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Effects of Pulsed Electromagnetic Field with Predatory Stress on Functional and Histological Index of Injured-Sciatic Nerve in Rat

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ABSTRACT

Objective: To assess the effect of combination of pulsed electromagnetic fields (PEMF) with predatory stress on transected sciatic nerve regeneration in rats.
Methods: In sham- operated group (SOG) the nerve was manipulated and left intact. The 10-mm rat sciatic nerve gap was created in rats. In transected group (Transected) nerve stumps were sutured to adjacent muscle and in vein graft group (VG) the gap was bridged using an inside-out vein graft. In VG/PEMF group the transected nerve was bridged using vein graft, phosphate buffered saline was administered into the graft and the whole body was exposed to PEMF. In VG/PS group the transected nerve was bridged using vein graft, phosphate buffered saline was administered into the graft and the rats underwent predatory stress (PS). In VG/PEMF/PS group the transected nerve was bridged using vein graft, phosphate buffered saline was administered into the graft, the whole body was exposed to PEMF and the rats underwent predatory stress. The regenerated nerve fibers were studied within 12 weeks after surgery.
Results: Functional, gastrocnemius muscle mass findings and morphometric indices confirmed faster recovery of regenerated axons in VG/PEMF and VG/PEMF/PS groups compared to those in the other groups (p=0.001). The whole body exposure to PEMF improved functional recovery. Predatory stress did not affect nerve regeneration in the animals undergone predatory stress (p=0.343).
Conclusion: Pulsed electromagnetic fields could be considered as an effective, safe and tolerable treatment for peripheral nerve repair in clinical practice.

Keywords: Nerve regeneration; Sciatic; PEMF; Predatory stress; Functional recovery.

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Introduction

The method of choice for repair of peripheral nerve gap is still autologous nerve grafting, however, autologous nerve grafts bear disadvantage of the loss of a functional nerve from donor site [1]. Thorough recovery is dependent on the regeneration of damaged nerve fibers and reestablishment of fully

functional connection with their targets. 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 [2]. 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 [3-5]. PEMF has also been shown to promote neurite outgrowth *in vitro* [6]. Whole body exposure to PEMF has improved functional recovery and morphometric indices of transected sciatic nerve [7].

Stress is a very general concept and relates to the responses of the body to external events that bring the physiological equilibrium out of balance. These external events can be of physical, chemical or psychological nature [8]. The stress response comprises those behavioural, neuroendocrine and neurochemical changes that are evoked as a kind of alarm system that is initiated when there is a discrepancy between what an organism is expecting and what really exists [9]. In other words, the stress response is meant to cope with stressors and to find a new equilibrated state [8].

In the present study combination of pulsed electromagnetic fields with predatory stress were applied to elicit the functional recovery in peripheral nerve injury in a short 10-mm gap defect in a rat model. The assessment of repair process was based on function, gastrocnemius muscle mass and histomorphometry in a 12-week period.

Materials and Methods

Whole Body 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 [3, 5]. 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 (AUTO, BMSC/PEMF and PEMF groups) or not activated (BMSC 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 [10]. 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 (Funktiongenerator, 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 Teslameter (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.

Predator Exposure

Cat exposure experiments were conducted on the second day after surgery based on a method described by others [10-12]. The rats were exposed to cat once a day for five consecutive days. Each stress session lasted for 1 hour started from 9 to 10 am. The rats were placed in cage with 30 cm distance from cat. Then the rats were removed and brought back to their facilities. In other groups the rats were transferred to the stress room in the same timing without presence of cat and brought back to their facilities. Blood was collected from all the study group animals before and after each test. To characterize the cortisol response to the stressor, samples were taken at fixed timings of 13 to 16 pm.

Analysis of Serum Level of Cortisol

Analysis of serum level of cortisol was based a method described by others [13]. In brief, one mL of blood sample was collected from each rat and was allowed to clot for 30 minutes at room temperature. The Serum was separated by centrifugation at 2500 rpm for 5 minutes and subsequently stored at -20 °C. The serum samples were then subjected to estimation of serum cortisol. The hormone was estimated by automated chemiluminescence immunoassay system Alpha Prime LS, France using Cortisol ELISA kit (DRG, USA). This immunoassay kit allows for in-vitro quantitative determination of endogenic cortisol concentration in serum. Principle of estimation of cortisol was competitive inhibition enzyme immunoassay technique. This assay has high sensitivity and specificity for estimation of cortisol levels in Wistar rats.

The Procedures

Fifty four male White Wistar rats approximately 260 g were randomized into six groups (n=9). Each group was subdivided into three subgroups of 3 animals each. Thirty six male White Wistar rats 300-350 g were used as donors of vein nerve guides. A 15- mm segment of right external jugular vein was prepared on a tube after the donor animals had been anesthetized, shaved and prepared aseptically. Grafts were washed in physiological solution and left at room temperature for 30- 40 min. A delicate retraction of 1mm was already predictable. Two weeks period was considered for acclimatization under 23 ±3 ºC and 12/12 light cycle. The animals had free access to food and water. Animals were anesthetized by

intraperitoneal injection of ketamine-xylazine (ketamine 5%, 90mg/kg and xylazine 2%, 5mg/kg). The operations were performed based on the Ethics Committee of the International Association for the Study of pain [14]. The University Research Council approved all experiments.

After surgical preparation in PEMF group sciatic nerve of the left side was exposed and excised next to peroneal bifurcation where a 7 mm segment was harvested. The cut ends of the nerve were each inserted 2 mm into a vein nerve guide and two 10/0 nylon sutures were put at each end of the cuff to fix the graft in place and leave a 10-mm gap between the ends. The nerve guide was injected with 20 µL phosphate buffered saline solution and sterile Vaseline was used to seal the ends of the tubes to avoid leakage. After careful hemostasis the muscles were closed with absorbable 4/0 sutures, and the skin with 3/0 nylon. The whole body was exposed to PEMF (0.3 mT, 2Hz) for 4 h/day within 1-5 days. In transected group (Transected) nerve stumps were sutured to adjacent muscle and in vein graft group (VG) the gap was bridged using an inside-out vein graft. In VG/PEMF group the transected nerve was bridged using vein graft, phosphate buffered saline was administered into the graft and the whole body was exposed to PEMF. In VG/PS group the transected nerve was bridged using vein graft, phosphate buffered saline was administered into the graft and the rats underwent predatory stress. In VG/PEMF/PS group the transected nerve was bridged using vein graft, phosphate buffered saline was administered into the graft, the whole body was exposed to PEMF and the rats underwent predatory stress (PS). The animals were killed humanely with transcardial perfusion of a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH=7.4) 4, 8 and 12 weeks after operation.

Sciatic Functional Index (SFI)

According to a method described by others the analysis of track was done in three time points of 4, 8 and 12 weeks during the study period [15]. The distances 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 operated site (E) and the un-operated normal site (N) in each animal. The Sciatic Function Index (SFI) in each rat was measured using the formula:

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

Overall, the SFI range falls between 0 to -100. Zero SFI shows normal nerve function and −100 SFI denotes complete dysfunction. The functional index was measured based on the BMSC group and 0 was considered for the normal level. In general, SFI is a negative value and a higher SFI denotes the improved function of the nerve [15]. The SFI index was made on both feet of rats before surgery.

Measurement of Muscle Mass

The weight ratio of the gastrocnemius muscles at the end of the study period was measured to assess recovery index. Instantly after euthanizing of animals, gastrocnemius muscles were isolated and dissected out carefully from both operated and un-operated limbs. They were weighed while still wet by an electronic balance. Two independent experts did the measurements.

Histological Assessments

The regenerated nerves from all groups were isolated and 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. An image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA) was used to perform morphometric analysis. To handle the sampling-related, fiber-location-related and fiber-size related biases an equal opportunity, systematic random sampling and two-dimensional dissector rules were followed [16].

Statistical Analysis

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 a factorial ANOVA ($p<0.05$) with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. Experimental results were expressed as means±SE.

Results

Findings of Serum Level of Cortisol

Comparison of serum cortisol levels after predatory stress was done in study group animals. It was observed that serum cortisol level was comparatively increased more after predatory stress. The mean value of serum cortisol levels in VG/PEMF/PS and VG/PS groups were 54.29±7.79 and 39.67±3.61 (ng/mL), respectively.

Sciatic Function Index and SFI Outcome

Sciatic function values in all animals are shown in Figure 1. Before operation, the values in all animals were nearly zero. Following operation, the mean value of the sciatic function got to -100 as a result of total dysfunction of sciatic nerve in all animals. The recovery of nerve function was significantly faster in VG/PEMF and VG/PEMF/PS groups compared to other groups groups ($p=0.001$).

Muscle Mass Measurement

Measurements of the mean ratios of gastrocnemius muscles showed statistically significant difference in comparison of VG/PEMF and VG/PEMF/PS and other groups ($p=0.001$). The percentage in VG/PEMF and VG/PEMF/PS groups was higher than in

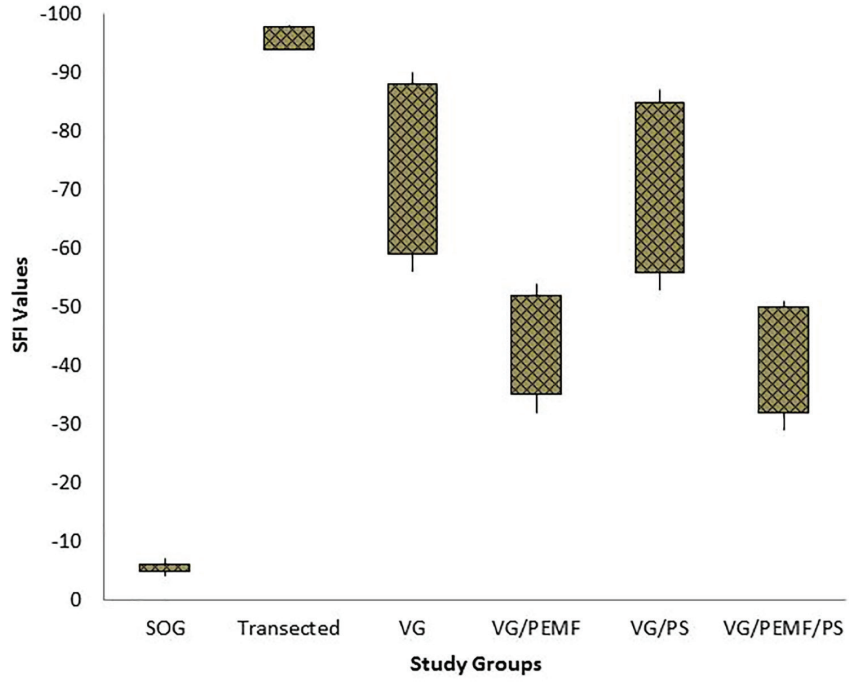

Fig. 1. Box-and-whisker plots of sciatic nerve function index values in each experimental group during the study period. Data are presented as mean±SE.

other groups and weight loss of the gastrocnemius muscle was improved by PEMF treatment (Figure 2).

Histological and Morphometric Findings

The animals of VG/PEMF and VG/PEMF/PS groups presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness during study period, compared to other groups (p=0.001) (Figures 3-6). In case of myelin thickness there was no significant difference among animals morphometrically (p=0.324).

Discussion

The present study demonstrated that whole body exposure to pulsed electromagnetic fields accelerated sciatic nerve regeneration in rats. Castaneda *et al*., [17] reported that contact of sprouts from the proximal stump to the distal nerve stump does not essentially indicate retrieval of nerve function.

Our results revealed that PEMF treated animals were enhanced in locomotion of the operated limb in comparison with the other groups within study

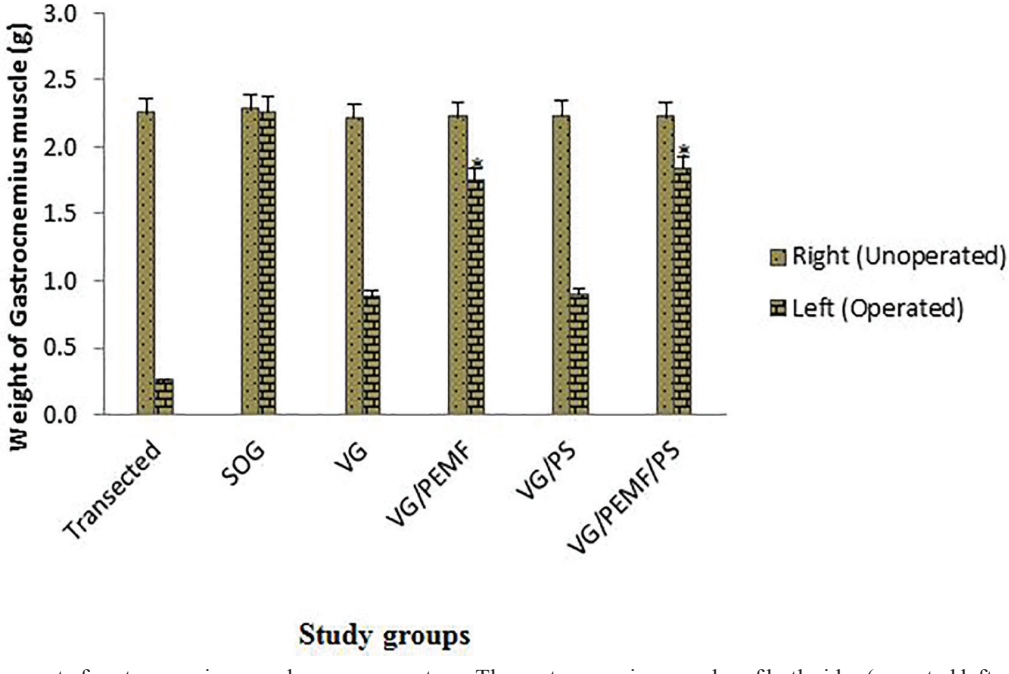

Fig. 2. Measurement of gastrocnemius muscle mass percentage. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery. *p<0.05 vs other groups. Data are presented as mean±SE.

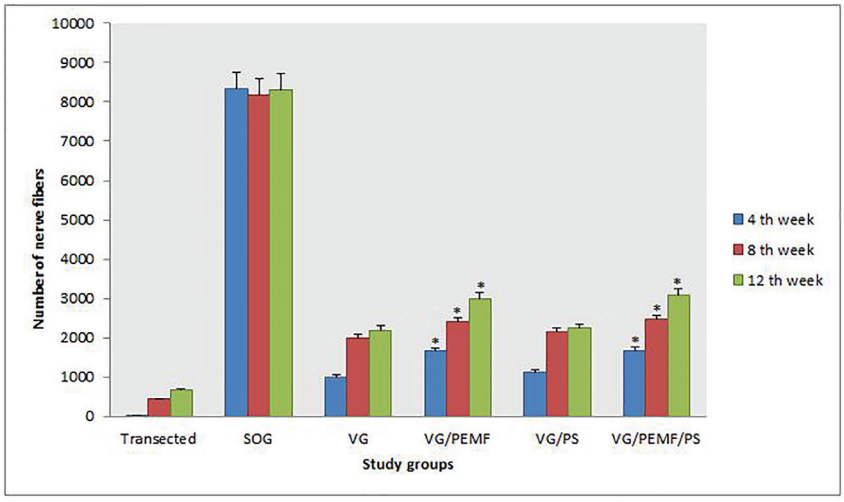

Fig. 3. Bar graph shows the results of number of nerve fibers. VG/PMEF and VG/PMEF/PS groups showed the greater number of fibers than other experimental groups at the end of the study period. Data are presented as mean±SD. * $p<0.05$ vs other groups.

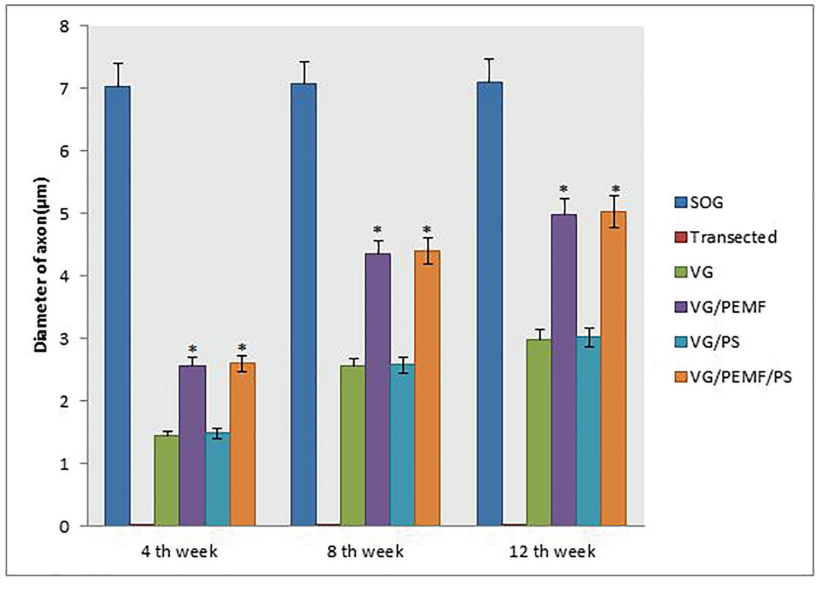

Fig. 4. Bar graph shows the quantitative results of mean diameter of axon. VG/PMEF and VG/PMEF/PS showed the greater mean diameter of axon than other experimental groups at the end of the study period. Data are presented as mean±SE. * $p<0.05$ vs other groups.

period. Analysis of tracks has commonly been adopted to reliably conclude functional recovery after nerve regeneration in rat models [15, 18]. It has been reported that it was a coordinated activity including input of sensory organ, response of motor organs and integration of cortical segment [17]. Our findings indicated there were similar finding between both SFI tests. To approve motor target organ reinnervation recording of wet muscle weight is alternatively utilized [19-21].

As the gastrocnemius muscle receives posterior tibial branch of the sciatic nerve, its mass will regain proportional to the quantity of axonal reinnervation [22, 23]. At the end of our study period animals of PEMF treated groups revealed that there were significant greater ratios of the mean gastrocnemius muscle weight compared to other groups. This indicated another evidence of effective end organ reinnervation.

Morphometry of the repaired nerve fibers indicated that there was significant difference between PEMF treated and other animals. Regarding better functional and morphometric indices in group, it could be stated that cell therapy combined with whole body exposure to pulsed electromagnetic fields both accelerated and improved the process of nerve regeneration.

The effect of PEMF on cells and organisms after short-term exposure has been reported in many studies. There are many potential mechanisms by which PEMF might affect neurotrophic factor levels in nerve tissue. 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. Previous studies have demonstrated that at 6 hr post-transection, increased levels of NGF in distal segments resulted from blocked retrograde

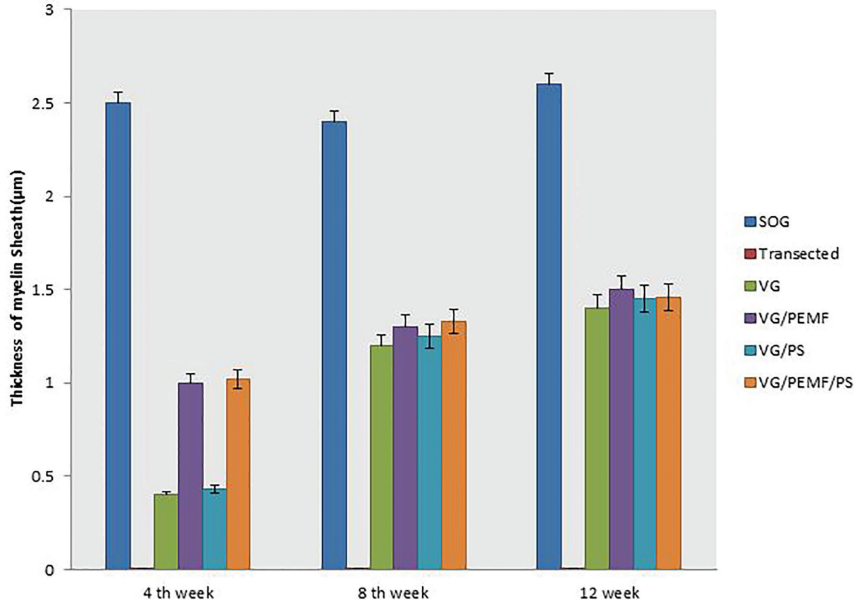

Fig. 5. The graph shows the quantitative results of mean thickness of myelin sheath. Regarding the myelin thickness no significant difference were observed among experimental groups.

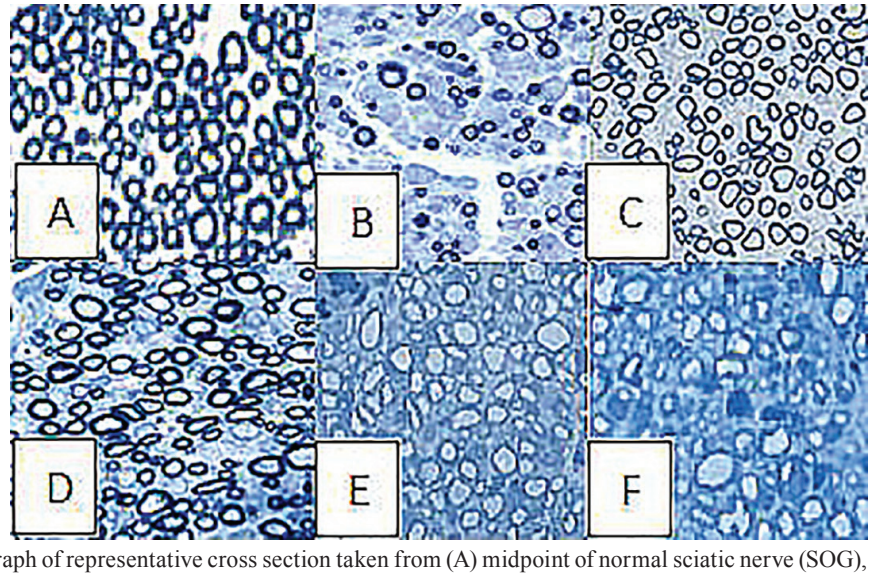

Fig. 6. Light micrograph of representative cross section taken from (A) midpoint of normal sciatic nerve (SOG), (B) regenerated cable (Transected), (C) middle cable (VG), (D) middle cable (VG/PEMF), (E) middle cable (VG/PS) and (F) middle cable (VG/PEMF/PS) 12 weeks after surgery. (Toluidine blue, ×400)

transport rather than local synthesis [24]. Thus, the significant effect of PEMF on reducing nerve growth factor-like activity and levels as early as 6 hr 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 might act include brain derived neurotrophic factor, ciliary neurotrophic factor insulin-like growth, fibroblast growth factor, and glia-derived neurotrophic factor [25-28]. 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 [29]. 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 [30]. Transcranial magnetic stimulation is used as an antidepressant, against migraine and also to enhance motor functions and it can interfere with human behaviour and also with cognitive tasks [30-32]. 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 [33].

Acute stress is well-known to trigger marked increase in glucocorticoid concentration in blood. The hypothalamic–pituitary–adrenal (HPA) axis is the key hormonal system that has well-characterized circadian pattern. Under the influence of stress, this pattern is altered and it exerts adverse impact on health. Also degree of activation of the HPA axis is related to the intensity of stress experienced by animals [34]. Rats are very sensitive to stress, and a mild stressor results in a robust increase of glucocorticoid levels that approximates to the amplitude of diurnal rhythm [35]. It has been reported that local administration of glucocorticoids had enhanced peripheral nerve regeneration [36].

In the present study the mean serum levels of cortisol indicated that PEMF caused additional stress to the animals. The predatory stress also resulted in significant increase in serum level of cortisol. Nevertheless, regarding improvement in functional recovery of the damaged nerve, no significant changes were observed between stressed animal and non-stressed animal. In other words, it could be concluded that stress was ineffective in the process of nerve regeneration.

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 [33]. In the present study the histological and molecular evidences for neuroprotective action of PEMF were not provided, therefore, detailed mechanism of neuroprotective actions remain to be investigated.

In conclusion, whole body exposure to pulsed electromagnetic fields offered a practical approach to accelerate nerve repair and may have clinical implications for the surgical management of patients after nerve transection.

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