

Effects of Pulsed Electromagnetic Fields on Peripheral Nerve Regeneration Using Allografts in Sciatic Nerve: An Animal Model Study

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Abstract

Current gold standard for the clinical treatment of severe peripheral nerve damage involves using an autologous nerve to bridge the defect in injured nerve. Effects of pulsed electromagnetic fields (PEMF) on nerve regeneration were studied using allograft in a rat sciatic nerve model. Thirty male white Wistar rats were randomized into three experimental groups (n = 10): Normal group, allograft group, and PEMF treated group. In normal group left sciatic nerve was exposed and after homeostasis muscle was sutured. In the allograft group the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibio-peroneal bifurcation where a 10 mm segment was excised. The same procedure was performed in the PEMF group. The harvested nerves of the rats of allograft group were served as allograft for PEMF group and vice versa. The PEMF group the whole body was exposed to PEMF (0.3 mT, 2Hz) for 4h/day within 1-5 days. Behavioral testing, sciatic nerve functional study, gastrocnemius muscle mass showed earlier regeneration of axons in PEMF than in allograft group (p < 0.05). Whole body exposure to PEMF improved functional recovery and morphometric indices of sciatic nerve. PEMF could be considered as an effective, safe and tolerable treatment for peripheral nerve repair and may have clinical implications for the surgical management of patients after nerve allografting.

Keywords: Peripheral nerve repair; Sciatic; PEMF; Allograft

Introduction

Current gold standard for the clinical treatment of severe peripheral nerve damage involves using an autologous nerve to bridge the defect in injured nerve [1]. This method has been shown to be effective, but has the several disadvantages, including an extra incision for removal of a healthy sensory nerve ultimately resulting in a sensory deficit at the donor site [2,3]. Surgical therapy in patients with peripheral nerve injuries has not presented changes over the last decades, especially due to the use of autologous grafts, to the development of intraoperative magnification, and to the proven deleterious effects of tension at neural repair site [4]. Despite all the advancements achieved, functional repair results are still imperfect. In addition, the collection of donor nerves produces a new neurological sequel. In extensive defects or in several nerves' defects on a same patient, there may not be enough autologous donor nerve to fill that neural failure. With the increasing understanding capacity and with the manipulation of the immune system, allografts have been proposed as an alternative method in peripheral nerve reconstructions [4].

Pulsed electromagnetic fields (PEMF) are reported to promote peripheral nerve regeneration to an extent similar to that observed with conditioning lesions, growth factors, and hormones [5]. Exposure to PEMF as a pretreatment prior to crush injury has resulted in acceleration of axonal regrowth, and consistent with the stimulation of regenerative neurite outgrowth increased functional outcomes such as walking behavior [6-9]. PEMF has also been shown to promote neurite outgrowth *in vitro* [7]. Others have demonstrated that prolonged PEMF regimen had led to delayed histological peripheral nerve regeneration and increased oxidative stress but no loss of function recovery [10]. These contradictory results were probably due to technical differences, specifically to different protocols for PEMF exposure. Therefore, the present investigators concluded that the issue was not clear and that more experiments were needed to assess the possible benefits of PEMF exposure on peripheral nerve regeneration. Furthermore, promising results regarding the beneficial effect of PEMF on transected

peripheral nerve regeneration are poor and not supported by functional tests, to the best of knowledge of the authors, which play a crucial role in the assessment of functional nerve recovery. Aimed to study systemic effects of whole body exposure to PEMF on nerve allografts, a study was designed to attempt to determine if PEMF treatment does in fact reduce dysfunction after nerve injury in the rat sciatic nerve allografting model.

Materials and Methods

Study Design and Procedures

Thirty male white Wistar rats weighing approximately 280 g were divided into three experimental groups (n = 10), randomly: Normal, allograft group and PEMF treated group. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages with an ambient temperature of 23±3 °C, stable air humidity, and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90mg/kg and xylazine 2%, 5mg/kg). All procedures followed a standard microsurgery technique under magnifying lenses (BIO-ART EQUIPMENTS ODONTOLOGICOS LTDA, Sao Carlos/SP- Brasil). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain [11]. The University Research Council approved all experiments. Following surgical preparation in the normal control group the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 nylon. In the allograft group the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibio-peroneal bifurcation where a 10 mm segment was excised. The same procedure was performed in the PEMF group. The harvested nerves of the rats of allograft group were served as allograft for PEMF group and vice versa. The proximal and distal stumps of transected sciatic nerve was sutured to the ends of the harvested allograft using 10/0 nylon (Figure 1). The animals were anesthetized (see above) and euthanized with transcardial perfusion of a fixative containing 2% par formaldehyde and 1% glutaraldehyde buffer (pH = 7.4) 12 weeks after surgery and gastrocnemius muscle mass measurement was performed.

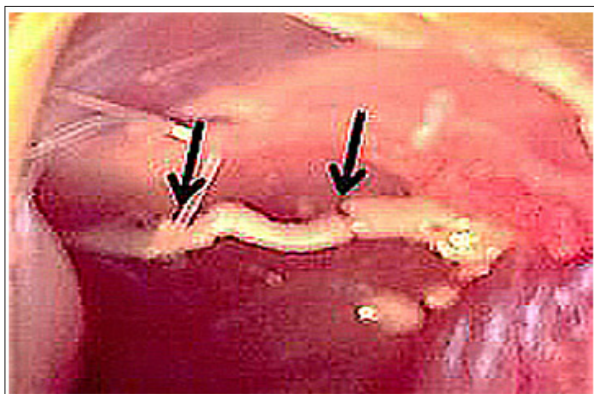


Figure 1: Intraoperative picture showing end-to-end anastomosis of harvested allograft (arrows) to distal and proximal stumps of transected sciatic nerve.

Exposure to Pulsed Electromagnetic Fields

Following recovery from anesthesia, rats were randomly assigned to control or experimental groups. Pulsed electromagnetic fields treatment was performed based on a method described by others [6-9]. In brief, on days 1-5, each animal was placed in an all-plastic restrainer located between Helmholtz coils and treated for 4 h each day with the PEMF signal generator either activated (PEMF group) or not activated (Normal group). In the present study whole body exposure was adopted because placement of the rats between the coils has assured that the site of the surgical lesion falls in the 90% homogeneity region of the magnetic field [12]. PEMF was applied using paired Helmholtz coils (PHYWE, 06514, Germany) 30 cm in diameter, placed 15 cm apart. The system was fed by a signal generator (Funktionsgenerator, PHYWE, Göttingen, Germany) producing a magnetic field amplitude of 0.3 mTesla with a pulse duration of 20 ms, repeated at a pulse repetition rate of 2 Hz. The output of the signal generator was amplified by a homemade audio amplifier (frequency width 20-10KHz, maximum power 600W) connected to a coil made of 50 turns of copper wire, with 4-cm Id and 2.5 cm length, producing 0.3 mTesla field. Intensity was measured by a Hall Effect Teslometer (HI-3550 Holaday Indus, Sofia, Bulgaria) in center of coils. The uniformity of magnetic field in the space was 0.05%. The rise time was 0.85 ms, the fall time 0.68 ms.

Behavioral Studies

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function [13]. Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries [14]. Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination. BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-minute exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 12 weeks.

Functional Assessment of Reinnervation

A. Sciatic Functional index (SFI): Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others [15]. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

$$SFI = -38.3 \times (EPL - NPL) / NPL + 109.5 \times (ETS - NTS) / NTS + 13.3 \times (EIT - NIT) / NIT - 8.8$$

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0.

SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

B. Measurement of Gastrocnemius Muscles Mass: Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance. Two independent observers unaware of the analyzed group made all measurements.

C. Histological Preparation and Quantitative Morphometric Studies: The harvested segments were fixed in 2.5 percent glutaraldehyde. The grafts were then embedded in paraplast paraffin, cut in 5 µm and were next stained with toluidine blue. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA).

D. Immunohistochemical Analysis: In this study, anti-S-100 (1:200, DAKO, USA) was used as marker for myelin sheath. Specimens were post fixed with 4% par formaldehyde for 2h and embedded in paraffin. Prior to immunohistochemistry nerve

sections were dewaxed and rehydrated in PBS (pH 7.4). Then the nerve sections were incubated with 0.6% hydrogen peroxide for 30 minutes. To block non-specific immunoreactions, the sections were incubated with normal swine serum (1:50, DAKO, USA). Sections were then incubated in S-100 protein antibody solution for 1h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1h. Horseradish peroxidase-labelled secondary antibody was applied for 1 h. After that all sections were incubated with 3,3'- diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope.

E. Statistical Analysis: Experimental results were expressed as means ± SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using repeated measures and a factorial ANOVA with two between-subjects factors and. Bonferroni test for pair wise comparisons was used to examine the effect of time and treatments. The differences were considered significant when $P < 0.05$.

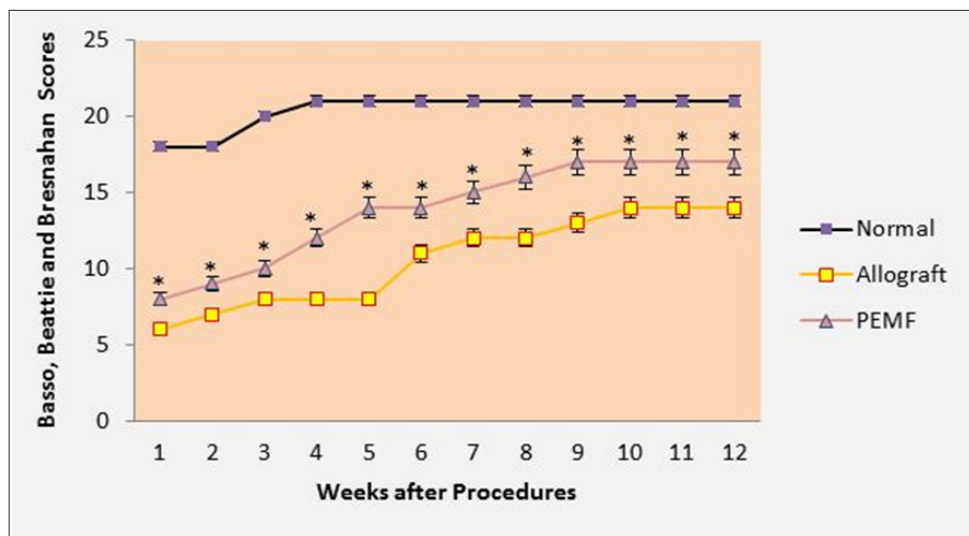


Figure 2: BBB score for all experimental groups. Exposure to PEMF with allografting gave better scores than in allograft group. Standard error at each data point is shown with bars. * $P < 0.05$ vs allograft group.

Results

BBB Recovery

In order to assess hind limb recovery, the open field locomotor was used. (Figure 2) shows BBB scores compared to the baseline. All experimental groups, except for sham, showed the greatest degree of functional deficit one week after surgery. The PEMF treated group showed significant improvement in locomotion of the operated limb compared to the control group during the study period ($P < 0.05$).

Recovery Of Sciatic Nerve Function And Reinnervation

a) SFI outcome: (Figure 3) shows sciatic function index (SFI) values in experimental groups. Prior to surgery, SFI values in

both groups were near zero. After the nerve transection, the mean SFI decreased to -100 due to the complete loss of sciatic nerve function in all animals. The statistical analyses revealed that the recovery of nerve function was significantly ($P < 0.05$) different between allograft and PEMF groups and exposure to PEMF improved functional recovery in the course of time.

b) Muscle Mass Findings: The mean ratios of gastrocnemius muscles weight were measured. There was statistically significant difference between the muscle weight ratios of allograft and PEMF groups ($P < 0.05$). The results showed that in PEMF group muscle weight ratio was bigger than allograft group and the gastrocnemius muscle weight loss was improved by exposure to PEMF (Figure 4).

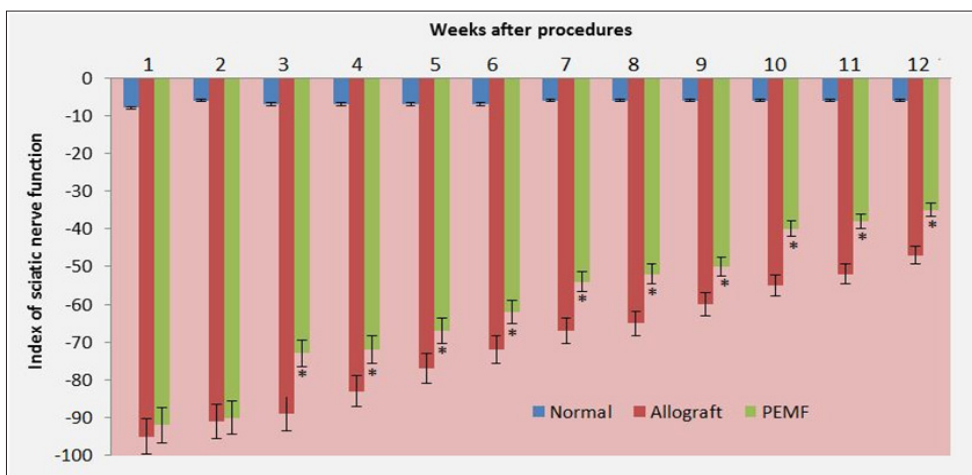


Figure 3: Diagrammatic representation of effects on index of the sciatic nerve function (SFI) in each experimental group during the study period. Statistically significant improvement ($P < 0.05$) was observed in functional recovery of the sciatic nerve in PEMF treated animals at the end of the study period. * $P < 0.05$ vs allograft group.

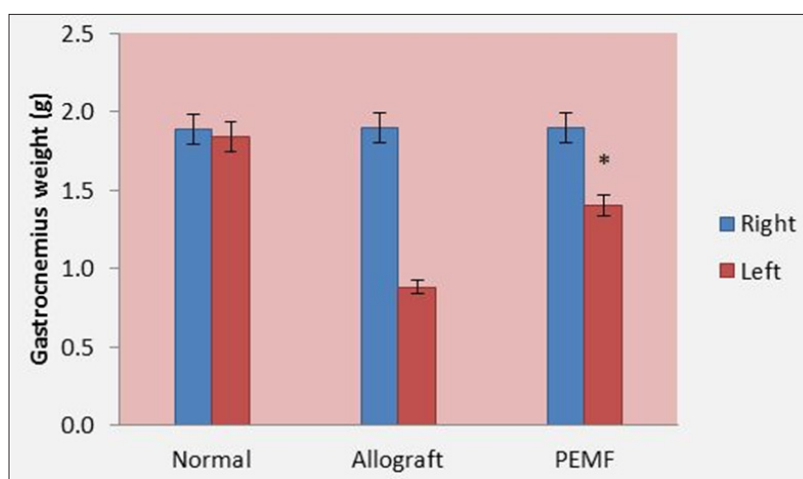


Figure 4: Measurement of gastrocnemius muscle mass. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery, * $P < 0.05$ vs allograft group. Data are presented as mean \pm SD.

c) Histological and Morphometric findings: The (Table 1) shows the quantitative morphometric analyses of regenerated nerves for each of the experimental groups. The PEMF treated group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness compared to allograft animals ($P < 0.05$).

Table 1: Morphometric analyses of sciatic nerve in each of the experimental groups. Values are given as mean \pm SD.

Groups	Axon counts fb/mm ²	Diameter of axon (μ m)	Thickness of myelin sheath (μ m)
Normal	28765 \pm 3256	11.57 \pm 0.15	2.67 \pm 0.05
Allograft	19856 \pm 3497	3.32 \pm 0.19	1.06 \pm 0.03
PEMF	25697 \pm 3014*	6.39 \pm 0.18*	1.29 \pm 0.03*

d) Immunohistochemistry: Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also

showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Figure 5). In both PEMF and allograft groups, the expression of S-100 and the findings resembled those of the histological evaluations.

Discussion

The results of the present study showed that whole body exposure to PEMF resulted in faster functional recovery of the sciatic nerve during the study period. The assessment and interpretation of the results achieved with nerve allograft are still controversial due to the uncertain histocompatibility between donor and receptor of different grafting technique and of the complexity of quantitative methods for neural regeneration assessment [16,17]. Proposed an experimental model to study neural regeneration with allograft in rats, applying a computer-based method for assessing results. Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [18-20]. Classical and newly developed methods of assessing

nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling [20] do not necessarily predict the reestablishment of motor and

sensory functions [19,21-23]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [19].

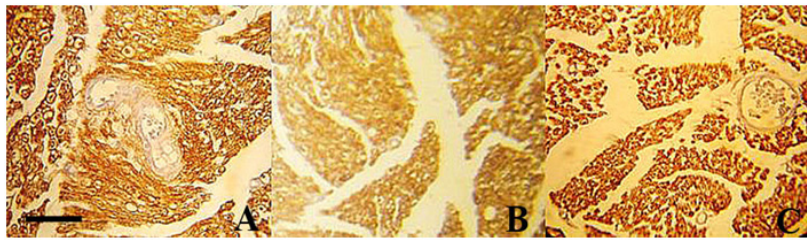


Figure 5: Immunohistochemical analysis of the regenerated nerves 16 weeks after surgery from middle cable (A) Normal, (B) Allograft and (C) PEMF. There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrow) within the periphery of nerve, indicating well organized structural nerve reconstruction in PEMF group. Scale bar: 50 μ m.

Therefore, research on peripheral nerve injury needs to focus on functional assessment. Castaneda et al. [23] suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Results of the present study showed that the PEMF treated animals had been improved in locomotion of the operated limb compared to the allograft group during the study period. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models [21].

Left gastrocnemius muscle weight was significantly greater in the PEMF group than in the allograft group, indicating indirect evidence of successful end organ reinnervation in the PEMF treated animals. Left gastrocnemius muscle weight was significantly greater in the PEMF group than in the allograft group, indicating indirect evidence of successful end organ reinnervation in the PEMF treated animals. It has been demonstrated that morphometric indices are measures of regenerated nerve maturity and quality of regeneration [24]. Larger diameters of axons and thicker myelination give rise to improved nerve function compared to smaller and thinner myelinated fibers [25]. At week 12 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the allograft and PEMF groups, indicating a beneficial effect of PEMF on the nerve regeneration. In immunohistochemistry the expression of myelin sheath special proteins was evident in both groups which indicate the normal histological structure. The location of reactions to S-100 in the PEMF group was clearly more marked than in the allograft group implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of remyelination and the structural recovery of regenerated nerve fibers.

The effect of PEMF on cells and organisms after short-term exposure has already been reported and there are many potential mechanisms by which PEMF might affect neurotrophic factor levels in nerve tissue [12]. The absence of PEMF effects in nerve segments isolated from non-transected rats raises the possibility that these mechanisms occur primarily in injured rather than in normal animals [10]. Previous studies have demonstrated that at 6 h post-transection, increased levels of NGF in distal segments resulted from

blocked retrograde transport rather than local synthesis [26]. Thus, the significant effect of PEMF on reducing nerve growth factor-like activity and levels as early as 6 h post-transection suggested that PEMF acts via mechanisms distinct from synthesis of nerve growth factor or nerve growth factor-like factors. Other growth factors potentially influence nerve regeneration and through which PEMF act might include brain derived neurotrophic factor, ciliary neurotrophic factor insulin-like growth, fibroblast growth factor, and glia-derived neurotrophic factor [27-30]. Following sciatic nerve transection, there is a gradual increase in brain derived neurotrophic factor mRNA expression in distal but not proximal nerve segments beginning at 3 days and reaching maximum levels 3-4 weeks later [31].

The delayed nature of this response suggests that brain derived neurotrophic factor is unlikely to influence early regenerative responses and is unlikely to constitute the activity measured in our studies. It has to be mentioned that this area is reaching from wound and bone healing over pain relief to transcranial magnetic stimulation. In the latter technique, neurons are actively stimulated by magnetic field-induced electric fields [32]. Transcranial magnetic stimulation is used as an antidepressant, against migraine and also to enhance motor functions and it can interfere with human behavior and also with cognitive tasks [33,34]. The cellular mechanisms underlying all these magnetic stimulations remain unclear. Although the positive influence of the fields is more and more recognized and used in therapeutic applications, the general effectiveness is still controversial. There are obvious knowledge gaps that make a conclusion of the risk for neurodegenerative diseases due to magnetic fields exposure very difficult [35]. Experimental research efforts should include a proper long-term perspective, possibly as life-long animal studies. Comprehensive and systematic studies regarding threshold identification as well as studies with non-activated and pre-activated cells could give more insight into the mode of action of field exposure and cells [35].

At 12 weeks postoperatively, results on the allograft group showed a normal growth speed, similarly to the PEMF group. This was probably due to the regeneration promoted by host' Schwann cells that entered into the graft. Schwann cells migration can be demonstrated by the induced rejection response that occurs in nerve segments implanted back into their original donor animals

[35]. The faster growth of neural fibers towards their target organ may result in a faster and more effective functional recovery. In clinical practice, functional results observed after peripheral nerve grafting depend on the extension of nerve defect and on its corresponding graft, on the time between injury and repair, and on the quality of functional rehabilitation; and these conditions cannot be simulated on an experimental model. Even though our preliminary study shows the neuroregenerative action of whole body exposure to PEMF in peripheral nerve injuries, determining the molecular mechanisms leading to the neuroregenerative action remains needs to be investigated. We have not given molecular evidence for neuroregenerative action of PEMF. This may be considered as a limitation to our study. Therefore, the authors stress that the aim of the current investigation was to evaluate clinical treatment potential of PEMF on nerve regeneration including functional assessments of the nerve repair, a case not considered in previous studies. The results of the present study indicated that whole body exposure to PEMF could be of benefit after sciatic nerve allografting. Detailed mechanism of neuroregenerative action remains to be investigated. The experimental model presented here is reproducible for the study of nerve allografting and the behavioral and functional methods could be effectively used for the study of peripheral nerve regeneration in allograft models.

Conclusion

The present study demonstrated that whole body exposure to PEMF could accelerate functional recovery after nerve allografting in sciatic nerve and may have clinical implications for the surgical management of patients after nerve transection.

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