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I. Introduction

The incidence of peripheral nerve crush injury is continuously increasing in clinical dentistry, possibly caused by dental implantations, maxillofacial traumas or orthognathic surgeries. Although regeneration usually occurs to some extent, proper regrowth of the axons with complete restoration of function is rarely obtained after severe peripheral nerve injury. Regardless of the capacity of peripheral nerves to regenerate after injury, and the advance of microsurgical techniques to re-unite nerve fascicles and guide the regenerating axons into the distal nerve stump, functional recovery is still far less than normal, especially when regenerating axons have to transverse long distances to reach their target organs. The reasons for poor functional recovery after peripheral nerve injury include; the progressive decline in the neuron ability to sustain axonal growth, failure of denervated Schwann cells to maintain a growth-supportive environment, and a slowed regeneration rate across the surgical gap\textsuperscript{[1]}.

Recently strategies including cell therapy\textsuperscript{[2, 3]}, growth hormones, neurotrophic factors\textsuperscript{[4-6]}, and electromagnetic stimulation\textsuperscript{[7-10]} have been used to improve these constraints. One of these strategies, pulsed electromagnetic field (PEMF), evolved from in vivo studies on bone healing\textsuperscript{[11]} was advocated as a potential candidate to promote nerve regeneration for several reasons. It has been shown to stimulate both peripheral and central nerve regeneration in vivo and in vitro experiments\textsuperscript{[12-17]}. PEMF exposure was reported to differentiate bone marrow mesenchymal stem cells (BMMSCs) into neuron-like cells by affecting the cell cycle\textsuperscript{[18]}. Raji and
Bowden[10] and Ito and Bassett [5] found that PEMF improved the functional recovery and increased number and diameter of regenerating axons following transection and re-approximation of the rat sciatic nerve. Although there’s a contradictory result indicating that the exposure to PEMF does not influence nerve regeneration[19], PEMF is generally believed to affect cell survival and propagation according to the intensity, time interval and frequency. PEMF can also induce changes in the cell cycle and propagation of exogenously delivered cells into the injury site as well as intrinsic cell sources. In these circumstances, nerve regeneration would be more enhanced by the addition PEMF to a cell delivery scheme.

Among the many potential stem cell sources for the nerve regeneration, human dental pulp stromal cells (hDPSCs) have received growing attention in recent years due to common characteristics in common with other mesenchymal stem cells, in addition to the ease of obtaining and propagating hDPSCs. There has been a continuous increase in the number of studies on proliferation and differentiation capacities since the first report by Gronthos et al. [20]. Dental pulp stem cells could differentiate into many types of cells especially neural cells [21]. Compared with human bone marrow mesenchymal stem cells (hBMMSCs), hDPSCs exhibited a higher proliferation rate in vitro, which could be explained by their origin [22]. Although the above experiment was on bone regeneration, a similar pattern is expected in peripheral nerve regeneration. hDPSCs probably have a lot of potential in the treatment of nerve regeneration. Also, hDPSCs are easy to isolate from
human third molars, are multi-potent, and express mesenchymal stem cell markers. hDPSCs can differentiate into various tissues. Therefore, hDPSCs would be an ideal material in clinical trials and tissue engineering for nerve regeneration.

FK506 was originally developed for the immunosuppressant in organ transplantation. It also has been shown to have neuroprotective and neurotrophic actions in experimental models by increasing neurite elongation and by accelerating the rate of nerve regeneration in vitro and in vivo [23-26]. Dawson et al. [27] have also described a neuroprotective effect of FK506 whereby neurite outgrowth is promoted in cultures of rat dorsal root ganglia. Furthermore, FK506 has been noted to enhance the rate of axonal regeneration after crush injuries in rat sciatic nerves [28]. Positive results in nerve regeneration in animals and humans immune-suppressed by FK506 have been reported in various studies [29-34]. The immunosuppressant FK506 can be used to alleviate the immune response of hDPSCs and to accelerate the nerve regeneration.

This study was conducted to examine if exogenously delivered hDPSCs with immune suppression and neuropromotion with FK506, combined with PEMF, would be more beneficial for peripheral nerve regeneration than PEMF only. We determined an effective stimulus condition for PEMF in terms of intensity and duration and then we evaluated the co-treatment effect of PEMF with hDPSCs and FK506 on nerve regeneration.
II. Materials & Methods

Surgical procedure

All surgical and experimental procedures were carried out in accordance with the care guidelines of the laboratory of animal resources of Seoul National University/Republic of Korea (SNU 100304-3). Animals were anesthetized with an intraperitoneal injection of a 4:1 mixture of ketamine HCl (100mg/kg, ketamine hydrochloride, Ketara®, Yuhan, Korea) and xylazine hydrochloride (5mg/kg, Rompun®, Bayer, Korea). The surgical field was prepared by hair shaving and the application of 10% povidone iodine to the lateral aspect of sacrum to thigh. The left sciatic nerve was exposed by a posterolateral longitudinal straight incision from the greater trochanter to the lateral condyle of the left femur and then by blunt dissection between the gluteus maximus and quadriceps muscles. When the sciatic nerve was exposed and detached from the surrounding tissues, a standard surgical needle holder engaged to the second clip of both arms was used to create a crushing injury at a distance about 12mm distal to the sciatic furcation (Figure 1). The nerve was clamped for 30 seconds to produce a crush-injury of 3mm in width. The injury site was marked under 16X magnification with a surgical microscope (Carl Zeiss, Germany) by introducing a single 9-0 nylon (Ethicon®, UK) epineural stitch at the distal limit of the injury for later identification.

The wound was closed in a single layer by the use of 4–0 Nylon sutures (Ethicon®, UK). Rats were kept in cages (3 rats/cage) with free access to specific rat chow and water prior to and after the surgery. Nerve regeneration was assessed
over a 4-week period.

**Animal Grouping**

Male Sprague-Dawley rats (200–250g) were randomly distributed into 5 groups (n=6 in each group); the crush only control, PEMF 15G2weeks, PEMF 15G4weeks, PEMF 30G2weeks and PEMF 30G4weeks groups, in order to determine the optimal stimulus conditions for PEMF intensity and duration. To evaluate the co-treatment effect, rats were distributed into 5 groups (n=18 in each group); the crush only control, PEMF, PEMF with FK506 (PEMF + FK 506), PEMF with hDPSCs (PEMF + hDPSCs) and PEMF with hDPSCs and FK506 (PEMF + hDPSCs + FK 506) groups.

**PEMF Exposure**

PEMF was generated with a pair of Helmholtz coils of 60 cm diameter and 30 cm distance. The coils were placed in a 90 × 90 × 50 cm Faraday cage in order to prevent environmental electromagnetic interaction. The Helmholtz coils were connected to a power supply and a microprocessor-controlled frequency generator developed in the Department of Electrical Engineering, HanKyoung University (Figure 2). The rats were put into a 30 × 30 × 25 cm plastic cage between the two coils, where they could freely wander. PEMF was carried out with pulse trains of 60Hz frequency and intensity of 15 and 30 Gauss in order to determine the optimal stimulus condition in terms of PEMF amplitude and duration. PEMF was applied for the period of 2 weeks or 4 weeks (1hour/day). To evaluate the co-treatment effect, PEMF was delivered with pulse trains of 60Hz frequency and 15Gauss for 2
weeks (1 hour/day) in a silent room at a temperature of 21–25°C.

**Isolation and Transplantation of hDPSCs**

hDPSCs were isolated and cultured according to previously reported protocols with minor modification [35]. Briefly, human 3rd molars were extracted from 10 adults (18-22 years of age, patients provided informed consent) at the Department of Oral & Maxillofacial Surgery, Seoul National University, Seoul, Korea and were used under approval by the Institutional Review Board of Seoul National University Dental Hospital. Human DPSCs were isolated as described previously [36]. Briefly, the teeth were cracked open to remove the pulp tissues gently with forceps. The pulp tissues were then minced into explants and placed in 60-mm culture dishes (NUNC, Rochester, NY, USA). The explants were cultured in DMEM supplemented with 10% FBS and antibiotics. The animals received an injection of hDPSCs (7-9 passages), at a concentration of 1X10^6 cells/rat immediately after injury by a 30-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA), at a distance of 5 mm proximal to the injury site. The total injection volume was 10μl. FK506 (Tacrolimus 5mg inj, Prograf®, Astelas, Korea) was injected intraperitoneally one day before surgery and thereafter for 7 days (0.5mg/kg/day).

**Evaluation of hDPSCs Transplantation using Cell Tracking with PKH26**

In order to trace the survival of transplanted hDPSCs, cells were labeled with PKH26 (Sigma-Aldrich) according to the manufacturer's protocol, and diluted to 1 × 10^5/μl in PBS. Briefly, cells were detached from the cultured dish by Trypsin
EDTA solution (Sigma USA) and pelleted. Cell count was done via trypan blue (Gibco USA) exclusion using a hemocytometer (Neubauer Improved-Germany) to determine the total number of cells and cell viability before staining. Approximately $5 \times 10^6$ to $2 \times 10^7$ human DPSCs (passage 3) were washed once with supplemented αMEM and centrifuged at 800 rpm for 5 min, 25°C, after which the supernatant was aspirated to leave no more than 25 μl in the pellet. The cells were resuspended in 1 ml of diluent C solution (included in the PKH26 Red Fluorescent Cell Linker Kit, Sigma, USA) and then incubated in 4 μM PKH26/1 ml diluent C dye solution for 5 min. The labeling reaction was stopped by the addition of 2 ml of FBS. Labeled cells were washed two times with αMEM/FBS by centrifugation for 10 min at 400 × g. And, PKH26-labeled hDPSCs were injected on the crushed nerve by a 30-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA), at a distance of 5 mm proximal to the injury site (Figure 2). After injection, the needle was left in place for 2 min prior to withdrawal, to minimize cell leakage [37, 38]. Rat sciatic nerves were harvested at 1, 2, 3 and 4 weeks after PKH26-labeled hDPSC injection, and sections were obtained at 20 μm thickness with a cryocut microtome (Leica, Ultracut, UCT, Austria). A laser scanning confocal microscope (Olympus, FV- 300, Tokyo, Japan) was used to capture images.

**Functional Assessment, SFI (Sciatic Function Index)**

Footprints were recorded preoperatively and postoperatively every week for all groups until the end of the follow-up period. Preoperative footprints were obtained.
by the conventional method described by Jolicoeur et al\textsuperscript{[39]} and the sciatic function index (SFI) was calculated by the widely accepted formula described by Bain et al\textsuperscript{[40]}. An average of 3 imprints for each rat was selected for the Sciatic Function Index (SFI) calculation at each time interval. Accordingly, each group was evaluated by the same observer during the study. For each footprint, the print length (PL, or the longitudinal distance between the tip of the longest toe and the heel), toe spread (TS, or the distance between the first and fifth toes), and the intermediate toe spread (IT, or the distance between the second and fourth toes), both in the normal (N) and the experimental (E) paws were all measured. Based on these parameters, SFI was calculated according the formula modified by Bain et al\textsuperscript{[40]}.

\[
\text{SFI} = -38.3 \times \text{EPL} - \frac{\text{NPL}}{\text{NPL}} \times 109.5 - \text{ETS} - \frac{\text{NTS}}{\text{NTS}} + 13.3 \times \text{EIT} - \frac{\text{NIT}}{\text{NIT}} - 8.8
\]

As an indicator of nerve function, SFI values around ‘−100’ indicate total loss of function. On the other hand, values close to ‘0’ indicate a normal function.

**Histomorphometric Analysis**

At the end of the 4-week follow-up period, 6 rats from each group were anesthetized. The sciatic nerve was exposed again and nerve segments including the crush injury site were harvested and seven specimens were prepared for histomorphometric analysis. The nerves were immediately immersed into a fixation solution containing 2.5% glutaraldehyde in PBS (pH 7.4) at 4°C for 24 hours. Only the distal portion (5mm distal to the injury) was used for histomorphometric
evaluation. The nerve segment was then post-fixed with 2% osmium tetroxide for 2 hours. Thereafter, it was washed with PBS (pH 7.4) solution and then routinely processed and embedded in epoxy resin. Serial transverse semi-thin sections of 1µm thickness were cut with a microtome (LEICA, Ultracut, UCT, Austria) and stained with 1% toluidine blue for light microscopy examination (Olympus, BX41, TF, Japan). Images were captured using a specialized system, SPOT RTTM-KE color mosaic (Diagnostic Instruments, Inc., USA) and digitized by SPOT software Ver. 4.6 (Diagnostic Instruments, Inc., CA. USA). For the purpose of axon counting, the total cross-sectional area of the nerve was measured at 40x (Olympus, BX41, TF, Japan), and three sampling fields were then randomly selected at 200x magnification using a protocol previously reported \cite{41, 42}. Mean fiber density was calculated by dividing the total number of nerve fibers within the sampling field by its area (N/mm2). Total fiber number (N) was then estimated by multiplying the mean fiber density by the total cross-sectional area of the whole nerve cross-section assuming a uniform distribution of nerve fibers across the entire section. The sections were analyzed using a transmission electron microscope (TEM; JSM 1200 IIEX, JEOL, Japan). After image capturing, images were analyzed by OPTIMAS Ver. 6.5 (Image Processing Solutions Inc., North Reading, MA, USA) software.

**Retrograde Labeling**

For retrograde labeling, bilateral sciatic nerves were exposed and retrograde tracer (fluorogold) was soaked at 4 weeks postoperatively and labeled neuron was counted at dorsal root ganglion 1 week later. Retrograde labeling and counting of
back-labeled sensory neurons were performed as described by Geremia et al. [43]. Briefly, in an attempt to increase the accuracy of the counting procedure, the person who was in charge of the counting was unaware of the groups and only clearly fluorescent cells were considered for counting. The sciatic nerves of six animals in each group were labeled with 4% Fluorogold (FG) (Fluorochrome, LLC, Denver, CO, USA), while a fourth rat served as a negative control using distilled water (DW). Sciatic nerves were sharply cut at a distance of 15 mm distally to the sciatic furcation, and soaked in 4% FG for 1 h in vaseline well, and then thoroughly irrigated with physiologic saline and reflected back and the wound was closed. The wound was covered with wet sterile gauze during the procedure. One week following retrograde labeling, L4 and L5 DRG were harvested and serially sectioned into fresh-frozen 20μm-thick sections using a Cryo-Cut microtome (Leica CM3050S Cryostat, Leica Microsystems, Wetzlar, Germany) at −18°C. A laser scanning confocal microscope (Olympus, FV-300, Tokyo, Japan) was used to capture images of the DRG sections at 20X magnification. For quantification of back-labeled neurons, we applied a simple method that takes into account the area of the DRG field in which the neurons are counted. The 3 largest sections from each DRG were considered and the sums of counted labeled neurons were compared between the groups.

**RT (Reverse transcriptase)-PCR for BDNF, TrkB, and P75NTR**

To follow-up on the molecular changes caused by PEMF combined with hDPSCs and/or FK506, we examined the expression of endogenous BDNF, its tyrosine
receptor kinase B (TrkB) and p75NTR in L4, L5 dorsal root ganglia (DRG) neurons in six animals/group, by means of quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) after 1,2,4,7 and 14 days postoperatively. Six animals of each group were anesthetized and DRGs were harvested. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The purified RNA was treated by RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). The mRNAs encoding BDNF, p75NTR and trkB receptor were reverse transcribed to cDNA using a first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA) and the amount of cDNA was also quantified using real-time PCR. The following primers were used to amplify specific cDNA regions of the transcripts of interest: BDNF (GeneBank Reference Sequence: XM_227523.3), p75NTR (GeneBank Reference Sequence: X05137.1), trkB (GeneBank Reference Sequence: M85214.1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GeneBank Reference Sequence no.: NM_017008.3). GAPDH quantification was used as an internal control for normalization. The percentage differences in mRNA levels over control values were calculated using the ΔCt method as described previously (Applied Biosystems Manual, Foster City, CA, USA) according to Chen et al[44].

Statistical Analysis.

Data analysis was carried out using StatView software (Version 5.0.1, SAS Institute, Cary, NC, USA). All data were presented as mean values with standard deviation (table) and standard error means (graph). Nonparametric one-way ANOVA test using Turkey’s test for multiple pairwise examinations was used to
compare data between different animal groups at specific times. Values of $p<0.05$ were considered statistically significant.
III. Results

Optimization of PEMF intensity and duration

One week following the crush injury, SFI for control, both 15G and both 30G groups showed injured level (-86.07±12.10 in control group, -84.98±9.26 in 15G2W group, -71.61±17.65 in 15G4W group, -93.29±2.33 in 30G2W group and -83.91±6.60 in 30G4W group) as a result of the axonotmesis and consequent loss of function (Figure 3-A). The recorded footprints were noticeably longer and the toe spread was smaller than in preoperative footprints. During the next two weeks, 15G2W, 15G4W and 30G2W groups showed progressive improvement in SFI (except for the 15G4W group), which was more pronounced between the second and third weeks, especially in the 15G2W group. But, control groups showed a little improvement. All PEMF groups showed a more pronounced effect on SFI. However, the comparison of the 2-week and 4-week groups showed no significant change in SFI after 4 weeks (Figure 3-A). By the end of the second week, the statistical difference between the control groups and 15G groups was clearly significant (p < 0.05; Figure 3-B). The 15G group slope increased more than any other group. Comparison of footprints between the 3 groups (control, 15G2W and 30G2W) 4 weeks postoperatively, showed that in the PEMF group, experimental and normal imprints were much alike, while in the control group, the experimental footprint was still longer with less toe-spreading potential than its normal counterpart. Gait analysis showed that the SFI's of the PEMF groups were higher than that of the crush injury only group at every interval with significantly higher
values in both the 15G2W group and 30G2W groups (Figure 3-B). PEMF is done at the beginning of the potential for nerve regeneration, and the promotion of nerve regeneration specifically found at 2 weeks.

Total axon counts and densities in order to determine the optimal stimulus condition of PEMF are shown in Table 1. For determining the PEMF intensity (15G or 30G), we compared only crush (control group), 15G and 30G groups under the same 2 weeks. The 15G2W and 30G2W groups reached the highest axon counts (12529.96±4034.18 in 15G2W group, 11123.15±2122.01 in 30G2W group). But no significant difference was observed in comparison to control groups (Figure 4-A), and none of the experimental groups showed differences in axon densities (17788.78±3040.46 fibers/mm² in the control group, 17774.62±2786.25 fiber/mm² in the 15G2W group and 17257.22±2695.38 fibers/mm² in the 30G2W group) (Figure 4-B). But, the 15G experimental group showed a slight elevation compared to the control and 30G experimental groups in axon counts and densities. These results were similar to those for SFI. The total fascicular area of the 15G group was greater than that of the 30G and control group (0.58±0.09mm² in control group, 0.71±0.22mm² in 15G2W group and 0.66±0.17mm² in 30G2W group). The 15G and 30G group were not significantly different, but the 15G group showed slightly better results. However, these results demonstrated that the PEMF group appears to have better results. So, we selected 15G and 2weeks of PEMF for assessing the co-treatment effect.

*Cell Survival (Cell Tracking with PKH 26) after hDPSCs Transplantation*
After sacrificing the rats, the fluorescence microscope analysis of the crushed nerve revealed an image of the red fluorescence of the PKH26 dye. PKH26-labeled hDPSCs were observed during the whole experimental period (Figure 5). Injected hDPSCs were observed on the crushed nerve segment, revealing their participation in the cell survival and no cell leakage. PKH26-labeled hDPSCs were present during the whole experimental period, however, only a few were observed in the 3rd week. PKH26-labeled hDPSCs gradually decreased in number, but they were still observed at 4 weeks. No obvious cytotoxicity was observed.

**Co-treatment with PEMF, hDPSCs and FK506**

One week postoperatively, control (only crush group) and all experimental groups showed injury level (-72.59±18.86 in control group, -86.25±9.13 in PEMF group, -80.95±12.58 in PEMF + FK506 group, -83.66±4.47 in PEMF + hDPSCs group and -84.11±5.38 in PEMF + hDPSCs + FK506 group) in SFI as a result of the axonotmesis and consequent loss of function (Figure 6). Two weeks postoperatively, the PEMF + FK506 and PEMF + hDPSCs group and control group showed a similar decrease. But, the PEMF and PEMF + hDPSCs + FK506 group showed a sharp increase at 2 weeks (p < 0.05).

Total axon counts and densities in order to evaluate the co-treatment effect of PEMF with hDPSCs and FK506 are shown in Table 2. In the PEMF group, the total axon counts were 10226.02±907.87 axons, and the densities were 17208.92±2758.04 fibers/mm^2. In the PEMF + FK506 group, the total axon counts were 18015.37±16734.36 axons, and the densities were 26085.57±23918.46
fibers/mm². In the PEMF + hDPSCs group, the total axon counts were 12163.15±1605.88 axons, and the densities were 15391.37±1417.55 fibers/mm². In the PEMF + hDPSCs + FK506 group, total axon counts were 17734.02±2545.57 axons, and the densities were 22052.76±1455.22 fiber/mm² (Figure 7-A and B). There were higher axon counts and densities in the PEMF + FK506 and PEMF + hDPSCs + FK506 group (p < 0.05). Also, there were statistically significant differences between the PEMF + hDPSCs and PEMF + hDPSCs + FK506 group against the PEMF only and PEMF + FK506 groups with respect to total fascicular area (p < 0.05). However, the difference was not significant when only PEMF and PEMF + hDPSCs groups were compared in axon number and density. As a result, the PEMF + hDPSCs + FK506 group showed higher axon number, density and total fascicular area than the other group. But, statistically significant differences were not noticed. In the PEMF + hDPSCs group, high total fascicular area and low total axon counts and densities were observed. The FK506-treated groups showed higher axon counts and densities.

The mean number of counted retrograde labeled neurons was higher in the PEMF + FK506 group, PEMF + hDPSCs group and PEMF + hDPSCs + FK506 groups (1148.83±327.59, 1174.33±79.44, and 1209.8±414.5) compared to the PEMF groups (899.68±295.01). All groups showed higher levels of sensory neurons in the DRG at 4 weeks postoperatively except the PEMF group. It was obvious that sensory neurons that have regenerated their axons through the crushing site were significantly augmented by the use of hDPSCs and FK506.
Quantitative RT-PCR showed higher expression of BDNF, TrkB, and p75NTR mRNA in the PEMF, PEMF + FK506, PEMF + hDPSCs, and PEMF + hDPSCs + FK506 group following crush injury. All experimental groups showed significantly higher expression compared with control group (Figure 9).

In terms of BDNF expression level (Figure 9-A and 9-B), all experimental groups showed higher expression levels than the control group. In the nerve segment, the expression level was higher than that in the control group. The PEMF group had increased expression levels from the first day, and the maximum was reached on the fourth day. In the PEMF group, the expression levels gradually decreased after the maximum was reached. DRG showed similar expression patterns compared with nerve segment in the PEMF group. In the PEMF + FK506 group, expression levels continued to decrease for 1 week in the nerve segment and DRG and then increased again at 2 weeks. In the PEMF + hDPSCs group, expression levels decreased gradually after 2 weeks. In PEMF + hDPSCs group, BDNF increased due to the differentiation of stem cells but expression levels decreased again after 2 weeks.

All groups showed increased TrkB level of expression compared to control (Figure 9-C and 9-D). In the nerve segment, the TrkB expression level gradually decreased in all experimental groups. But, the expression level was pronounced after 2 weeks in all groups. Especially, the expression level in the PEMF + hDPSCs + FK506 group remained high. But, the PEMF + FK506 group expression level
decreased gradually with time in the nerve segment and DRG. The expression levels in the PEMF + hDPSCs group expression levels slightly gradually increased over time. The PEMF group did not show a regular pattern. PEMF did not seem to affect the level of TrkB expression. However, the expression of TrkB associated with the expression pattern of the p75NTR should be considered.

The PEMF + hDPSCs group showed high expression of p75NTR (Figure 9-E and 9-F) and low expression of TrkB from day 1 to day 7. But, the expression level of p75NTR and TrkB was high after 2 weeks in the PEMF + hDPSCs group. In the PEMF + hDPSCs + FK506 group, the expression level of p75NTR was high the entire time. The PEMF + hDPSCs + FK506 group showed the highest expression patterns in all fields.
IV. Discussion

Our data showed that 15G and 30G PEMF groups showed progressive improvement in function, axon count and density. No complications or side effects were observed at the dose used in this study. Also, the 15G PEMF group showed earlier recovery patterns than the 30G PEMF group, although a statistically significant difference was not found. However, across all experiments, the 15G PEMF group showed the most improvement in nerve regeneration.

The major difficulty with PEMF as a potential approach to enhance nerve regeneration consists of selecting the optimal intensity and duration at the injury site and protecting against the toxicity of biodegradation mechanisms. The influence of low-frequency PEMF on peripheral nerve regeneration in experimental animals has been studied using a variety of protocols, with amplitudes generally between 0.3 and 300mT, and frequencies between 2 and 1,000Hz [45-49]. Some articles have shown that PEMF between 25Hz and 100Hz is most effective in stimulating bone regeneration [50]. However, other studies using in vitro models of chick embryo explants found that PEMF at lower frequencies was most effective, resulting in the use of pulsed electromagnetic fields between 0.5Hz and 3Hz for in vitro and in vivo studies [51]. Static electromagnetic fields have been tested on cultured cells [52] and animal preparations with varying measures of success. Previous studies using PEMF applications have reported profound effects on a large number of biological processes and have suggested that non-invasive PEMF can be effective and practical for clinical applications [53, 54]. Also, Sisken et
said the fields of high electromagnetic strength (45mT–90mT) with added NGF stimulate neurite outgrowth at levels comparable to the response obtained with low electromagnetic strength with added NGF. In contrast to Sisken et al, low intensity and duration in our study (1.5mT or 3mT and 60Hz, 2weeks and 4weeks) showed a pronounced effect on nerve regeneration. However, it is still unclear and controversial which intensity and duration are best for nerve regeneration. And, a high PEMF potentially toxic, risk estimation comprises identification of mechanisms of effect and evaluation of dose response. Therefore, we selected (1.5mT & 3mT and 60Hz) for PEMF conditions with hDPSCs and/or FK506 based on previous studies and our data.

Despite the clinical success of PEMF, which have been reported to have negative effects on the proliferation and differentiation of bone cells. Under specific PEMF conditions, it has been observed that the proliferation of osteoblasts is limited and that alkaline phosphatase (ALP) activity is elevated. Recent research in MG-63, ROS 17/2.8, and MLO-Y4 cell lines has shown that PEMF treatment either causes no effect or has a negative effect on cell proliferation. But, the mechanism of action underlying how PEMF promotes the formation of bone in an in vitro environment remains elusive. Similarly, its effects on nerve regeneration have not even been confirmed yet. Our experiments demonstrated the effect of PEMF on nerve regeneration although a detailed mechanism was not evaluated. But, PEMF is generally believed to affect cell survival and propagation according to the intensity, time interval and frequency.
PEMF can also induce changes in the cell cycle and propagation of exogenously delivered cells into the injury site as well as intrinsic cell sources. Among many potential stem cell sources for nerve regeneration, hDPSCs have received growing attention in recent years due to their ease of obtainment and propagations. hDPSCs are highly advantageous as potential sources for cell therapies because they are easily accessible and there are fewer ethical problems regarding their use, as long as donors provide informed consent. Furthermore, they are advantageous because of their proliferative capacity and ability to differentiate into other cell types. Notably, hDPSCs are a promising candidate source of proliferative cells and can be prepared from wisdom and premolar teeth extracted for orthodontic reasons, a major practical advantage for a wide range of clinical applications. Cells within the primary tooth pulp can promote epithelial cell differentiation toward an ameloblast phenotype, suggesting the potential use of this heterogeneous population of cells in cell-mediated enamel tissue engineering [60-62].

Li et al. [59] found that BMMSCs still have differentiation potential after PEMF treatment, especially in neuron-like cells. The change in cell cycle progression of BMMSCs under PEMF treatment might be attributed to the alteration of cell membrane potential resulting from PEMF exposure. Although our experiment did not reveal the mechanism of action, it is likely the same for BMMSCs and hDPSCs. Actually, Gronthos et al. [20] identified hDPSCs, displaying similar features to BMMSCs, with the ability to develop into odontoblasts, which are the cells that form the mineralized matrix of dentin. Adipogenic and chondrogenic
differentiation potentials of hDPSCs were weaker than those of BMMSCs [20, 63]. But, the neurogenicity of hDPSCs might be more potent than that of hBMMSCs due to their neural crest origin [64]. Therefore, hDPSCs would be an ideal material in nerve regeneration. Therefore, we focused on PEMF added hDPSCS rather than hBMMSCs for nerve regeneration.

PKH26-labeled hDPSCs were observed during the whole experimental period in our results (Figure 5). Unfortunately, cytoplasmic PKH26 labeling did not allow us to quantify survival of transplanted hDPSCs. But, PKH26 can be used to track the differentiated and non-dividing cells from BMSCs and adipocytes for many months [65]. PKH26-labelled donor cells were detectable at least up to 1 month after transplantation in the rat sciatic nerve. Various types of cells are easily loaded with PKH26, and no obvious cytotoxicity is observed [66]. However, a few PKH26-labeled hDPSCs were observed in the 3rd week, although the labeled cells were present during the whole experimental period. Coppe et al [67] reported that primary tooth dental pulp cells contain less than 2 percent stem cells. But, hDPSCs are easily obtained and propagated and could differentiate into many types of cell, especially neural cells [21]. Although hDPSCs might exert their supportive effect by providing an initial regenerative boost, which is less important in the subacute phase of nerve regeneration, new strategies need to be developed to improve stem cell survival and nerve regeneration mediated by hDPSCs. Furthermore, it is important to determine the amount of cells required by transplanted stem cells to exert their regenerative effect. In our study, we chose the injection concentration of
1.0x10^6/cell per rat, which is the same as that used by Erba et al.\cite{68}, who reported regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells.

Co-treatment with PEMF, hDPSCs and FK506 showed a synergistic effect accelerating the rat sciatic nerve regeneration especially in the early stage, although, surprisingly, PEMF with hDPSCs did not show a significant improvement. Karaoz et al\cite{22} showed that hDPSCs and hBMMSCs spontaneously produce high levels of TGF-β1 and VEGF, but not IL-10, TNF-α, and IFN-γ using ELISA. Stem cells may exert protection by the secretion of protective factors. But, high volume and protective factors induce ischemia and cell cytotoxicity. The negative effects of the PEMF + hDPSCs group may be due to ischemia and cell cytotoxicity. Also, recent studies have suggested that MSCs possess the dual ability to suppress or activate immune responses depending on the stimulus to which they were exposed. This fact would have affected the results of our experiments. Contrary to what was expected, PEMF with hDPSCs did not show a significant improvement. However, there are a number of possible reasons for this, which we discuss below.

In this experiment, the immunosuppressant FK506 was used to alleviate the immune response of hDPSCs and to accelerate the nerve regeneration. In fact, there are many reports of positive results regarding nerve regeneration in animals and humans immunosuppressed by FK506\cite{33,34}. Although previous studies found that FK506 is maximally effective when administered in high doses (5-10mg/kg/day) during the entire regeneration period in rat sciatic nerve models\cite{69}, prolonged
systemic immunosuppressant might not be justified for ensuring the success of nerve regeneration. It is therefore important to determine the optimal treatment dosage for FK506 after allograft transplantation. In addition to supporting previous findings, Wang et al. [69] examined the effect of low-dose FK506 on nerve regeneration in a model more applicable to the severe peripheral-nerve injuries seen in clinical settings and found that it was more feasible to reduce the high doses of FK506 accompanied with high rates of side effects for a non-vital indication. Therefore, in this study we choose the dose of (0.5mg/kg/day) based on side effects for a non-vital indication and nerve regeneration. In addition to supporting previous findings, we found that a low-dose of FK506 was more feasible than a high dose, which is accompanied by high rates of side effects for a non-vital indication [70].

FK506 is widely used in organ transplantation operations and is believed to cause immuno-suppression via a calcineurin-dependent mechanism [30]. FK506 has been noted to enhance the rate of axonal regeneration after crush injuries in rat sciatic nerves [28]. Positive results in nerve regeneration in animals and humans immune-suppressed by FK506 have been reported in various studies [29-34]. Although neurotoxic complications resulting from immune-suppressant therapy with FK506 have been noted in the CNS and the peripheral nervous system [27], no neurotoxic complications were observed our study. Our data showed that there were higher axon counts and densities in the FK506-injected groups. Compared with the PEMF + hDPSCs group, the PEMF + hDPSCs + FK506 group and PEMF + FK506 group
showed more regenerative results. This result may be due to suppressed neurotoxicity. But, with the passage of time, there was not a significant difference between the FK506-injected groups and the non-treated groups. FK506 seems to play an important role in early nerve regeneration.

Kapil et al. \cite{71} reported that axotomized motor neurons, within in just a few days, get surrounded by reactive microglial and astroglial cells. Activation of microglial cells can cause either cytotoxic or neuroprotective effects (or a combination of both), depending on the manner in which the activation is controlled. In most circumstances the cytotoxic state induces apoptotic neuron loss. Gold et al. \cite{28} first reported that systemic administration of FK506 accelerates functional recovery following a nerve crush lesion by increasing the rate of axonal regeneration in sciatic nerve in adult rats. Saxena et al. \cite{72} reported that FK506 caused early proliferation of microglia at 1 and 3 days after sciatic nerve injury. FK506 also significantly restricted transformation of these cells in to phagocytes. Following peripheral nerve damage the immediate central responses are proliferation and/or migration and activation of glial cells. FK506 promotes neurite outgrowth in cultures of rat DRG. The increased axon counts and the lower myelin debris observed in the FK506 group after 2 weeks indicate a faster Wallerian degeneration process and higher axon sprouting into the graft initially \cite{73}. But, it is less effective after a regeneration period of 6 weeks or later, when there was no significant difference between the normal and FK506-injected group. Whether this is related to a simple pruning mechanism of sprouts or down-regulation of regenerative
processes in the nerve due to possible neurotoxic effects of FK506 remains unknown \[74\]. Our experimental results were the same, although a longer period (4-6 weeks) was not examined in this experiment. FK506 showed a regenerative effect, especially in the early stage (within 2 weeks) in our experiment.

All experimental groups showed a higher expression level of BDNF than that in the control group, which would give a beneficial effect on nerve regeneration. The expression levels in PEMF increased from the first day to the fourth day, when the maximum was reached. Although FK506 helps to promote the nerve regeneration cycle after 2 weeks, it seems to be helpful within 1 week, and expression levels increased again at 2 weeks. All groups showed increased TrkB expression levels compared to the control levels. The increased expression of TrkB means that there is an increase in the incidence of nerve regeneration. In the nerve segment, TrkB expression was pronounced after 2 weeks in all groups. Especially, the high expression in the PEMF + hDPSCs + FK506 group was maintained. The expression levels in the PEMF + hDPSCs group gradually increased over time. However, the expression of TrkB associated with the expression of p75NTR should be considered. The PEMF + hDPSCs group showed a high expression of p75NTR and low expression of TrkB from 1 day to 7 days. Ibanez et al \[75\] reported that cells expressing the Low-Affinity Nerve Growth Factor Receptor (LNGFR or p75NTR) in the absence of Trk receptors may die rather than live in the presence of a neurotrophin. So, this result indicates that there may be apoptosis of neuronal cells. But, the expression level of p75NTR and TrkB was high after 2 weeks in the PEMF.
+ hDPSCs group. The expression level of p75NTR was high throughout the experiment in the PEMF + hDPSCs + FK506 group, and showed the highest expression in the PEMF + hDPSCs + FK506 group. This indicates that the combination of PEMF with hDPSCs and FK506 is beneficial for nerve regeneration. However, the fact that the results vary depending on the presence or absence of FK506, indicates that it appears critical.

This study was conducted to examine the benefits of using exogenously delivered hDPSCs with immune suppression and neuropromotion from FK506 combined with PEMF for peripheral nerve regeneration in comparison to a PEMF only modality. In our study, we observed improvement and a synergistic effect in the PEMF + hDPSCs + FK506 group. Although the exact mechanism was not identified in the present study, each modality (PEMF only, PEMF + hDPSCs, PEMF + FK506) provided some enhancement over control, and evidence of enhanced regeneration was not observed. The following three reasons may have contributed to our result. First, FK506 has solely been shown in experiments have an effect on nerve regeneration and PEMF, hDPSCs and FK506 have a synergistic effect because FK506 has been reported to increase the rate of axonal regeneration and to promotes neurite outgrowth \(^{27}\). Second, the efficacy of FK506 in the prevention of graft rejection following hDPSC injection into rat has been shown. But, the PEMF + hDPSCs group showed a slightly regenerative effect in comparison to control group (crush only) and the PEMF group, although there was not a statistically significant difference. If hDPSCs induced an immune response in
rat, the PEMF + hDPSCs group would have had a negative effect on nerve regeneration. So, immunosuppression of FK506 was not important in this experiment. Third, the use of FK506 to prevent an immune response may affect cell survival and propagation of hDPSCs. Effects of FK506 on T cell proliferation affect stem cell function significantly inhibiting SCF (stem cell factor)/c-kit-dependent MC (mast cell)/9 cell proliferation and survival \(^76\). The cyclic undecapeptide, cyclosporin A (CsA), and FK506, are powerful immunosuppressant. So, the use of an immunosuppressant other than FK506 would be helpful to evaluate interactions between hDPSCs and FK506. The precise mechanism of action between PEMF, hDPSCs and FK506 remains to be elucidated. The effects of FK506 on hDPSCs proliferation and survival, however, are of profound significance in nerve regeneration. Some researchers reported on the approach of combining two different modalities to enhance nerve regeneration. A definite explanation is lacking at this stage and further studies are required to search for possible mechanisms of interaction between PEMF, hDPSC and FK506. This experiment is meaningful in the evaluation of the PEMF, hDPSCs and FK506 on nerve regeneration.
V. Conclusions

Exposure to 15G PEMF during the early stage (0-2 weeks) after nerve damage seems to have more practical significance compared with 15G with a longer exposure (4 weeks), or 30G with a shorter (2 weeks) or longer exposure (4 weeks). Histomorphometrically PEMF with hDPSCs and FK506 showed a co-treatment effect accelerating the rat sciatic nerve regeneration, while PEMF with hDPSCs did not show a significant improvement effect. We conclude that 15G PEMF co-treated with hDPSCs and FK506 during early stage of damage revealed effective nerve regeneration of crush-injured sciatic nerve in rats.
References

2. Kilmer SL, Carlsen RC. 1987. Chronic infusion of agents that increase cyclic AMP concentration enhances the regeneration of mammalian peripheral nerves in vivo. Exp Neurol. 95(2)357-367.
12. Freeman JA, Manis PB, Snipes GJ, Mayes BN, Samson PC, Wikswo JP, Jr,


33. Snyder AK, Fox IK, Nichols CM, Rickman SR, Hunter DA, Tung TH,
43. Geremia NM, Gordon T, Brushart TM, Al-Majed AA, Verge VM. 2007. Electrical stimulation promotes sensory neuron regeneration and growth-


Figures and Figure Legends

Fig 1. Schematic diagram of sciatic nerve crush injury model. A standard surgical needle holder engaged to the second clip of both arms was used to create a crushing injury at a distance about 12mm distal to the sciatic furcation. The nerve was clamped for 30 seconds to produce a crush-injury of 3mm in width.

Fig 2. Overview of the PEMF device and Helmholtz coil structure. PEMF was generated in a pair of Helmholtz coil of 60 cm diameter and 30 cm distance. The coils were placed in a 90 × 90 × 50 cm sized Faraday cage in order to prevent environmental electromagnetic interaction. PEMF was delivered with pulse trains at 60 Hz frequency and an intensity of 15 or 30 Gauss.
Fig 3. Graph of weekly sciatic function index (SFI) of the each group. Superior recovery was noticed in the PEMF 15G group by the end of the follow-up period.

A. Graph of SFI in order to determine the optimal PEMF condition. Exposure to 15G PEMF during early stage (0-2 weeks) after the nerve damage seemed to be more helpful (practical significance) compared to 15G with longer exposure (4 weeks) or 30G with early (2 weeks) or longer exposure (4 weeks).

B. Graph of SFI within 2 weeks. Superior recovery was noticed in PEMF 15G group by the end of the two weeks postoperatively.
Fig 4. Histomorphometric results in control, 15G2weeks and 30G2weeks group. Higher axon counts (A) were seen in 15G2W group and axon densities (B) were seen in all group. All values are presented as mean ± SEM.

A. Total axon numbers at different PEMF amplitudes.

B. Total axon density at different PEMF amplitudes.
Fig 5. **Cell tracking with PKH26 labeling.** hDPSCs with PKH26 were observed during the experimental period (Scale bar=200μm)

![Cell tracking images](image)

Fig 6. **Graph of SFI in order to evaluate the co-treatment effect.** One week postoperatively, control (only crush group) and all experimental groups decreased sharply in SFI as a result of the axonotmesis and consequent loss of function. Two weeks postoperatively, PEMF + FK506, PEMF + hDPSCs group and control group showing a similar degree of slope showed. But, PEMF and PEMF + hDPSCs + FK506 group showed a sharp increase at 2 weeks (* represents p<0.05 PEMF group and PEMF + hDPSCs + FK506 group compared with other groups)

![Graph of SFI](image)

- Control
- PEMF
- PEMF+FK506
- PEMF+hDPSCs
- PEMF+hDPSCs+FK506
Fig 7. Histomorphometric results in all experimental groups. Significantly higher axon counts (A) and axon densities (B) were seen in the PEMF + FK506 group and PEMF + hDPSCs + FK506 group, indicating a better histomorphometric recovery. All values are presented as mean ± SEM.

A. Total axon number at PEMF co-treatment with hDPSCs and FK506 (* represents p<0.05. PEMF vs PEMF+FK506, PEMF vs PEMF+hDPSCs+FK506, PEMF+hDPSCs vs PEMF+FK506, PEMF+hDPSCs vs PEMF+hDPSCs+FK506)

B. Total axon density at PEMF co-treatment with hDPSCs and FK506 (* represents p<0.05. PEMF vs PEMF+FK506, PEMF vs PEMF+hDPSCs+FK506, PEMF+hDPSCs vs PEMF+FK506, PEMF+hDPSCs vs PEMF+hDPSCs+FK506)
Fig 8. FG (Fluorogold) labeled DRG (Dorsal root ganglia) neurons. Co-treatment of hDPSCs and FK506 significantly augmented regeneration of the sensory neurons at the crush site (* represents p<0.05. PEMF vs PEMF+FK506, PEMF vs PEMF+hDPSCs, PEMF vs PEMF+hDPSCs+Fk506).

Fig 9. Expression levels of BDNF, TrkB, and p75NTR in RT-PCR. To follow-up the molecular changes caused by PEMF added with hDPSCs and/or FK506, the expression of endogenous BDNF, its tyrosine receptor kinase B (TrkB) and p75NTR in L4, L5 dorsal root ganglia (DRG) neurons and nerve segments were evaluated RT-PCR after 1, 2, 4, 7 and 14 days postoperatively. Higher expression of BDNF, TrkB and p75NTR are seen in all experimental groups. Expression levels are normalized to GAPDH.

A. BDNF expression levels in DRG.
B. BDNF expression levels in nerve segment.

C. TrkB expression levels in DRG.

D. TrkB expression levels in nerve segment.
E. p75NTR expression levels in DRG.

F. p75NTR expression levels in nerve segment.
Tables

Table 1. Total axon counts and densities in order to determine the optimal stimulus condition of PEMF intensity and duration. Higher axon counts were seen in 15G2W group. All values are presented as mean ± SEM. No significant difference was observed in comparison to the control groups, and both experimental groups showed no differences in axon densities.

<table>
<thead>
<tr>
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<th>Total Axon Counts</th>
<th>Axon Density (fibers/mm²)</th>
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<tbody>
<tr>
<td>Control</td>
<td>9842.74 ± 970.67</td>
<td>17788.78 ± 3040.46</td>
</tr>
<tr>
<td>15Gauss 2Weeks</td>
<td>12529.96 ± 4034.18</td>
<td>17774.62 ± 2786.25</td>
</tr>
<tr>
<td>30Gauss 2Weeks</td>
<td>11123.15 ± 2121.01</td>
<td>17257.22 ± 2695.38</td>
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Table 2. Total axon counts and densities in order to evaluate the co-treatment effect. Significantly higher axon counts and axon densities were seen in the PEMF + FK506 group and PEMF + hDPSCs + FK506 group, indicating a better histomorphometric recovery. All values are presented as mean ± SEM. (* represents p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Total Axon Counts</th>
<th>Axon Density (fibers/mm²)</th>
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<tbody>
<tr>
<td>PEMF</td>
<td>10226.02 ± 907.87</td>
<td>17208.92 ± 2758.04</td>
</tr>
<tr>
<td>PEMF + FK506</td>
<td>18015.37 ± 16734.86*</td>
<td>26085.57 ± 23918.46*</td>
</tr>
<tr>
<td>PEMF + hDPSCs</td>
<td>12163.15 ± 1605.88</td>
<td>15391.37 ± 3169.74</td>
</tr>
<tr>
<td>PEMF + hDPSCs + FK506</td>
<td>17929.12 ± 2545.57*</td>
<td>22315.77 ± 1455.22*</td>
</tr>
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