the 60-min ES group is elevated 34% in respect to the control group, which is a similar study that placed ES electrodes on the proximal stump in a crush injury, the number of the regenerated axons in ES group is elevated approximately 7% to 18% concerning control group [15].

As seen in Figure 4, inconsistent with the gold standard (one-hour ES application) the fiber number count in the silicon tube is significantly increased in the 60-min ES group. A higher number of nerve fibers may indicate the number of survived neurons. The increased thickness of myelin sheath in the silicon tube segment is highly likely to suggest to faster regeneration of fibers thereby, as early as the fibers enter and then pass through the silicon tube the myelin synthesis is being initiated [34]. This may lead to higher values of myelin sheath thickness (Figure 4B). Furthermore, the increased values of axonal and fiber diameters in tibial branch of the regenerated sciatic nerve in 60-min ES group (Figure 4C and D) can be attributed to the "compensatory mechanism". This mechanism occurs after transection injury when some motor nerve fibers fail to innervate the appropriate muscle fibers. In this case, the regenerated motor fibers innervate nerve-less muscle fibers by increasing the number of end-branches. As the thickness of the nerve fiber increases with the number of end branches, the diameter of the restored motor fibers increases [35]. Furthermore, the mean number of nerve fibers and myelin sheath thickness in the tibial branch are in a same level for 60-min ES and control group (Figure 5A, B).

In general, it looks like that the ES directly or by retrograde transportation of some biochemical signals not only prevent the neuronal death but also increase the rate of regeneration. Finally, it seems that compared with 10- and 30-min ES, the 60-min ES has a better effect on the speed of regeneration.

Conclusion

The aim of this study was to investigate the effect of brief ES application on the nerve regeneration and compare it with the "gold standard scheme" (60-min ES duration). The results obtained from this study confirmed that 60-min ES has a better outcome than shorter ESs. On the other hand, the results of the present study showed that the placement of positive and negative electrodes on proximal and distal stumps respectively has a greater outcome than the placement of both electrodes on the proximal stump of the injured nerve. In future studies, the effect of other electrical specifics such as frequency, pulse width, and waveform (monophasic or biphasic) can be investigated.

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