치의학박사 학위논문

Regeneration of crush-injured mental nerve using Schwann-like cells derived from dental pulp stem cells combined with pulsed electromagnetic field

전자기장과 치수줄기세포에서 분화된 슈반형세포를 이용한 압박손상 턱신경 재생

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서울대학교 대학원

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Wei-Hong Hei의 박사 학위논문을 인준함

2015 년 7 월

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Abstract

Regeneration of crush-injured mental nerve using Schwann-like cells derived from dental pulp stem cells combined with pulsed electromagnetic field

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Background

Peripheral nerve injury is relatively common in clinical situation which may result in serious disturbance such as dysesthesia, persistent paresthesia and pain. Various techniques have been investigated to enhance peripheral nerve regeneration including the application of pulsed electromagnetic field (PEMF) and administration of Schwann cells from mesenchymal stem cells as the potential sources.

Purpose of study

The purpose of study was firstly to clarify the influence of frequency and duration of PEMF and find the suitable condition of PEMF on the peripheral nerve regeneration; and secondly to investigate the effects of combining PEMF and local injection of Schwann-like cells, in comparison

with each single modality, on peripheral nerve regeneration in a rat model with crush-injured mental nerve.

Materials & Methods

The experiments were divided into two parts, part I and part II.

Part I: Immortalized rat Schwann cells (iSCs) ($1x10^2$/well) were exposed at 4 different conditions (50 Hz 1 Hr/day, 50 Hz 12 Hr/day, 150 Hz 1 Hr/day and 150 Hz 12 Hr/day). Cell proliferation and mRNA expression of S100 and BDNF (brain-derived neurotrophic factor) were analyzed. Sprague-Dawley rats (200–250 g) were divided into 6 groups (n=10 each): Control, Sham, 50 Hz 1 Hr/day, 50 Hz 12 Hr/day, 150 Hz 1 Hr/day and 150 Hz 12 Hr/day groups. Mental nerve was crush-injured and exposed at condition of PEMF for 3 weeks. Nerve regeneration was evaluated with functional test, histomorphometry and retrograde labeling of trigeminal ganglion (TG).

Part II: Schwann-like cells derived from human dental pulp stem cells (hDPSCs) (1×10^5 cells per well) were used. Integrin β4 (CD104), S100, glial fibrillary acidic protein (GFAP), laminin, $P75^{NTR}$ were detected with Immunocytochemistry. $P75^{NTR}$ and S100 mRNA were analyzed using Quantitative Real-time polymerase chain reaction (qRT-PCR); Male Sprague-Dawley rats (200-250 g, six-week-old) were divided into 7 groups (n=10 each): Control, Sham, PEMF, hDPSCs, hDPSCs + PEMF, Schwann-like cells, Schwann-like cells + PEMF group. Cells were transplanted (106 cells/6μl) after crush injury with or without PEMF exposure. Outcomes of nerve regeneration were evaluated with functional test, histomorphometry and retrograde labeled neurons.

Statistical analyses were performed using SPSS program version 21 (SPSS Inc., Chicago, IL, USA). All data were presented as mean values with standard error means (SEM). The normal distribution of the measurements was verified by One-Sample Kolmogorov-Smirnov normality test. One-Way ANOVA test (axon counts, labeled neurons result) and Repeated Measure ANOVA test

(sensory test, MTT and qRT-PCR results) followed by post-hoc Tukey test was used to compare data between different groups. P value ≤.05 was considered statistically significant.

Results

Part I: iSCs proliferation with 50 Hz, 1 Hr/day was increased from 4th to 7th day; mRNA expression of S100 and BDNF was significantly increased at the same condition from first week to third week ($p<.05$, vs. control); Difference score was increased at the second and third week, and Gap score was improved at the third week under 50 Hz 1 Hr/day PEMF compared with control while other conditions showed no statistical significance. Axon counts and the number of retrograde labeled neurons were significantly increased under PEMF of four different conditions compared with control. Although there was no statistical difference, 50 Hz, 1 Hr/day PEMF showed highest regeneration ability than other conditions.

Part II: Schwann-like cells differentiated from hDPSCs expressed CD104, S100, GFAP (glial fibrillary acidic protein), laminin, $P75^{NTR}$ (P75 neurotrophin receptor). $P75^{NTR}$ and S100 mRNA expression was the highest in Schwann-like cells exposed with PEMF. Difference and Gap scores were increased significantly at the first and third week than control while other treatment groups showed no significance. Axon and retrograde labeled neuron counts were obviously increased in all interference groups. Schwann-like cells + PEMF group showed the highest ability and significances were found when compared with PEMF (P=.033, axon count; P=.035, retrograde labeling); hDPSCs (P=.042, axon count) and Schwann-like cells group (P=.038, axon count).

Conclusion

1. PEMF enhanced peripheral nerve regeneration, and that it may be due to cell proliferation and increase in BDNF and S100 gene expression. Among the various conditions, 50 Hz, 1 Hr was revealed to be the most suitable inferred from the data of in Vitro and in Vivo study.

2. Schwann-like cells and hDPSCs with or without PEMF in addition to PEMF alone improved peripheral nerve regeneration after crush injury. Among these, the Schwann-like cells combined with PEMF showed the highest regeneration ability. PEMF had a synergic effect on the ability for hDPSCs and Schwann-like cells to regenerate nerves in Vitro and, after transplantation, in Vivo.

Keywords pulsed electromagnetic fields (PEMF), peripheral nerve regeneration, crush injury of nerve, Schwann-like cell, human dental pulp stem cell (hDPSCs), mental nerve

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Contents

I. Introduction

Peripheral nerve injury is relatively common in clinical situation which may result in serious disturbance such as dysesthesia, persistent paresthesia and pain [1]. Peripheral nerve regeneration may benefit from pulsed electromagnetic field (PEMF) stimulation with unique advantage of non-invasive application [2-4].

PEMF therapy, was evolved from in vivo studies on bone healing, which was also advocated as a potential candidate to promote peripheral nerve regeneration [5]. It has been shown to stimulate both peripheral and central nerve regeneration in Vivo and in Vitro experiments [6-11]. PEMF exposure was reported to differentiate bone marrow mesenchymal stem cells (BMMSCs) into neuron-like cells by affecting the cell cycle [12]. Previous study reported that PEMF improved the functional recovery and increased number and diameter of regenerating axons following transection and re-approximation of the rat sciatic nerve [13,14]. Although there is a contradictory result indicating that the exposure to PEMF did not influence nerve regeneration [15-17]; PEMF, which had biological windows, was generally believed to affect cell survival, propagation and peripheral nerve regeneration according to the intensity, time interval and frequency [18-25].

However, the influence of PEMF on peripheral nerve regeneration has been studied using varieties of protocols, with intensity generally between 0.3 to 300 mT [18-

25], frequencies from 2 to 2000 Hz [18-25], time between 10 minutes/day and 24 Hr/day [18-27]; which made it difficult to choose in clinical treatment. Among them, previous study reported that one of the biological "windows" was 0.5-2 mT [20] and also 1 mT was suitable in others' study [28-30], and it was reported that 50 Hz, 150 Hz played positive roles [26, 27, 31-35]; what's more, 1 Hr/day, 12 Hr/day was suitable exposure time in many protocols [27, 36-38]. The most effective, shortest and exact parameters were needed in clinical treatment. Based on above previous data, we selected short time (1 Hr/day) and low frequency (50 Hz) as experiment conditions, and long time (12 Hr/day) and higher frequency (150 Hz) as control; and set intensity at 1 mT.

Schwann cells, a main contributor of endogenous repair after injury, played a pivotal role in peripheral nerve regeneration, making them an attractive therapeutic target [39]. They not only reconstitute myelin, which was essential for fast neural action potential propagation, but also provided physical guidance (bands of Bungner) and trophic support for axonal regeneration [39, 40]. However, autologous Schwann cells had limited clinical application. The requirement for nerve donor material evoked additional morbidity and the time required to culture and expand the cells would delays treatment [41].

There has been a substantial amount of research that focuses on deriving Schwann-like cells from multi-potent stem cells such as bone marrow stromal cells

(BMSCs) [42], Adipose-derived stem cells (ADSCs) [41, 43] and human dental pulp stem cells (hDPSCs) [44]. Isolation of BMSCs and ADSCs needed surgical procedure to procure tissues such as bone marrow aspiration process and liposuction process, which was invasive and painful for patients.

hDPSCs, multi-potent stem cells in pulp, not only expanded well in culture [45, 46] but also could differentiated toward Schwann-like cells [47]; But, unlike primary Schwann cells, Schwann-like cells were not sustainable and would revert back to their primitive states upon withdrawal of the cocktail of extrinsic induction factors from culture [48].

PEMF (pulsed electromagnetic field) was reported that it was helpful for mesenchymal stem cell proliferation, differentiation in vitro [49-51] and protective towards apoptosis in a neuroblastoma cell line [52]. In addition, it could promote neurite outgrowth in the dopaminergic MN9D and PC12 cell line [53, 54].

The purpose of this study was to clarify which frequency and duration of PEMF was more suitable for the peripheral nerve regeneration using Schwann cell line and rat mental nerve crush model firstly. And then to explore the effect of Schwann-like cells (derived from hDPSCs) combined with PEMF on peripheral (mental) nerve regeneration after crush-injury.

II. Experiment body (Part I & Part II)

Part I: Effects of Electromagnetic Field (PEMF) Exposure at Different Frequencies and Durations on the Peripheral Nerve Regeneration: in Vitro and in Vivo Study

1. Materials and Methods

1.1. Pulsed electromagnetic field (PEMF) exposure

PEMF device with two identical Helmholtz coils of 30 cm diameter and 15 cm distance was placed in the incubator for in vitro study (Fig. 1). The density was set to 1 mT, four different parameters (50 Hz, 1 Hr; 50 Hz, 12 Hr; 150 Hz, 1 Hr; 150 Hz, 12 Hr) were used as the exposure hertz (Hz) and duration every day (Hr.). PEMF in vivo study was generated with a pair of Helmholtz coils of 60 cm diameter and the distance of two coils kept 30 cm, which was same as described before [3].

1.2. Cell line

Immortalized rat Schwann cell line (iSC) [55] was obtained from professor Sung Joong Lee's lab, Department of Oral Physiology, Seoul National University, they were grown and maintained in a-MEM (alpha modified minimal essential medium, Gibco BRL, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Carlsbad, California, USA) and antibiotics (Penicillin-G 100 U/ml, streptomycin 100 mg/ml, fungizone 2.5 mg/ml, Gibco BRL) at 37℃ in a 5% CO_2 humidified atmosphere. Culture medium was replaced every 3 days.

1.3. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay for cell proliferation

MTT was performed as described previously [56]. Briefly, cells in different groups were exposed according to PEMF parameter. There was no exposure for control group. The proliferation of iSCs was evaluated by MTT assay. Briefly, iSCs were seeded into 96-well plates at a density of 1×10^2 cells/well and cultured. The culture medium was removed on the 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} and 7^{th} day respectively. After washing with PBS, 20 μL of MTT (5 mg/ml) was added to each well and incubated for 4 hours at 37°C. After removing the MTT solution, the converted dye was dissolved in Me_2SO and measured by reading the absorbance at a wavelength of 540 nm with a microplate reader (Multiskan

EX; Thermo, Seoul, Korea). Triplicate samples were analyzed from three independent experiments.

1.4. Quantitative real-time polymerase chain reaction (qRT-PCR) for S100, BDNF

Quantitative real-time polymerase chain reaction (qRT-PCR) was done as described before [3]. Briefly, to identify whether PEMF expose could impact iSC marker gene expression, the expression of S100, endogenous brain-derived neurotrophic factor (BDNF) were tested in 0 week, 1st week, 2nd week and 3rd week post-exposure. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantification was used as an internal control for normalization. The percentage differences in mRNA levels over control values were calculated using ΔCt method as described previously (Applied Biosystems Manual, Foster City, CA, USA) according to Chen et al [57].

1.5. Surgical procedures and grouping design

Sixty Sprague-Dawley rats (males, 6 weeks-old, 200-250 g) were used in this experiment; about 3 mm crush injury was made at a distance about 2 mm distal to the mental foramen. All the animal surgery and experimental procedures were carried out in accordance under the care guidelines of the laboratory animal resources of Seoul National University, Republic of Korea. Animals were anesthetized with an intraperitoneal

injection of a 4:1 mixture of ketamine HCl and xylazine hydrochloride. A submandibular skin incision was made and the left mental nerve was exposed to the mental foramen. A standard surgical needle holder engaged to the second clip of both arms was used to create a crushing injury at a distance about 10 mm distal to the mental foramen. The nerve was clamped for 30 seconds to produce a crush-injury of 3 mm in width. The injury site was then marked by introducing a single 9-0 Nylon (Ethicon®, UK) epineural stitch under 16 X magnification using a surgical microscope (Carl Zeiss, Germany) at the distal limit of the injury for later identification.

Rats were divided into 6 groups (n=10 each): sham, control and experimental group which was sub-grouped into E1 group (50 Hz, 1 Hr), E2 group (50 Hz, 12 Hr), E3 group (150 Hz, 1 Hr) and E4 group (150 Hz, 12 Hr). In sham group, the mental nerve was exposed and closed without any injury or treatment. The mental nerve of control group was crush-injured without exposure to the PEMF. In experiment group (E1-E4), mental nerve was crush-injured and received PEMF treatment of different frequencies and durations for 3 weeks. The mental nerve of control group was crush-injured without exposure to the PEMF. In experiment group (E1-E4), mental nerve was crush-injured and received PEMF treatment of different frequencies and duration for 3 weeks.

1.6. Sensory test

Sensory test (Fig. 5) was performed and scores were calculated according to Seino et al [58]. Briefly, a series of von Frey filaments (n=20 in a set) (Semmes-Weinstein monofilaments, North Coast Medical, Inc., Arcata, CA, USA) used to compare the behavioral response sensitivities to mechanical stimulation. The difference score was defined as the difference between the mechanical touch thresholds (grams) of the ipsilateral and contralateral sides of the injury, and calculated as the value of the ipsilateral mental area (b) minus the value of the contralateral area (d). The gap score was defined as the difference between the mechanical touch thresholds of the medial and distal parts of the mental nerve, and calculated as the value of the ipsilateral lip area (a) minus the value in the vicinity of the mental foramen (b). By definition, high score means poor recovery. As the damaged nerve recovers functionally, then the scores approach "score zero". A series of von Frey filaments (Semmes-Weinstein Monofilaments, North Coast Medical, Inc., CA, USA) were used to determine pain sensitivity to mechanical stimulation. Von Frey filaments (bending force; 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, and 6.0 g) were delivered from above to the center of the whisker pad. Head withdrawal, touching or scratching the facial regions upon von Frey filament applications was considered as positive pain response. A negative response was defined as a lack of two sequential withdrawal responses elicited by three sequential

stimulations. Statistical comparisons of differences in the mean scores at post-operative weeks 1, 2 and 3 within each group were recorded.

1.7. Retrograde labeling and quantification of sensory neurons

Trigeminal ganglion (TG) neurons were retrograde labeled with a fluorescent dye, DiI (Molecular Probes, OR, USA). The mental nerves were cut distally to the crush-injured lesion at 4 weeks postoperatively, and DiI was placed onto the distal end of the transected mental nerve, sutured and the rats were allowed to recover. Five days later, the animals were deeply anesthetized and perfused with saline and paraformaldehyde (PFA) solutions according to Savignat et al [59]. After craniotomy, the ipsilateral trigeminal ganglia were removed and post-fixed overnight with the same solution of PFA. The ganglia were immersed in a 20% sucrose solution for 2 days, then embedded in Tissue Tek (Sakura, Japan) and frozen in liquid nitrogen. Serial 35 um longitudinal sections were made at – 20°C in a cryostat microtome (Leica CM30505 Cryostat, Italy). Sections were then observed under a fluorescence microscope equipped with a rhodamine filter (Olympus FV-300, Japan). Those sections obtained from the top and bottom of the TG and those showing sign of technical or processing errors were excluded from counting. The number of DiI back-labeled neurons in each TG was counted for each animal and the means were then compared among the groups. The

labeled neurons at each TG section were randomly selected and their area (soma size of neuron) was measured and averaged with computer software (OPTIMAS Ver. 6.5, Japan) [59,60].

1.8. Histomorphometric evaluation

At the end of the 4 weeks follow-up period, 6 rats from each group were anesthetized. Mental nerve was exposed again and the nerve segment including the crush-injury site was harvested. 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 (5 mm 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 1 μm thickness were cut with an 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 simplifying purpose of axon counting, the total cross-sectional area of the nerve was measured at 40 x (Olympus, BX41, TF, Japan). And three sampling fields were then randomly selected at 200 x magnification using a protocol previously reported [61,62].

2. Statistical analysis

The statistical analyses were performed using SPSS program version 21 (SPSS Inc., Chicago, IL, USA). All data were presented as mean values with standard error of mean. The normal distribution of the measurements was verified by One-Sample Kolmogorov-Smirnov normality test. One-Way ANOVA test (axon counts, labeled neurons result) and Repeated Measure ANOVA test (sensory test, MTT and qRT-PCR results) followed by post-hoc Tukey test was used to compare data between different groups. P values ≤.05 were considered statistically significant.

3. Results

3.1. Effect of PEMF exposure on the proliferation of iSCs

To determine whether different parameters of PEMF exposure effect the proliferation of iSCs, cell proliferation was assessed and compared among different groups. The absorbance value at a wavelength of 540 nm showed that cell proliferation (iSCs) was increased significantly at 4^{th} (p=.024; vs. control), 5^{th} (p=.041; vs. control), 6^{th} (p=.036; vs. control), 7^{th} day (p=.042; vs. control) in 50 Hz 1 Hr group than control. The proliferation was increased in other groups, however, there was no statistical meaning compared with control (Fig. 2).

3.2. Effect of PEMF exposure on gene expression in Vitro

qRT-PCR analysis after exposing iSCs in PEMF with different exposed parameters for 0, 1^{st}, 2^{nd}, 3^{rd} weeks was done to determine whether PEMF exposure could impact nerve regeneration related gene expression. The expression of S100 and BDNF mRNA were increased in all groups with the time elapsed, and there was statistical difference at 1^{st} (p=.02; vs. control); 2^{nd} (p=.021; vs. control) and 3^{rd} (p=.005; vs. control) week for S100 mRNA in 50 Hz 1 Hr group (Fig. 3); in addition, there was significant increase in 50 Hz 1 Hr group compared with control at 1^{st} (p=.03); 2^{nd} (p=.041) and 3^{rd} (p=.046) week for

BDNF mRNA (Fig. 4).

3.3. Functional recovery after PEMF exposure

The Difference score (Fig. 6) was same on 0 week which was done before the day of surgery. Then it significantly increased because of the crush injury on the 1st week, with time lapsed, all the groups decreased and there was statistically significant on the 2nd week (p=.045; vs. control) and 3rd week (p=.032; vs. control) in 50 Hz 1 Hr group.

As for the Gap score (Fig. 7), the change was similar with the Difference scores. Data of all the groups on 0 week was same, which was got before the day of surgery. Then it increased because of the crush injury on the 1st week. There was statistical significance on the 3rd week in 50 Hz 1 Hr group (p=.031; vs. control).

3.4. Effect of PEMF exposure on the axonal regeneration of mental nerve in Vivo

Photomicrographs of histologic features in semi-thin sections at distal to the crush injury site was shown (Fig. 8). Total axon counts were shown in Table 1. With 3-week exposure, myelinated axons were increased in all experimental groups and 50 Hz 1 Hr group showed the biggest axons number than other groups. There was statistical meaning in 50 Hz 1 Hr group (p=.036; vs. control), 50 Hz 12 Hr group (p=.034; vs. control), 150 Hz 1 Hr group (p=.041; vs. control), 150 Hz 12 Hr group (p=.045; vs.

control), there was no significance than sham group in all experimental groups (Fig. 9).

3.5. Effect of PEMF exposure evaluated with retrograde labeling

Fluorescence photomicrographs from TG (trigeminal ganglion) in different group was shown (Fig. 10). The mean value of retrograde labeled neurons and statistical statistical result was shown in table 1. With 3 week recovery period, the positive trigeminal ganglion axons in control group, 50 Hz 1 Hr group (p=.046; vs. control), 50 Hz 12 Hr group (p=.034; vs. control), 150 Hz 1 Hr group (p=.026; vs. control) and 150 Hz 12 Hr group (p=.019; vs. control) were significant increased, there was no significance than sham group in all experimental group (Fig. 11).

4. Discussion

In this study, all the different conditions of PEMF showed the potential of improving the nerve regeneration judged by in vitro and in vivo test including immortalized Schwann cell proliferation and relative genes (S100 and BDNF) expression, function recovery, axon and retrograde labeled neuron count; 50 Hz 1 Hr group showed higher regeneration ability than other groups, which resulted in beneficial effect while ensuring compliance from patients and health care providers.

During last decades, exposure of magnetic fields for nerve regeneration had received considerable interests and various protocols were applied. It was reported that the stimulatory effect of magnetic fields on regeneration of the rat sciatic nerve was frequency dependent from 2 Hz to 2000 Hz [18-25]; Also exposure times from 10 min/day to 24 Hr/day were reported to be useful [18-27]; In addition, amplitudes were detected between 0.3 to 300 mT [18-25], however the exact condition was not clear for the clinical use, so we selected two exposure time (short 1 Hr/day, long 12 Hr/day) and two frequencies (low 50 Hz, high 150 Hz) based on previous reports as our protocol and also set PEMF density at 1 mT [28-30]. Finally, 50 Hz, 1 Hr/day 1 mT was proved as the most ideal one among these 4 different conditions; which was suitability for a clinical setting, saving both time and costs comparing with long time exposure.

PEMF multifaceted effects were also reported in molecular level. Longo et al [63]

reported that electromagnetic field influenced NGF activity and levels following sciatic nerve transection. Previous study reported that pulsed electromagnetic fields decreased pro-inflammatory cytokine secretion (IL-1β and TNF-α) on human fibroblast-like cell culture [26]. It was descripted that pulsed electromagnetic fields induced peripheral nerve regeneration and endplate enzymatic changes [64]. What's more, PC12 cells had been widely used in both neurobiological and neurotoxicological studies. It was reported that the effects of flux density and frequency of pulsed electromagnetic field could impact PC12 cell neurite outgrowth [53]; also, PEMF had the positive effective on the osteogenesis of MC3T3-E1 Cells [65], Human Dental Pulp Stromal Cells [3], steoprogenitor cells [66], human MG-63 osteosarcoma cell line and human osteoblast-like cells [67]. However, Schwann cells were few studied yet under PEMF exposure; the effect of PEMF to iSCs proliferation was explored herein. Interestingly, positive result was got, the reason of it may due to its function to the ion channels in cell membrane. A paper published by Panagopoulos [68] suggested a hypothesis where by the externally applied electromagnetic field caused the ions to vibrate and when this vibration reaches a critical point, this gave a false signal to the voltage gated channels present in the membranes of eukaryotic cells. Once the voltage gated channel received a false signal, the gate might be forced to either open or perhaps close but theoretically affecting the physiology of the cell [68].

PEMF treatment increased iSCs mRNA expression of S100 and BDNF. Previous study demonstrated that PEMF exposure improved BDNF expression in vivo both in nerve segment and DRG (dorsal root ganglia) [3]. Another paper reviewed that electromagnetic fields increased in vitro and in vivo angiogenesis through endothelial release of FGF-2 [69], Pulsed electromagnetic field enhanced brain-derived neurotrophic factor (BDNF) expression through L-type voltage-gated calcium channel-and Erk-dependent signaling pathways in neonatal rat dorsal root ganglion neurons [2]. S100, "classic" Schwann cell marker, which stimulated and induced axonal regeneration [48], was present in developing chicken neurons and Schwann cells promoting motor neuron survival in vivo [70]. S100 was Ca^{2+}-binding proteins [71], which was located and played possible role in the snail nervous system [72] and stimulated glial cell proliferation [73]. One of the mechanisms for nerve regeneration of PEMF was Ca^{2+} channel change [17, 74, 75]. So herein, PEMF could regulate Ca^{2+} expression and promote binding with S100 protein, which had positive role to Schwann cell proliferation and nerve regeneration.

The remarkable point of this study was PEMF of short exposure time and lower frequency (50 Hz 1 Hr) at 1 mT can be a suitable condition for the crush-injured sensory nerve regeneration and can be used in the clinical situation. However, there were some limits in this study. A few frequencies, exposure time and only one density in 1mT was used; various intensities, frequencies and exposure time from low to high have to be

tested to generalize our findings in the future.

Part II. Schwann-like Cells differentiated from human dental pulp stem cells (hDPSCs) combined with Pulsed Electromagnetic field (PEMF) can improve Peripheral Nerve Regeneration

1. Materials and Methods

1.1. In vitro study

1.1.1. Isolation and differentiation of hDPSCs into Schwann-like cells.

Human third molars from 10 adults (18–22 years of age) were collected at the Seoul National University Dental Hospital (Seoul, Korea), the experimental protocol was approved by the Institutional Review Board and informed consent was obtained. Human dental pulp stem cells (hDPSCs) were isolated and cultured as described previously [47, 76].

At passage 2, Schwann-like cell differentiation was done as previously [47, 77]. hDPSCs were incubated with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Seoul, South Korea) containing 1 mM beta-mercaptoethanol (β-ME, Sigma-Aldrich, Seoul, South Korea), then replaced with media consisting of DMEM, 10% fetal bovine serum (FBS, Sigma-Aldrich, Seoul, South Korea) and 35 ng/mL all-trans-retinoic acid (RA, Sigma-Aldrich, Seoul, South Korea). Then, cells were transferred to DMEM containing 10% FBS, 5 μM forskolin (FSK, Sigma-Aldrich, Seoul, South Korea), 10 ng/mL recombinant human

basic fibroblast growth factor (b-FGF, Sigma-Aldrich, Seoul, South Korea), 5 ng/mL recombinant human platelet-derived growth factor-AA (PDGF, Sigma-Aldrich, Seoul, South Korea) and 200 ng/mL recombinant human heregulin-beta 1 (HRG, Immunotools, Friesoythe, Germany) and cultured for 6 days.

1.1.2. Schwann cell character analysis: Immunocytochemistry

To examine the stemness and origination of hDPSCs in the whole pulp cells, immunostaining was performed with STRO-1 primary antibodies (R&D Systems, Minneapolis, MN) and Nestin (Millipore, Billerica, MA) as previously described. Pulp cells were fixed with 4% paraformaldehyde, incubated primary antibodies and fluorescent-labeled secondary anti-mouse antibody (Invitrogen overnight at 4℃ with, Carlsbad, CA, USA). A similar process was performed to analyze glial makers. CD104 (Abcam, Cambridge, England), S100 (DAKO, Carpinteria, California), GFAP (Chemicon, Temecula, California, USA), laminin (Abcam, Cambridge, England) and $P75^{NTR}$ (Dakocytomation, Glostrup, Denmark) were used as the primary antibody. Alexa 488-conjugated anti-rabbit IgG, anti-mouse IgG were used as secondary antibodies (Molecular Probes, Invitrogen, Eugene, OR). To identify cell nuclei, 40, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Seoul, South Korea) was used (1:1,000 dilution). The cells were then visualized using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

1.1.3. S100 and P75^{NTR} mRNA expression change of schwann-like cells treated with PEMF

Real-time polymerase chain reaction (qRT-PCR) was done as described before [3]. Briefly, to identify PEMF effect to Schwann-like cell differentiation. S100 and P75 mRNA expression were quantified at the 0 week, 1st week, 2nd week and 3rd week post-PEMF exposure. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantification was used as an internal control for normalization. The percentage differences in mRNA levels over control values were calculated using ΔCt method as described previously (Applied Biosystems Manual, Foster City, California, USA) according to Chen et al [57].

1.2. In Vivo study

1.2.1. Evaluation of cell transplantation using cell tracking with PKH26

In order to trace the survival of transplanted hDPSCs and Schwann-like cells, target cells were labeled with PKH26 (Sigma-Aldrich, Seoul, South Korea) (1 × 10^5/μl in PBS) according to the manufacturer's protocol. Labeled cells were injected into the crushed nerve by a 30-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA), at a distance of 2 mm proximal to the injury site (10^6/6μl/rat). After injection, the needle was left in place for 2 min prior to withdrawal, to minimize cell leakage. Rat mental nerves

were harvested at 1st, 2nd and 3rd week after PKH26-labeled Schwann-like cell injection and sections were obtained at 20 μm thickness with a cryocut microtome (Leica, Ultracut, UCT, Austria).

1.2.2. Experimental design and PEMF exposure

Sprague-Dawley rats (male, 6-week-old, 200-250 g) were used; the surgical technique was same with part I. About 3 mm crush injury was made at a distance about 2 mm to the mental foramen. Rats were divided into 7 groups (n=10 each): control, sham, PEMF, hDPSCs, hDPSCs + PEMF, Schwann-like cell group and Schwann-like cell + PEMF groups. Experimental groups were exposure into PEMF (3 weeks) or combined with cell transplantation. All the groups were done sensory test before surgery and then weekly postoperatively until 3rd week; half of every group was used for retrograde labeling; the other half was used for axon counting. PEMF devise was same as previously [31, 79]. The condition of PEMF was set with 50 Hz, 1 Hr/day and 1 mT for three weeks.

1.2.3. Cell transplantation in Vivo

After 3 week PEMF exposure, hDPSCs and Schwann-like cells (10^6/6μl/rat) were transplanted immediately after injury by a 30-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA) at a distance of 2 mm proximal to the injury site. hDPSCs

+PEMF and Schwann-like cell + PEMF group exposed into PEMF (50 Hz, 1 Hr/day, 1 mT) for 3 weeks after cell transplantation.

1.2.4. Evaluation

The methods of real time RT-PCR, sensory test, histomorphometric evaluation and retrograde labeling and quantification of sensory neurons were same as described in part I.

2. Statistical analysis

The statistical analyses were performed using SPSS program version 21 (SPSS, Chicago, USA). All data were presented as mean values with standard error means. The normal distribution of the measurements was verified by One-Sample Kolmogorov-Smirnov normality test. One-Way ANOVA test (axon counts, labeled neurons result) and Repeated Measure ANOVA test (sensory test, MTT and qRT-PCR results) followed by post-hoc Tukey test was used to compare data between different groups. P value $\leqslant .05$ was considered statistically significant.

3. Results

3.1. In Vitro study

3.1.1. Characterization of hDPSCs and Schwann-like cells

STRO-1 and Nestin were identified in the dental pulp cell culture (Fig. 12). Six days after induction, differentiated hDPSCs were analyzed morphologically for the expression of Schwann cell proteins - CD104, S100, GFAP, laminin and $P75^{NTR}$ (Fig. 13). After differentiation, cells changed from a fibroblast-like morphology to an elongated spindle shape with positive staining for CD104, S100, GFAP, laminin and $P75^{NTR}$.

3.1.2. Change in S100 and P75 mRNA expression in Schwann-like cells treated with PEMF

After the 1st week, the Schwann-like cells + PEMF group showed significantly greater P75NTR mRNA expression than the hDPSCs group (p=0.036) and the Schwann-like cells group (p=0.023). In addition, the Schwann-like cells + PEMF group maintained elevated $P75^{NTR}$ expression at the 2nd (p=0.021) and 3rd week, compared to the hDPSCs group (p=0.036). Among both groups with Schwann-like cells, PEMF demonstrated an added advantage at the third week (p=0.020) (Fig. 14). For S100 mRNA expression, significances were found in the hDPSCs group (p=0.035, 1st week; p=0.034, 2nd week; and

p=0.037, 3rd week) and the Schwann-like cells group (p=0.023, 1st week; p=0.035, 2nd week; and p=0.036, 3rd week) when compared with the Schwann-like cells + PEMF group (Fig. 15).

3.2. In Vivo study

3.2.1. Cell survival analysis after transplantation: cell tracking with PKH26

PKH26-labeled hDPSCs and Schwann-like cells were observed until the 3rd week despite a decrease in cell count with time. No obvious cytotoxicity was observed. Injected cells were observed on the crushed nerve segment, revealing their participation in the cell survival and the absence of cell leakage (Fig. 16). A difference in cell viability between Schwann-like cells and hDPSCs was not observed.

3.2.2. Peripheral nerve regeneration analysis in vivo: sensory test

The difference score was significantly increased at the 1st week compared to baseline (before injury), and then decreased (Fig. 17). Significances were found in the hDPSCs + PEMF group (p=0.023, 1st week and p=0.036, 3rd week) and the Schwann-like cells + PEMF group (p=0.047, 1st week and p=0.030, 3rd week) compared with the control. Statistical differences were also observed when comparing the PEMF group with the Schwann-like cells + PEMF group (p=0.027, 3rd week).

The changes in gap scores (Fig. 18) mirrored the changes in difference scores; scores were elevated after injury and then decreased. Significances were observed in the hDPSCs + PEMF group (p=0.018, 1st week and p=0.031, 3rd week) and Schwann-like cells + PEMF group (p=0.012, 1st week; p=.038, 2nd week; and p=.018, 3rd week) compared with the control. Also, the gap scores of the PEMF group significantly differed from those of the Schwann-like cells + PEMF group (p=0.027, 3rd week).

3.2.3. Peripheral nerve regeneration analysis in vivo: histomorphometry

Photomicrographs of histologic features in semi-thin sections at distal to the crush injury site was shown (Fig. 19). Total axon counts were shown in Table 2. With 3-week exposure, myelinated axons were increased in all treatment groups; and Schwann-like cells + PEMF group showed the biggest axons number. There was statistical meaning in PEMF group (p=.033 vs. control; p=.021 vs. sham; p=.032 vs. Schwann-like cells + PEMF), hDPSCs group (p=.023 vs. control; p=.031 vs. sham; p=.042 vs. Schwann-like cells + PEMF), hDPSCs + PEMF group (p=.040; vs. control), Schwann-like cells group (p=.042 vs. control; p=.045 vs. sham; p=.038 vs. Schwann-like cells + PEMF) and Schwann-like cells + PEMF group (p=.039; vs. control) (Fig. 20).

3.2.4 Peripheral nerve regeneration analysis in vivo: retrograde labeling

Fluorescence photomicrographs from TG in different group was shown (Fig. 21). The retrograde labeled neurons were shown in table 2. With 3-week recovery period, the positive trigeminal ganglion axons in all treatment groups were increased. Significances were found in PEMF group (p=.034 vs. control; p=.026 vs. sham; p=.035 vs. Schwann-like cells + PEMF); hDPSCs group (p=.035 vs. control; p=.029 vs. sham); hDPSCs + PEMF group (p=.042; vs. control), Schwann-like cells group (p=.040 vs. control; p=.033 vs. sham) and Schwann-like cells + PEMF group (p=.029; vs. control) (Fig. 22).

4. Discussion

In this study, glial character of Schwann-like cells differentiated from hDPSCs was explored using marker genes CD104, S100, GFAP, laminin and $P75^{NTR}$. All treatment groups can improve peripheral nerve regeneration after crush injury, Schwann-like cells + PEMF group showed highest ability; PEMF seemed to play an important role for hDPSCs and Schwann-like cell differentiation in vitro and nerve regeneration using cells transplantation in vivo.

Since hDPSCs originate from migrating neural crest cells, they were thought to be predisposed toward differentiating into peripheral glial cells under the correct environmental conditions [46]. The high proliferation capacity, multi-potency, plasticity, and immune-modulatory properties of DPSCs made them excellent candidates for regenerative medicine purposes, especially in the field of neural tissue engineering [46, 80, 81] .

Previous studies based on Schwann cell development and cell fate specification had been explored to commit MSC to a Schwann cell fate [48,77, 82, 83]. β-mercaptoethanol (β-ME), all-trans retinoic acid (RA), cytokine cocktail including forskolin (FSK), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and neuregulin-1 (HRG) were used herein [43, 47, 48]. β-ME and RA were considered triggering factors to induce changes in morphological and transcriptional characteristics of MSCs toward the

neuronal lineage. Then, a combination of FSK, bFGF, PDGF, and Neuregulin-1 were used [48] to promote the differentiation of hDPSCs into cells with Schwann cell characteristics [47, 48].

To confirm the Schwann-like cell phenotype of differentiated hDPSCs, multiple markers was evaluated. CD104 (integrinβ4), S100, GFAP, laminin and $P75^{NTR}$, were stained strongly positive in Schwann-like cells similar to Schwann cells but hardly detectable in undifferentiated hDPSCs. These results demonstrated the successful morphological differentiation of hDPSCs toward Schwann-like cells. Although $P75^{NTR}$, robust marker of neural stem cells, was detected in the hDPSCs because $P75^{NTR}$ was existed in various stem cell such as bone marrow stem cells, muscle stem cells, live stem cells, stem cell of the oral mucosa and played various roles [84].

A previous study reported that, unlike primary Schwann cells, the ability of differentiated MSCs to support regeneration decreased over time [85]. Also induced Schwann-like cell states derived from MSCs were not sustainable and would revert back to their primitive states without the extrinsic induction factors from the culture [48]. However, pulsed electromagnetic fields (PEMF) can lead to significantly increase cell numbers in the S+G2+M cell cycle phase [51], induce secretion of RA [52], FSK [86], b-FGF [87], PDGF [88], increase nerve growth factor (NGF) secretion [63], improve mesenchymal stem cell proliferation [49-51], differentiation [49-51], promote neurite

outgrowth [53, 54]. So, herein, the effect of PEMF to Schwann-like cell differentiation was explored here and hDPSCs were used as control. Interestingly, Schwann-like cell character was stable after differentiation which was contrary with previous study [48, 86]; however PEMF increased mRNA expression of S100 and P75 of hDPSCs and Schwann-like cells in vitro and promoted peripheral nerve regeneration ability after transplantation in vivo, which was reported first time herein.

The number of positive PKH26-labeled hDPSCs and Schwann-like cells were decreased with time but were still presented at the 3rd week, the reason for this decline in surviving cells may be related to the de-differentiation and re-differentiation process [48, 89]. In addition, both of the cell types originated from human, so the immune response may have prevented cell survived in this study [79].

Schwann-like cells were successfully differentiated from hDPSCs. PEMF positively influenced cell differentiation, glial mRNA expression of S100 and P75NTR and peripheral nerve regeneration after cell transplantation. Schwann-like cells had more influence on nerve regeneration than hDPSCs either with or without PEMF. In this study, only one set of parameters for PEMF was used- 50 Hz, 1 hr/day, 1 mT. Other conditions using various frequencies, durations and intensities of PEMF were tested in a parallel study. Also, the effect of PEMF on hDPSCs and Schwann-like cell needs to be more comprehensively evaluated by more sophisticated methods such as Western blot and 3D analysis through

co-culture with dorsal root ganglion (DRG) to analyze neurite length. For true clinical applicability of cell therapy plus PEMF as a treatment for peripheral nerve regeneration, the long-term effects need to be evaluated.

III. Conclusion

1. PEMF enhanced peripheral nerve regeneration, and that it may be due to cell proliferation and increase in BDNF and S100 gene expression. Among the various conditions, 50Hz, 1Hr was revealed to be the most suitable inferred from the data of in vitro and in vivo study.

2. Schwann-like cells and hDPSCs with or without PEMF in addition to PEMF alone improved peripheral nerve regeneration after crush injury. Among these, the Schwann-like cells combined with PEMF showed the highest regeneration ability. PEMF had an additive effect on the ability for hDPSCs and Schwann-like cells to regenerate nerves in vitro and, after transplantation, in vivo.

IV. References

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Osteoblasts stimulated with pulsed electromagnetic fields increase HUVEC proliferation via a VEGF-A independent mechanism. Bioelectromagnetics. 2009; 30(3):189-97.

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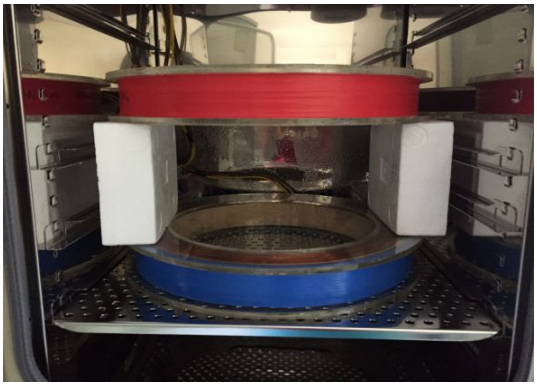
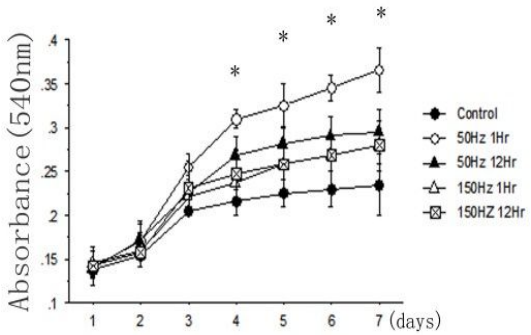
V. Figures and Tables

Figure 1



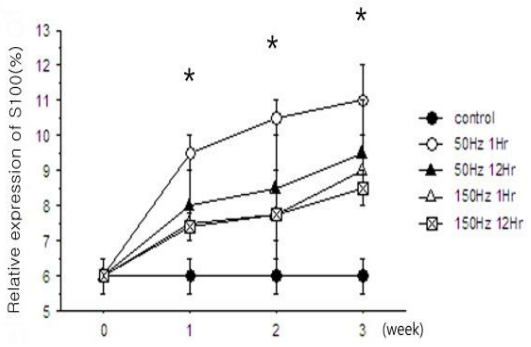
Overview of PEMF device in vitro study, two identical Helmholtz coils of 30 cm diameter and 7 cm width; PEMF was carried out with density of 1 mT, 4 conditions (50 Hz 1 Hr; 50 Hz 12 Hr; 150 Hz 1 Hr; 150 Hz 12 Hr) were set according to the exposure protocol.

Figure 2



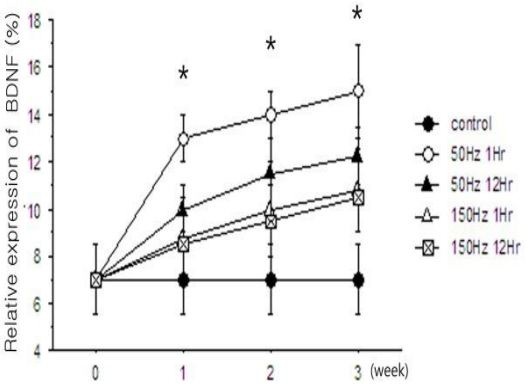
Cell Proliferation assay, immortalized rat Schwann cell Line (iSCs) were seeded into 96 well plates at a density 1×10^2 cells/well and cultured. Cell proliferation was evaluated by the MTT assay at 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day. Values are presented as the mean ± SEM. of three independent experiments (*P<.05, vs. Control).

Figure 3



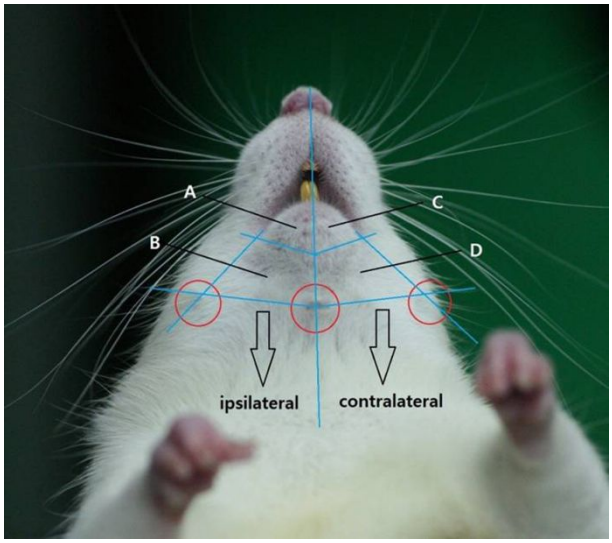
S100 mRNA expression in different groups were analyzed by qPT-PCR. S100 mRNA was increased in all groups with time development. Values are shown as mean ± SEM normalized to GAPDH from at least 3 independent experiments (n≥3)(*P<.05, vs. Control).

Figure 4



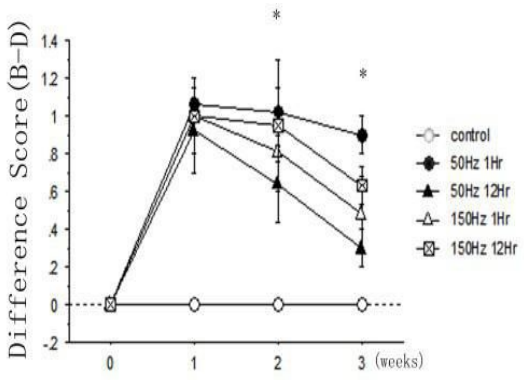
BDNF mRNA expression in different groups were analyzed by qPT-PCR. The relative gene BDNF was expressed in all groups and increased with time development. Values are shown as mean ± SEM normalized to GAPDH from at least 3 independent experiments (n≥3)(*P<.05, vs. Control).

Figure 5



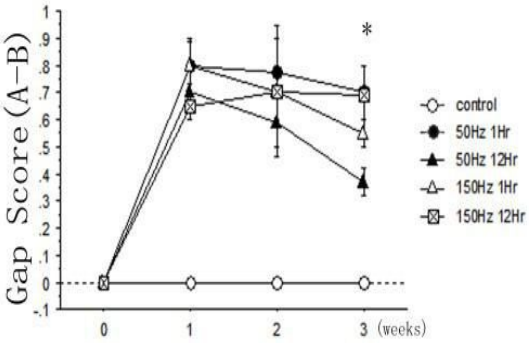
Sensory test, A, a lip area on the ipsilateral side; B, an area over the foramen on the ipsilateral side; C, a lip area on the contralateral side; D, an area over the foramen on the contralateral side. Difference score=B-D; Gap score=A-B.

Figure 6



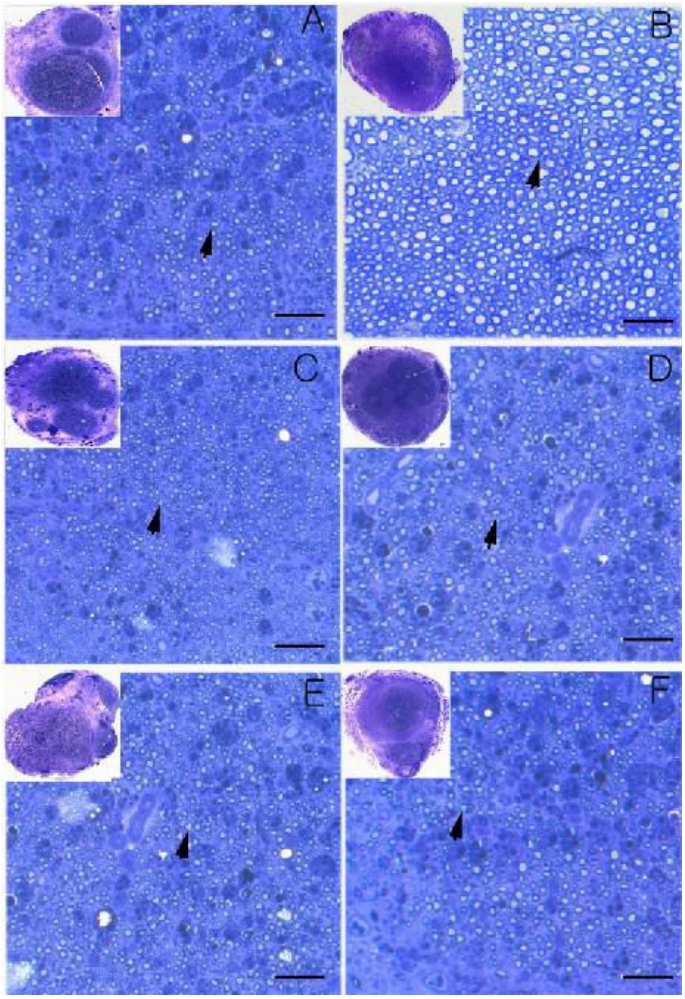
Difference score graph of weekly sensory test about all groups. Values are presented as the mean ± SEM. (*P<.05, vs. Control). There were significant difference on the 2nd and 3rd week between 50 Hz 1 Hr and control group, 50 Hz 1 Hr group showed higher ability to regenerate peripheral nerve.

Figure 7



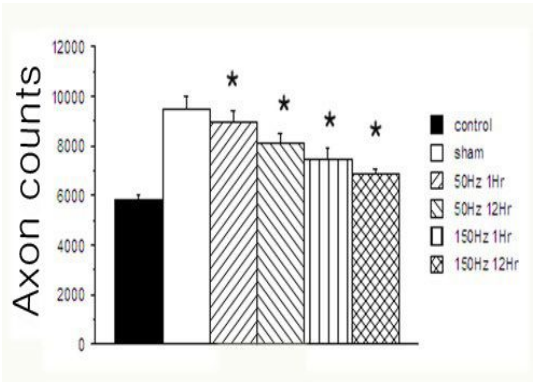
Gap score graph of weekly sensory test about all groups. Values are presented as the mean ± SEM. (*P<.05, vs. Control), 50 Hz 1 Hr showed higher ability to regenerate peripheral nerve. And there was significant difference on the 3rd week between 50 Hz 1 Hr and control group.

Figure 8



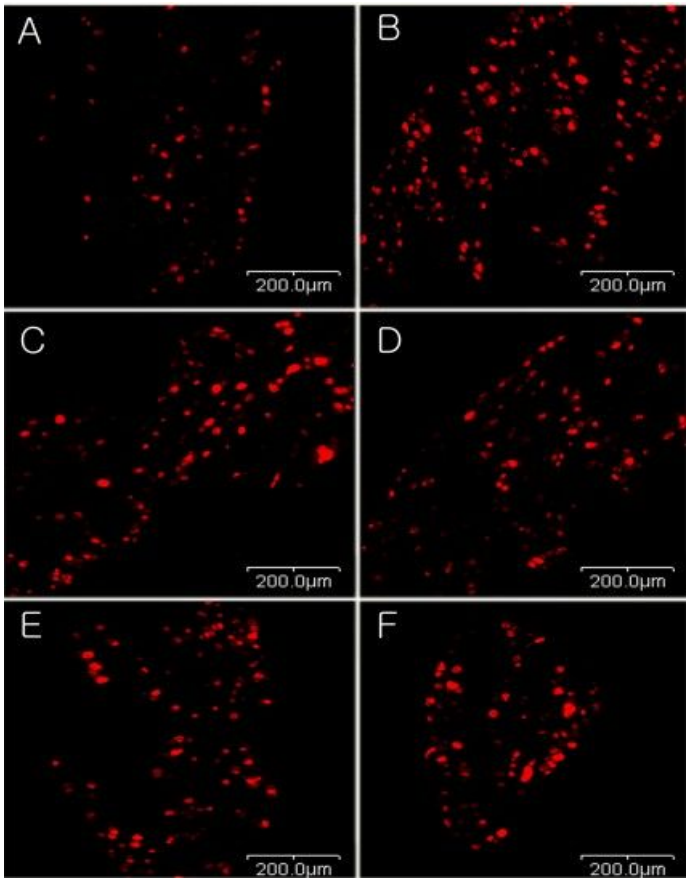
Photomicrographs of histologic features in semi-thin sections at distal to the crush injury site (3 weeks postoperatively), representative microphotographs showed the typical regenerating nerve with myelinated fibers of smaller size than in intact nerves, which tended to cluster in minifascicles. A, control group; B, sham group; C, 50 Hz 1 Hr group; D, 50 Hz 12 Hr group; E, 150 Hz 1 Hr group; F, 150 Hz 12 Hr group; Scale bar=50 μm.

Figure 9



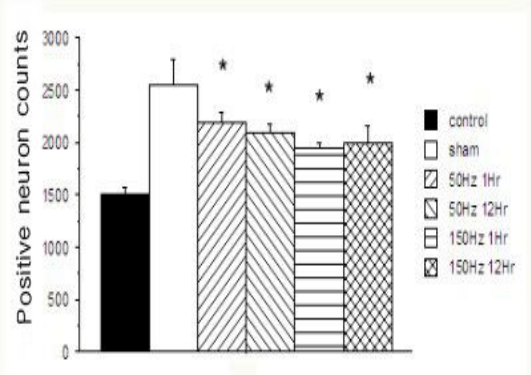
Statistical result and histograms of axon counting in each group, the axon number in 50 Hz 1 Hr was larger than other groups. Values are presented as the mean ± SEM (*P<.05, vs. Control).

Figure 10



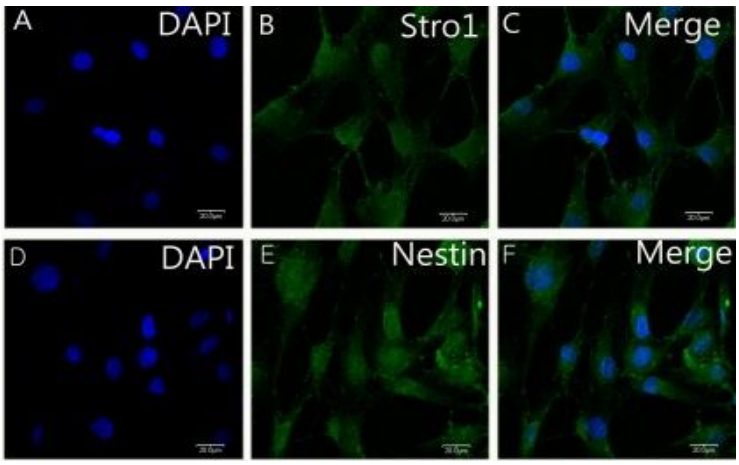
Representative photomicrographs of retrograde trigeminal ganglion labeling with DiI. More positive TG neuron was found in 50 Hz 1 Hr group. A, control group; B, sham group; C, 50 Hz 1 Hr group; D, 50 Hz 12 Hr group; E, 150 Hz 1 Hr group; F, 150 Hz 12 Hr group; Scale bar=200 μm.

Figure 11



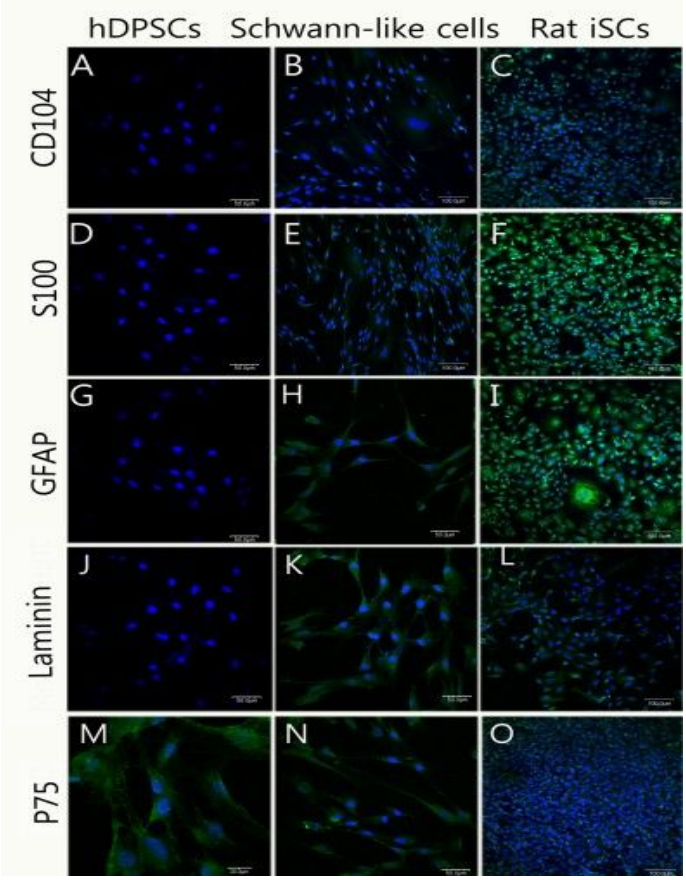
Statistical result and histograms of the total number of labeled sensory neurons in each group, the positive neuron counts were larger in 50 Hz 1 Hr groups than other groups. Values are presented as the mean ± SEM (*P<.05, vs. Control).

Figure 12



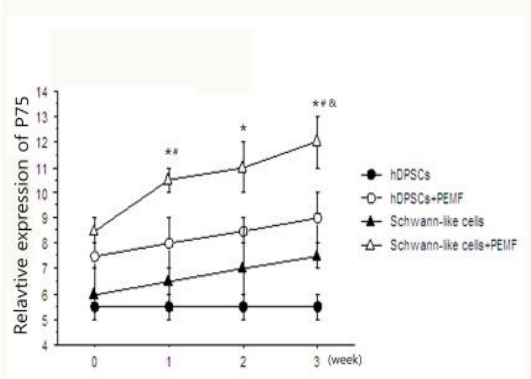
Immunostaining of isolated hDPSCs with STRO-1 and Nestin, Scale bar: 20 μm. STRO-1 and Nestin positive results were found in the result.

Figure 13



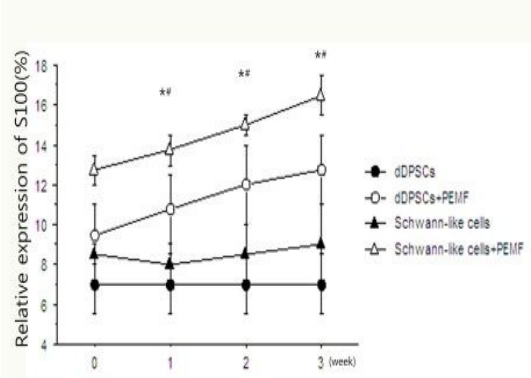
hDPSCs differentiated to Schwann-like cell phenotype; hDPSCs (A, D, G, J, M); Schwann-like cells (B, E, H, K, N); Immortalized Schwann cell line (C, F, I, L, O). CD104 (A-C); S100 (D-F); GFAP (G-I); Lamin (J-L); P75 (M-O), Scale bar=50 μm (A, D, G, H, J, K, M, N), Scale bar=100 μ m (B, C, E, F, I, L, O). All markers positive staining were found, P75 positive staining was also found in the hDPSCs group.

Figure 14



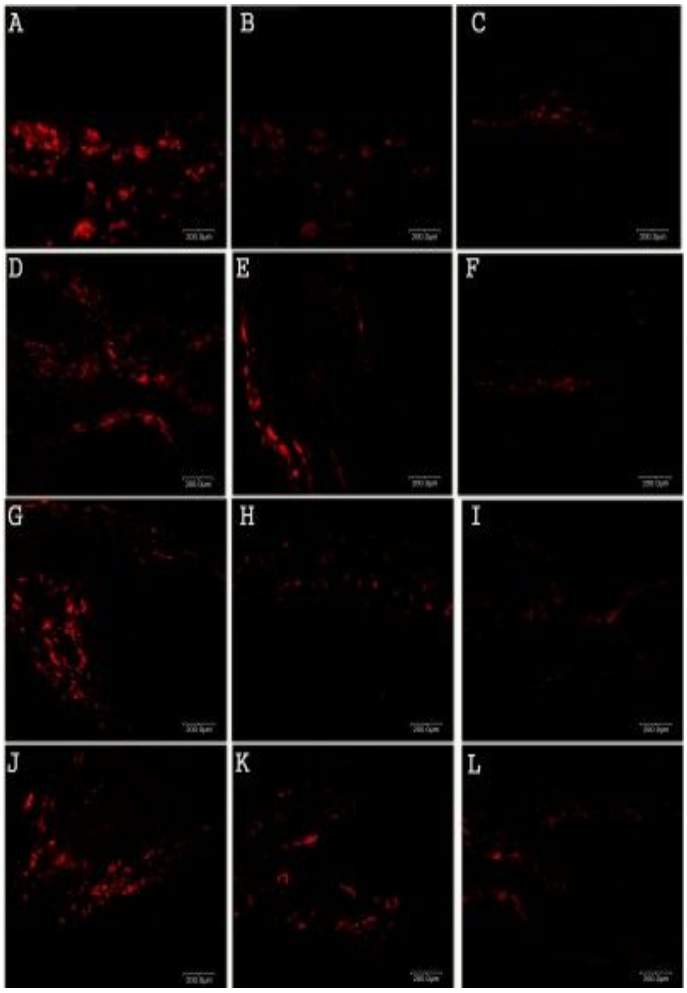
P75 mRNA expression, values are shown as mean ± SEM normalized to GAPDH from at least 3 independent experiments (n≥3)(*P<.05, hDPSCs group vs. Schwann-like cell + PEMF group; #P<.05, Schwann-like cell group vs. Schwann-like cell + PEMF group; &P<.05, hDPSCs group vs. hDPSCs + PEMF group)

Figure 15



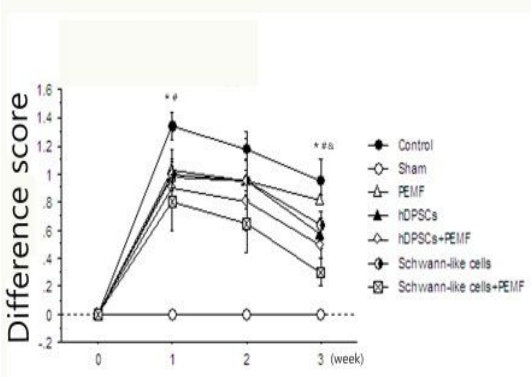
S100 mRNA expression, it was expressed in all groups. Values are shown as mean ± SEM normalized to GAPDH from at least 3 independent experiments (n≥3)(*P<.05, hDPSCs vs. Schwann-like cell + PEMF; #P<.05, Schwann-like cell vs. Schwann-like cell + PEMF)

Figure 16



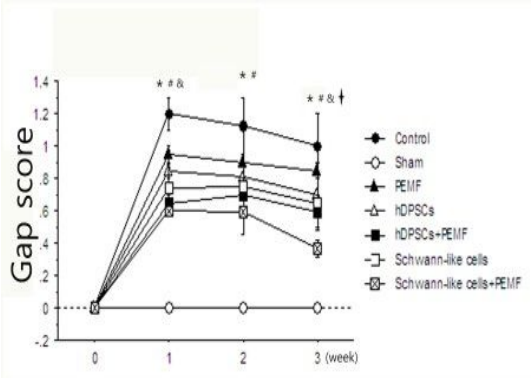
Cell tracking with PKH26, labeled cells were observed until the 3rd week. 1st week (A, D, G, J); 2nd week (B. E, H, K); 3rd week (C, F, I, L) Schwann-like cell group (A-C); Schwann-like cell group + PEMF (D-F); hDPSCs group (G-I); hDPSCs + PEMF group (J-L). Scale bar= 200 μm.

Figure 17



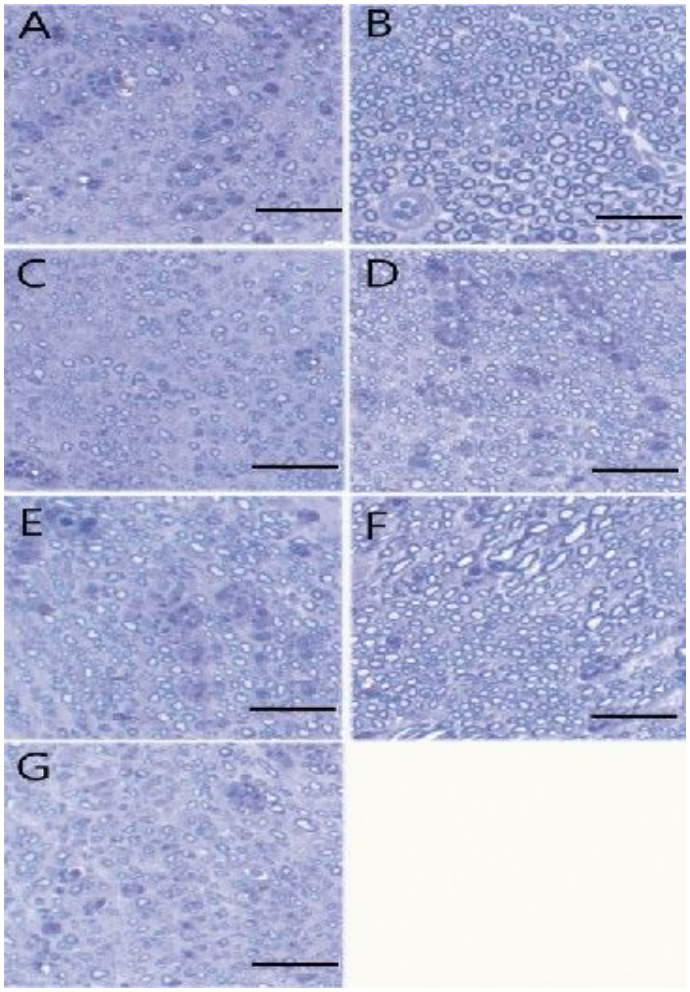
Difference score graph of weekly sensory test. Values are presented as the mean ± SEM. (*P<.05, Schwann-like cell + PEMF vs. Control; #P<.05, hDPSCs + PEMF vs. Control; &P<.05, PEMF vs. Schwann-like cell + PEMF).

Figure 18



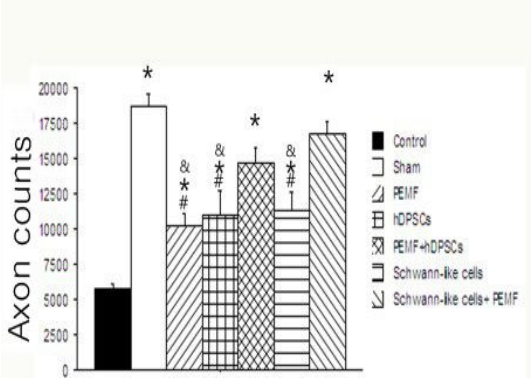
Gap score graph of weekly sensory test. Values are presented as the mean ± SEM. (*P<.05, Schwann-like cell + PEMF vs. Control; &P<.05 hDPSCs + PEMF vs. Control; #P<.05, PEMF vs. Schwann-like cell + PEMF).

Figure 19



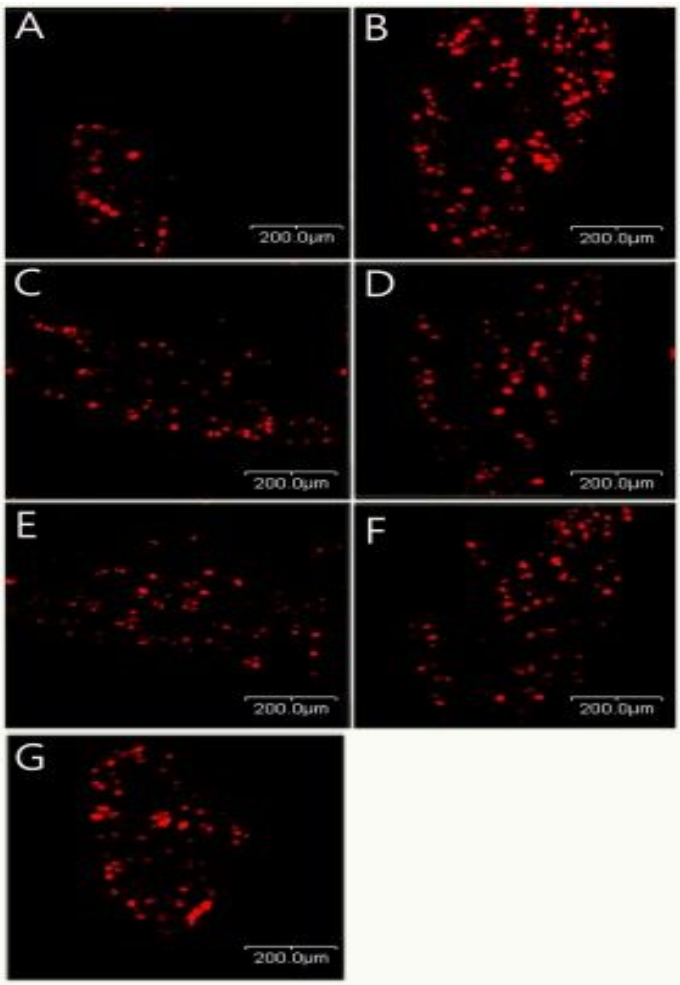
Photomicrographs of histologic features in semithin sections at distal to the crush injury site (3 weeks postoperatively A, control; B, sham; C, hDPSCs; D, hDPSCs + PEMF; E, Schwann-like cell; F, Schwann-like cell + PEMF; G,PEMF. Axon numbers were increased in all treatment groups. The Schwann-like cells + PEMF group showed the largest increase in axon number than other interval groups. Scale bar=50 μm.

Figure 20



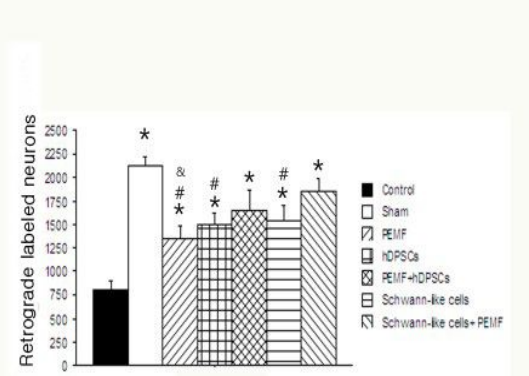
Statistical result and histograms of axon counting, values were presented as the mean ± SEM. (*P<.05, vs. control; #P<.05, vs. sham; &P<.05, vs. Schwann-like cell + PEMF). Axon numbers were increased in all treatment groups. The Schwann-like cells + PEMF group showed the largest increase in axon number than other interval groups.

Figure 21



Representative photomicrographs of retrograde trigeminal ganglion labeling with DiI. A, control; B, sham; C, hDPSCs; D, hDPSCs + PEMF; E, Schwann-like cell; F, Schwann-like cell + PEMF; G,PEMF. Scale bar=200 μm. With the 3 week recovery period, the positive neurons of the TG were increased in all treatment groups.

Figure 22



Statistical result and histograms of the total number of labeled sensory neurons, values were presented as the mean ± SEM. (*P<.05, vs. Control; #P<.05, vs. Sham; &P<.05, vs. Schwann-like cell + PEMF) With the 3 week recovery period, the positive neurons of the TG were increased in all treatment groups.

Table 1 Comparison of axon counts and retrograde labeled neurons

Group	Axon Counts	Retrograde labeled neurons
Control	5790±339	1450±212
Sham	9510 ±692*	2550±339*
50Hz 1Hr	8948±700*	2183±162*
50Hz 12Hr	8128±544*	2100±132*
150Hz 1Hr	7455±643*	1933±115*
150Hz 12Hr	6904±197*	2000±264*

Significantly higher axon counts and retrograde labeled neurons were seen in all the groups, 50 Hz 1 Hr group has higher axon count and retrograde labeled neuron than other groups. All values are presented as mean ± SEM (*P<.05, vs. Control).

Table 2 Comparison of axon counts and retrograde labeled neurons

Groups	Total Axon Counts	Retrograde labeled neurons
Control	5790±479	803±134
Sham	18780 ±1142*	2117±132*
PEMF	10200 ± 1282*#&	1353±182*#&
hDPSCs	11004±2411*#&	1500±171*#
PEMF + hDPSCs	14704± 1562*	1650± 299*
Schwann-like cells	11325±1842*#&	1536±231*#
Schwann-like cells + PEMF	16780 ±1142*	1850±196*

Significantly higher axon counts and retrograde labeled neurons were seen in sham and all experimental groups than control. Among these, Schwann-like cells combined with PEMF group showed highest total axon counts and retrograde labeled neurons. All values are presented as mean ± SEM. (*P<.05, vs. Control; #P<.05, vs. sham; &P<.05, vs. Schwann-like cells + PEMF)

VI. Abstract in Korean

전자기장과 치수줄기세포에서 분화된 슈반형세포를 이용한 압박손상 턱신경

서울대학교 대학원 치의학과 구강악안면외과학 전공

(지도교수 이종호)

黑伟红 (Wei-Hong Hei)

연구목적

본연구는 말초신경 재생에 효과적인 PEMF의 주파수 및 조사시간을 찾고, 그리고 치수줄기세포에서 분화된 슈반형세포 주입과 전자기장 조사가 백서 압박손상 턱신경 재생에 미치는 효과를 평가하는 것을 목적으로 하였다.

재료 및 방법

Part I: 불멸화 백서 슈반세포(iSCs), $1x10^2$ /well)를 각 4가지 자기장 조건 (50 Hz 1 Hr/day, 50 Hz 12 Hr/day, 150 Hz 1 Hr/day and 150 Hz 12 Hr/day)으로 조사한 뒤 세포 증식 , S100 및 BDNF (brain-derived neurotrophic factor)의 mRNA 발현을 분석 하였다, 동물 평가를 위해 SD 백서를 (n=10, each) control, sham, 50 Hz 1 Hr/day, 50 Hz 12 Hr/day, 150 Hz 1 Hr/day and 150 Hz 12 Hr/day 6개군으로 나누였다, 백서 턱신경에 압박손상을 가한 후 상기 4가지 조건을 3주동안 조사하였다. 감각기능테스트, 삼차신경절의 retrograde axon 전달 그리고 이식 4주째에 조직형태학적 관찰을 수행하여 신경재생 정도를 평가하였다.

Part II: 치수줄기세포에서 슈반형세포를 분화 유도하였으며, CD104, S100, GFAP, laminin, $P75^{NTR}$의 세포면역화학법으로 분석하였다. $P75^{NTR}$ 및 S100의 mRNA 발현은 Quantitative Real-time polymerase chain reaction (qRT-PCR)을 이용하여 분석하였다; 백서의 하악 턱신경에

3mm 압박손상을 주고 7개 그룹으로 나누어 (control, sham, PEMF, hDPSCs, hDPSCs + PEMF, Schwann-like cells, Schwann-like cells + PEMF) 실험을 진행 하였다.

연구결과

Part I: PEMF의 50 Hz,1 Hr/day 조건에서 4 일부터 7 일 까지 불멸화된 백서 슈반세포가 증가하였다. $P75^{NTR}$ 및 S100의 mRNA 발현은 같은 조건에서 1 주부터 3 주까지 유의하게 증가하였다. 대조군과 비교할 때, 다른 조건은 통계 학적 유의성을 보이지 않은 반면 50 Hz 1 Hr 조건에서 difference score는 2 주째와 3 주에, gap score는 3 주째에 통계적으로 유의하게 증가하였다. 그리고 대조군과 비교할 경우, 4 조건 모두 axon 수와 retrograde labeled neuron을 증가시켰다. 통계학적으로 유의한 차이는 없었지만, 50 Hz, 1 Hr/day 조건의 PEMF는 다른 조건에 비해 가장 큰 신경재생 능력을 보여주었다.

Part II: 치수줄기세포에서 분화유도된 슈반형세포에서 CD104, S100, glial fibrillary acidic protein (GFAP), laminin, p75 neurotrophin receptor ($P75^{NTR}$)항체가 검출되었다. $P75^{NTR}$ 및 S100의 mRNA 발현은 PEMF를가한 슈반형세포에서 가장 높았다. 대조군과 때 다른 조건은 통계학적 의미가 없는 반면, difference score와 gap score는 1 주째와 3 주째에 증가하였다. Axon 수와 retrograde labeled neuron는 모든 군에서 유의하게 증가하였는데, PEMF + 슈반형세포 군에서 가장 높았으며,, PEMF 군 (p=.033 axon count; p=.035 retrograde labeling) 그리고 hDPSCs군과 (p=.042 axon count), Schwann-like cells group과 (p=.038 axon count) 비교할 때 유의성이 나타났다.

결론

1. 1 mT 4 가지 주파수 및 노출 시간 자기장의 PEMF은 말초신경 재생을 촉진하였으며, 이 중에서, 50 Hz, 1 Hr/day 가장 신경재생 효과가 있었다.

2. 전자기장을 준 슈반형세포, hDPSCs 세포와 자기장을 주지 않은 슈반형세포, hDPSCs 에서 압박신경손상 후 신경재생효과가 나타났다. 자기장을 준 슈반형세포에서 가장 우수한 신경재생능력이 있었으며, 전자기장 조사는 in vitro 및 in vivo 실험에서 슈반형세포와 hDPSCs의 신경재생능에 추가적인 효과를 나타냈다.

주요어 전자기장, 말초신경재생, 압박신경손상, 슈반형세포, 치수줄기세포, 이(턱)신경.

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