

Effect of electrical stimulation on motor nerve regeneration in sciatic nerve ligated-mice

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Abstract

The purpose of this study was to investigate the effect of electrical stimulation on sciatic nerve regeneration and functional recovery of target muscles. Mice were randomly divided into 3 groups: ligated without electrical stimulation, ligated with electrical stimulation and control (non-ligated). The unilateral peripheral mononeuropathy was produced on the right hind limb. Sciatic nerve was then electrically stimulated daily for a period of 2 weeks (duration: 0.2 msec, frequency: 100Hz, amplitude: 15mA). Evoked surface EMG was recorded from biceps femoris (BF) and gluteus maximus (GM) muscles on the 3rd, 7th, 10th and 14th day after sciatic nerve ligation. Muscle force and sensitivity was determined by processing of the recorded EMG signals in time and frequency domains respectively. The results showed electrical stimulation (ES) produced a significant increase in the EMG response of BF, and muscle force significantly increased on the 14th day ($p < 0.001$), however no significant difference was found in GM muscle force between experimental groups. This may be due to possible innervation by inferior gluteal nerve. Frequency analysis of BF signals indicates that hyperalgesia remained after 14 days in both ligated groups. On the 14th day no difference in GM muscle sensitivity was found between groups. In conclusion, the results of this study have shown that the electrical stimulation of sciatic nerve accelerates nerve repair and indirectly improves BF muscle force to a comparable level with control without effect on muscle sensitivity. However, ES had no effect on GM muscle force and sensitivity.

Key Words: EMG, electrical stimulation, muscle force, muscle sensitivity, ligated mice

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Partial or complete loss of sensory and motor function may be induced by injury to peripheral nervous system. Peripheral nerves are able to regenerate following lesion or disease. However, regeneration is not complete and the function rarely returns to pre-injury levels.^{1,2,3} Depending on the age and the severity of the lesion, the nerve repair is limited and different.^{4,5} Electrical stimulation (ES) is a popular treatment, useful to recover from muscle atrophy and also used to evoke functional contractions. Different types of ES such as weak direct current,⁶ high-voltage electrical currents,⁷ alternating currents,⁸ transcutaneous nerve stimulation,⁹ neuromuscular electrical stimulation,¹⁰ and electromagnetic fields¹¹ are also used to promote nerve regeneration and to improve neuromuscular functions, but their precise effects on nerve regrowth are debated. Recent studies in the field of peripheral nerve repair are focused on histological methods.^{2,7,9} Furthermore, a number of investigations regarding neuromuscular

recovery following nerve injury, have focused on electrical stimulation of muscles,^{12,13,29} or neuromuscular junction.¹⁰ Effect of nerve electrical stimulation on related muscle electrophysiology has however received less attention. Surface electromyography (SEMG) is a non-invasive method which is an indirect indicator of the central drive. On the other hand, this method is directly associated with activity of the motor units which are affected by physiological parameters such as motor unit discharge rates, and muscle fiber membrane characteristics.^{15,16} So, the purpose of this study was to investigate the effect of electrical stimulation on sciatic nerve regeneration and functional recovery of target muscles.

Materials and Methods

Animals

Male NMRI mice (20-25g) were housed in groups of 10 in conditions of constant temperature ($20 \pm 2^\circ\text{C}$) with

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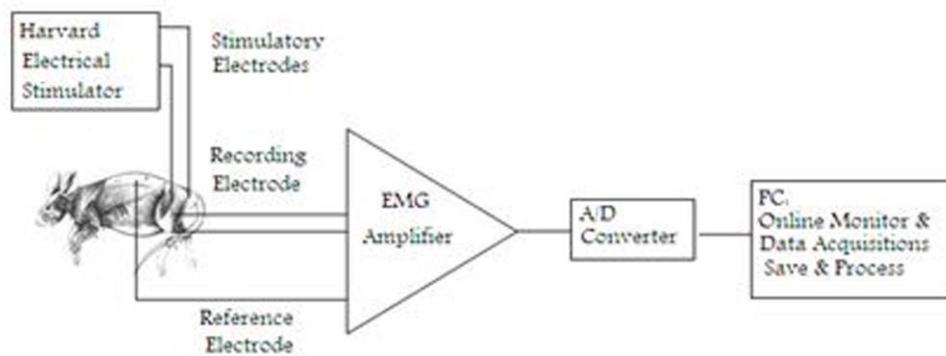


Fig 1. Diagram of the experimental set-up.

12L:12D photoperiod, and were allowed free access to food and water except during the experimental sessions. Each animal was used once only, and was euthanized immediately after the experiments. All experiments were carefully conducted according to the ethical guidelines for the use of experimental pain in conscious animals put forth by the International Association for the Study of Pain.

Surgical procedure for nerve ligation.

The animals were anaesthetized with intraperitoneal injection of sodium thiopental (40mg/kg). The unilateral

peripheral mononeuropathy to study regeneration of peripheral nerve was produced on the right hind limb, based on the method of Bennet & Xie (1988)¹⁷ and Attal et al. (1990).¹⁸ Then the animal's right sciatic nerve was exposed and freed from the surrounding tissues attachment, and a 2-3 mm long nerve segment was dissected. Only one ligature with fine metal wire was secured around the nerve and the nerve was returned to its bed.

Electrical stimulation of nerve

This process started from the day of surgery. Under

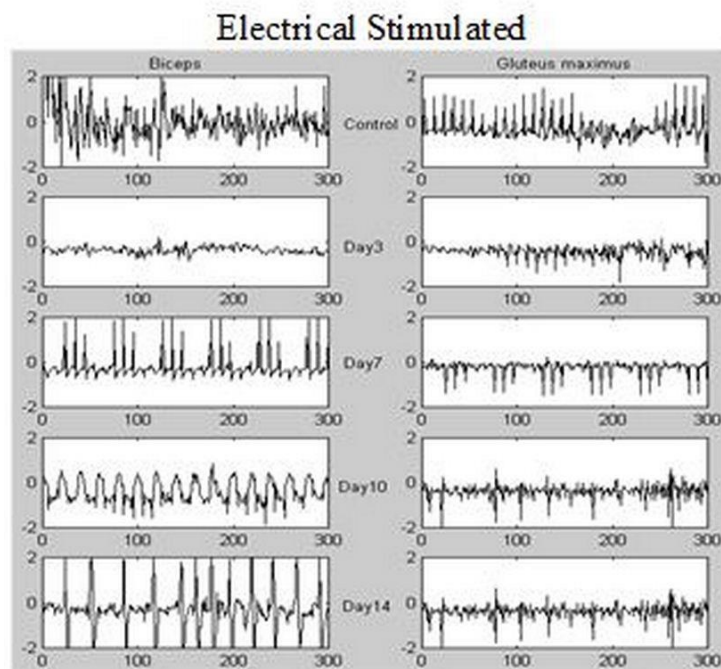


Fig 2. Raw electromyograms (EMGs) of biceps femoris (left side) and gluteus maximus (right side) muscles after electrical stimulation of sciatic nerve on the 3rd, 7th, 10th and 14th day in treated group after nerve ligation in mice. Control EMG signals of biceps femoris and gluteus maximus muscles are shown in upper row.

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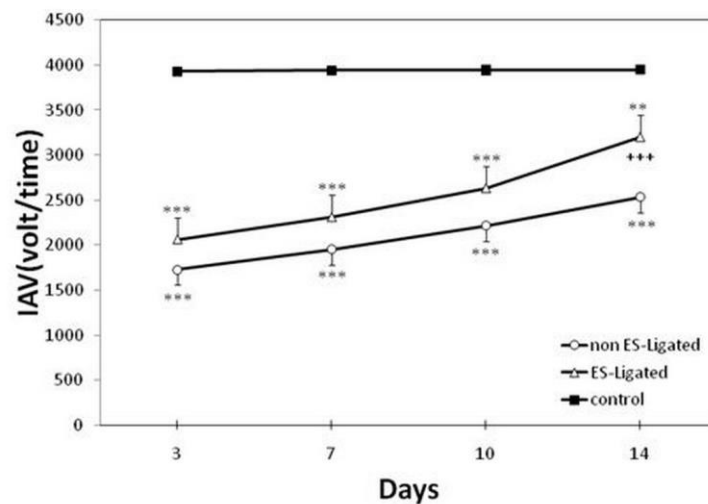


Fig 3. Effect of electrical stimulation on Biceps femoris muscle force. The EMG signal was recorded on the 3rd, 7th, 10th, 14th day after ligation in non ES-Ligated group (o), ES-Ligated group (Δ) and control group (■). Each point is mean $\pm$ SEM of Integral Absolute Value (IAV) for 10 mice. (** $p < 0.01$, *** $p < 0.001$ different from respective control group, ++ $p < 0.001$ different from respective non ES-Ligated group).

light anesthesia with ether, the sciatic nerve was re-exposed and gently suspended on a pair of stainless-steel stimulatory electrodes. The proximal nerve stump was then electrically stimulated for 40 seconds per day (20 pulses/ contraction). The stimulatory pulse (duration: 0.2 millisecond, frequency: 100 Hz, amplitude 15 milliAmpere) was delivered by a stimulator (Harvard, U.K) being connected to a voltage-to-current convertor circuit using IC (LM 134, U.S.A). The amount of current delivered to each animal was monitored by an oscilloscope (Tektronix, TDS 1002, TEXAS) to ensure that 15mA current was being delivered.

Electromyography.

Evoked surface EMG was recorded from Biceps Femoris (BF) and Gluteus Maximus (GM) muscles on the 3rd, 7th, 10th and 14th day after sciatic nerve ligation on the right hind limb. To record, first the electrode sites were shaved and cleaned with alcohol, and the lubricant gel was applied for better conductivity. A pair of recording disposable adhesive Ag-AgCl electrodes (Biopac Company) placed on each end of the muscle (totally 4 electrodes). The fifth electrode (reference) was placed on the vertebral column; the electrodes were taped securely to avoid excessive lead movements. Snap leads were used to connect the electrodes to the amplifier (2 channels). All raw myoelectric signals were amplified (band width: 8-1600Hz). Evoked EMG was recorded for 5 sec (5000 samples) during the final 10 sec of the sciatic nerve stimulation. EMG signals were digitized at 1KHz with an A/D card (ADVENTECH, 818 PCL -818 HG). Data were collected and saved on a personal computer for statistical analysis. The schematic

drawing of the experimental set-up is shown in Figure 1.

Treatment protocols

To study the effect of electrical stimulation (ES), mice were randomly divided into 3 groups of 10. Group 1: Ligated without electrical stimulation (non ES-Ligated). In this group sciatic nerve was ligated and allowed to recover naturally. On the 3rd, 7th, 10th and 14th day, surface EMG of BF and GS muscles were recorded in this group. Group 2: Ligated with electrical stimulation (ES-Ligated) or treated group. In this group sciatic nerve was ligated and it was under electrical stimulation daily for a period of 2 weeks. The first ES was just after the surgery. Surface EMG signals were recorded on the 3rd, 7th, 10th and 14th day after nerve ligation. Group 3: In this control group the sciatic nerve was intact and EMG signals of BF and GS muscles were recorded.

Signal processing

The raw EMG data were stored and processed off line. The digitized data were viewed before processing to reject those with insufficient signal level or with artefacts. All signal processing was performed using custom programs written with LabView (version 6, National Instruments, Austin, TX). To study muscle power or force, EMG signals were processed in the time domain (Integral Absolute Value: IAV). In this method first signal was rectified and then root mean square (RMS) was obtained and finally linear envelope was determined in order to derive muscle power from the EMG signal. The frequency domain of the surface EMG signals was used to study muscle sensitivity and the number of frequency components was considered as muscle sensitivity index. Increased frequency components of the EMG signal was interpreted as

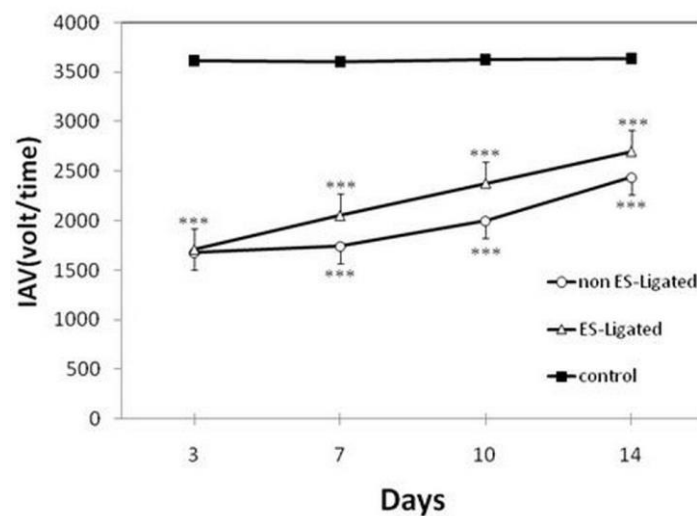


Fig 4. Effect of electrical stimulation on Gluteus maximus muscle force. The EMG signal was recorded on the 3rd, 7th, 10th, 14th day after ligation in non ES- Ligated group (o), ES-Ligated group (Δ) and control group (■). Each point is mean $\pm$ SEM of Integral Absolute Value (IAV) for 10 mice. (***) $p < 0.001$ different from respective control group).

muscle hyperalgesia or muscle hypersensitivity. First Fast Fourier Transform (FFT) of signal was obtained, rectified, filtered, analyzed and finally the number of frequency component were counted using a peak detector. A sample of raw EMG is shown in Figure 2.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey test was used to evaluate the significance of the results. All data were expressed in terms of mean $\pm$ standard error of mean (SEM), and $p < 0.05$ was considered to be significant.

Results

Effect of sciatic nerve stimulation on the muscle force of Biceps Femoris

The effect of electrical nerve stimulation on biceps femoris EMG signals is shown in Figure 3, in which ES-Ligated (ligated with electrical stimulation or treated) and non ES-Ligated (ligated without electrical stimulation) groups compared with each other and with control group. Analysis of variance revealed significant difference between ES-Ligated and non ES-Ligated groups ($p < 0.001$). There is also a significant difference between the two experimental groups versus control group ($p < 0.001$). Further analysis showed that there is no significant difference between ES-Ligated and non ES-Ligated groups on the 3rd, 7th, 10th day after ligation. However, the main difference occurred on the 14th day after treatment with ES. A significant increase in the EMG response and muscle force was also obtained ($p < 0.001$) and reaches to 80.9% of that of control group. Further analysis showed that in ES-Ligated group, there is a significant gradual increase in

muscle force from the 7th day onward. Significant differences between the 7th and 10th day ($p < 0.05$), 10th and 14th day ($p < 0.001$), 3rd and 10th day ($p < 0.001$) were observed.

Effect of sciatic nerve stimulation on the muscle force of Gluteus Maximus

The results are shown in Figure 4. There is a significant difference between experimental groups (ES-Ligated and non ES-Ligated) as compared with control group ($p < 0.001$), but no significant difference was found in muscle force between the two experimental groups. Further analysis showed that in ES-Ligated group there is a significant gradual increase in muscle force, i.e., that there are significant differences between the 3rd and 7th day, 7th and 10th day, 10th and 14th day ($p < 0.05$). In non ES-Ligated group there is significant differences between the 10th and 14th day ($p < 0.01$), 3rd and 10th day ($p < 0.01$), and 7th and 14th day ($p < 0.01$), but no significant differences were found between the 3rd and 7th day or 7th and 10th day ($p > 0.05$). No significant differences were found between the other groups

Effect of sciatic nerve stimulation on the sensitivity of Biceps Femoris

The effect of ES on the number of frequency components of EMG signals of BF muscle is shown in Figure 5. The results of ANOVA showed that there is a significant difference between the two experimental groups versus the control group ($p < 0.001$), indicating that the number of frequency components is significantly higher in experimental groups versus control group. There is also significant difference between ES-Ligated and non ES-Ligated groups

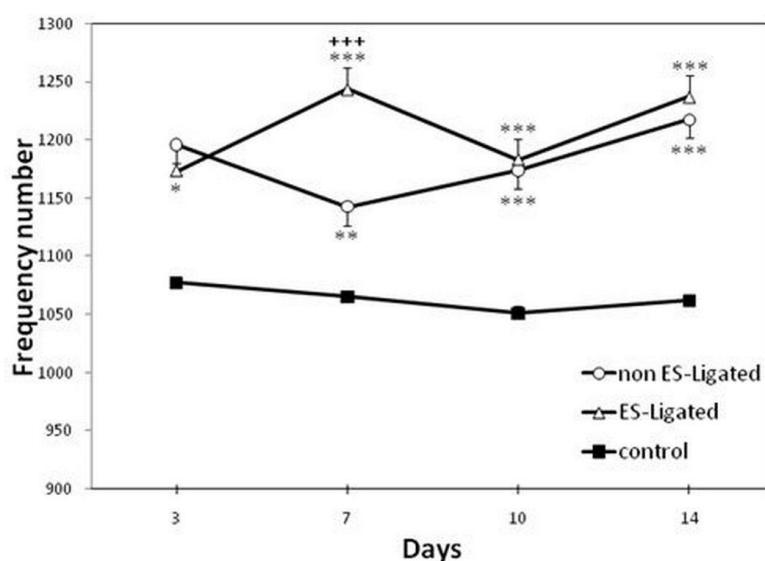


Fig 5. Effect of electrical stimulation on Biceps femoris muscle sensitivity. The EMG signal was recorded on the 3rd, 7th, 10th, 14th day after ligation in non ES-Ligated group (○), ES- Ligated group (△) and control group (■). Each point is mean $\pm$ SEM of the frequency number for 10 mice. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ different from respective control group, +++ $p < 0.001$ different from respective non ES-Ligated group).

($p < 0.001$). Although no difference between these two groups were found on the 14th day ($p > 0.05$), on the 7th day there was a significant difference between experimental groups ($p < 0.001$), thus the number of frequency components is higher in ES-Ligated group. Further analysis between experimental groups showed in non ES-Ligated group there is only significant difference between the 3rd and 14th day ($p < 0.05$), however in ES-Ligated group there is significant difference between the 3rd and 7th day ($p < 0.05$).

Effect of sciatic nerve stimulation on the sensitivity of Gluteus Maximus

Figure 6 shows the comparison of the effect of ES on the number of frequency components of GM muscle among groups. Analysis of variance did not show significant difference in the number of frequency components between non ES-Ligated with control group ($p > 0.05$), or between ES-Ligated group with control group ($p > 0.05$). No difference was found between the two experimental groups ($p > 0.05$).

Discussion

Surface electromyography (SEMG) is a non-invasive method for evaluation of muscle physiology. The time and frequency domains of the signal have been used to examine central and peripheral aspects of neuromuscular physiologic function, as well as the motor unit activation modulate muscular force production.^{15,19,20} The EMG signal amplitude reflects the number of activated motor units and their firing rates, and its frequency contents may be associated with the number of muscle fiber action potentials.^{21,22} Hence

evaluation of the repeatability of EMG variables is of considerable relevance for the clinical use of this technique.²³ In the present study, we have found a significant reduction in muscle power after sciatic nerve ligation. This finding confirms previous data that when muscles lose neural drive, they lose ability to generate force due to atrophy of their muscle fibers.^{2,10,24,25} Our results revealed that sciatic nerve electrical stimulation produced a significant increase in the EMG response of biceps femoris, indicating that its muscle strength increased over time and reached 80.9% of that of normal muscle at the 14th day after sciatic nerve ligation, thus motor function was recovered better in ES-treated mice. However, non-ES ligated (non-treated) group did not show significant increase in BF muscle power on the 14th day. These results confirm Xu et al. (2014) study that recorded gastrocnemius muscle needle EMG, and reported nerve ES one day after surgery increases muscle action potential parameters such as motor nerve conduction velocity and peak amplitude and decrease latency onset of muscle action potentials.²⁷ It seems that ES induces structural changes in the muscle that, in turn, improves muscle function. Our hypothesis confirms other studies indicating that following nerve ES the number of myelinated fibers in distal nerve stump,²⁷ the number of regenerated axons, the number of motoneurons and the thickness of myelin sheath were significantly increased as compared to control group.²⁸ In accordance with our results, there is evidence indicating that neuromuscular electrical stimulation improved muscle strength.¹⁰ There is more evidence indicating that ES restores the paralyzed

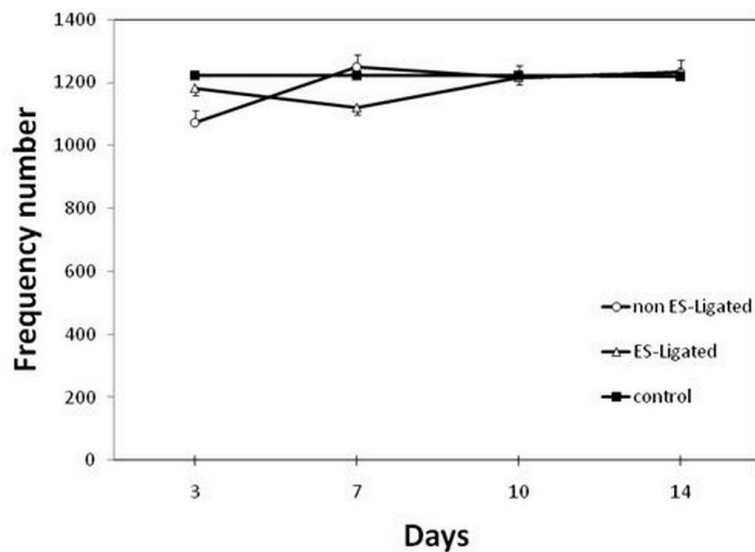


Fig 6. Effect of electrical stimulation on Gluteus Maximus muscle sensitivity. The EMG signal was recorded on the 3rd, 7th, 10th, 14th day after ligation in non ES-Ligated group (○), ES-Ligated group (△) and control group (■). Each point is mean $\pm$ SEM of the frequency number for 10 mice.

muscle mass,²⁹ and evoked visible rhythmic muscle contractions in these muscles.²⁴ Even in old muscles, the electrical stimulation can generate tetanic contractions maintaining their mass and maximum force.²⁵ Willand et al. (2015) reported that ES of muscle following peripheral nerve injury accelerates muscle reinnervation and its functional behaviors.¹³ The mechanisms by which the electrical stimulation exerts its effect are not clear. However, on the basis of available evidence we conclude that ES may improve muscle function through the following mechanisms. Muscle power is a mechanical quantity which in our experiments was evaluated by evoked EMG. When a nerve is transected and sutured, axonal regeneration from the proximal stump into inappropriate distal pathways after nerve lesion has been long recognized as a factor contributing to poor functional recovery.³⁰ Therefore innervation is a critical factor for the support of the structural and functional integrity of skeletal muscles.²⁶ A possibility is that in our experiments sciatic nerve ES increases the number of correct projections to the distal stump and to related muscles. This is consistent with the results of Al-Majed et al. (2000) and Brushart et al. (2002) who found that, if the proximal stump of a cut nerve is stimulated at the time of its surgical repair, the speed of reinnervation of target muscles and sensory-motor precision were improved.^{4,31} Electrical activity may influence the axonal path finding of developing neurons,³² and enhance the speed and accuracy of nerve regeneration.²⁷ Therefore ES may be effective in promoting nerve regeneration after peripheral nerve injury. From our

results, one may suggest that ES could function as a mechanism to bridge the gap between the nerve stumps electrically and improve muscle force. In other words, the possibility may exist that the ES result in changes in the excitation-contraction coupling mechanism probably be mediated through muscle membrane ion channels which can accelerate depolarization and results synchronized activation of motor units and/or increase of force generating capacity on the 14th day, which is in accordance with finding of Ashley et al. (2007).³³ Along with these results, it has been suggested that bridging is the best type of peripheral nerve repair.³⁴ But what is the molecular mechanism behind this bridging? Another explanation for the effect of ES in our study could be that gene expression of nerve growth factors is increased. Nerve growth factor (NGF) is normally produced in muscle on local injury and inflammation and is known to play a role in regeneration after muscle injury.³⁵ Denervation leads to increased NGF production by skeletal muscle,³⁶ and ES induces up-regulation of muscular neurotrophic factors.^{13,37} It has been shown that expression of proteins such as neurotrophins whose release at the level of the muscle might play a key role in determining the accuracy of reinnervation are increased.^{1,38} On the other hand, NGF is involved in regulation of muscle strength.^{39,40} Barmptsioti et al. (2011) reported that NGF administration ensured a significant increase of average number of myelinated axons per μ m and lead to better EMG results.⁴¹ Dose and duration of NGF administration are factors that determine the extent of recovery following peripheral nerve injury.⁴² Zhao et al. (2015) confirmed efficacy

and safety of nerve growth factor for the treatment of neurological diseases.⁴³ Indeed, there is evidence indicating that electrical muscle stimulation increases expression of genes of motor endplate, calcium binding proteins and acetylcholine receptors in muscle following spinal cord lesion.²⁹ The present study revealed that ES did not affect GM muscle force. This muscle seems to function differently from BF muscle. This is probably because of its innervation, in fact BF and GM muscles are innervated by different nerves. BF is a double muscle receiving 2 nerves supplies. The nerve to the short head of the BF comes from the common peroneal part of the sciatic nerve, while long head of the muscle is innervated by tibial branch of the sciatic nerve. Whereas, GM is innervated by inferior gluteal nerve.⁴⁴ According to our data, the number of frequency components of BF muscle that represents muscle sensitivity significantly increased in experimental groups, indicating both of them were hyperalgesic as compared with control group, however there is no difference between experimental groups on the 14th day. This increased EMG activity, muscle hyperalgesia, may be secondary (increased responsiveness to nociceptive stimuli outside the site of injury). Whether the hyperalgesia responses of biceps femoris muscle in the present study is mediated through a cutaneous or visceral mechanism should be clarified. In the present study as can be shown in both ES-Ligated and non ES-Ligated groups, ligation can induce hyperalgesia. According to our data, ES-treated group were becoming hyperalgesic from the 3rd day, and there was a significant difference between treated and non-treated groups on the 7th day (Figure 5). There are data from Hirayama et al. (2001) indicating that electrical stimulus applied to the sciatic nerve in rats was high enough to activate C-fibers and nerve reflexively excited muscles.⁴⁵ However, we do not know whether pain existed on the 7th day of the experiment. Pharmacologic studies are needed to show if this effect will be reverse by analgesic agents used clinically to treat muscle pain. On the other hand, there is some evidence indicating that intramuscular injection of NGF induces hyperalgesia.⁴⁶⁻⁴⁸ Furthermore, NGF increases voltage-gated Na⁺ channels activity in excitable cells.^{49,50} It has been reported that the activity of these channels is linked to neuronal excitability in chronic pain states.⁴⁹ As we mentioned before; ES increases NGF production. So, we hypothesize that NGF production is at its maximum level of production at day 7 after ES which result in significant difference between ES-ligated and non ES-ligated mice. This is consistent with the results of Brushart et al. (2002) who found that the use of ES significantly increased the number of axons crossing at the 4 and 7th day, with only a few crossing after 2 weeks.³¹ It is possible that after this day a form of muscle adaptive behavior occurs, indicating repetitive input results in a negative feedback, and the muscle sensitivity return to non-treated level. It has

been shown NGF generates secondary hyperalgesia via a central mechanism.⁴⁶ Our results have shown that there was no significant difference in muscle sensitivity of GM between experimental groups with control or between experimental groups. Thus ligation could not induce muscle hyperalgesia in experimental groups. This can be due to innervation of this muscle as mentioned earlier or may be because of less production of mediators involved in hyperalgesia. It seems that both mechanisms may account for this response. Some evidence showed that frequency of ES is an important factor that may determine electrical stimulation effectiveness.^{51,52} In fact, a limitation of our study is that we did not examined the effect of different frequencies. Further research is required to confirm the effectiveness of the nerve electrical stimulation (without stimulation of muscle nociceptors and producing pain) for nerve repair, and precise molecular mechanisms involved.

On the other hand, our results support recent clinical application of Functional Electrical Stimulation (FES) in Spinal Cord Injury (SCI) and aging patients.⁵³⁻⁶⁰

In conclusion, the results of this study have shown that electrical stimulation of sciatic nerve accelerates through local mechanisms sciatic nerve repair and indirectly improves BF muscle force to a comparable level of the control without affecting muscle sensitivity. ES of sciatic nerve, indeed, had no effect on force and sensitivity of the GM muscle that is innervated by the inferior gluteal nerve.

List of acronyms

BF – Biceps Femoris
EMG - electromyography
ES – electrical stimulation
FES - Functional Electrical Stimulation
FFT - First Fast Fourier Transform
GM – Gluteus Maximus
IAV - Integral Absolute Value
RMS - root mean square
SCI - Spinal Cord Injury
SEM - standard error of mean
SEMG - Surface electromyography

Author's contributions

Dr. F. Samiee conceived the work, performed the bibliographical search and drafted the manuscript. Dr. M.-R. Zarrindast approved the research and the final manuscript.

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Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of Interest

None of the authors have conflicts of interests.

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