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The Effect of Pulsed Electromagnetic Field (PEMF) Pre-treated Mesenchymal Stem Cells on the Regeneration of Crush - Injured Rat Mental Nerve

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서 나 리

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Abstract

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1. Purpose

Peripheral nerve injury is often encountered in clinical situations, and cell therapy is a promising regeneration technique. For this purpose, cells must be rapidly expandable, easily harvestable, and immunologically tolerable. Mesenchymal Stem Cells (MSCs), which satisfy these conditions, are a new tool in the regeneration of damaged peripheral nerves. Another non-cellular approach for the injured peripheral nerve regeneration, Pulsed Electromagnetic Field (PEMF), has been suggested. Past in vitro application of PEMF to MSCs increased cell proliferation and growth factor release, and it specifically induced MSC differentiation into Schwann-like cells. Also, PEMF in vivo helps in the regeneration of damaged peripheral nerve, motor, and sensory neurons.

The aim of present study was to evaluate the effect of PEMF on the proliferation

and growth factor release of MSCs and to evaluate the effect pf PEMF pre-treated MSCs (PMSCs) on peripheral nerve regeneration.

2. Methods

MSCs were harvested from the long bones of five-week-old Sprague Dawley rats (200 - 250g) and primary cultured. The effect of PEMF pre-treatment (50 Hz, 0.1 mT, 1 hour/day) on MSCs was evaluated by cell proliferation assay and the change of S100, GFAP, BDNF, and NGF mRNA expression. Cell survival was observed for two weeks by injecting the DiI-labeled MSCs into the injured nerve. Animal surgery was carried out in four groups: Sham, PBS, the MSC-injected group, and the PEMF pre-treated MSCs (PMSCs) -injected group. Crush injury was performed on the left mental nerve using a needle holder with a width of 3 mm for one minute. MSCs and PEMF pre-treated MSCs (PMSCs) ($1x10^6$) were suspended in 5 μL PBS and injected into the injury-induced nerve using a Hamilton syringe. Functional recovery of the nerve was carried out by measuring the gap and difference scores at pre-surgery and 1 and 2 weeks post-surgery. Axonal regeneration was evaluated with histomorphometric analysis and retrograde labeling of the trigeminal ganglion (TG).

3. Results

The PEMF pre-treated MSCs (PMSCs) group showed higher cell proliferation and growth factor expression than the MSC group. In the mental nerve sensory test, the PMSC injection group showed faster recovery than the untreated MSC injection group. Also, the PEMF pre-treated MSCs (PMSCs) exhibited significantly higher myelinated axon count and density than the MSC and PBS

groups. Furthermore, using TG retrograde labeling, a greater number of dyed neurons was counted in the PEMF pre-treated MSCs (PMSCs) group compared to the MSC group.

4. Conclusion

PEMF pretreatment on MSCs (PMSCs) in vitro increased not only the growth rate of the cells, but also the expression of nerve growth factors. Moreover, when these PEMF pre-treated MSCs (PMSCs) were injected into an injured nerve, they were more effective in regeneration than untreated MSCs. This suggests that PEMF pre-treated MSCs (PMSCs) are an enhanced strategic tool in cell therapy for recovery of injured nerves.

Keyword: Mesenchymal Stem Cells (MSCs), Pulsed Electromagnetic Field (PEMF), Peripheral Nerve Regeneration, Mental Nerve, Crush-Injury

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Contents

Ⅰ. Introduction

Peripheral nerve damage is not uncommon and can result in severe dysesthesia, persistent paresthesia, and post-traumatic pain (1). Injured peripheral nerves can regenerate in time, but the regeneration is sometimes not complete or takes a long time. Patients might benefit from surgical interventions such as nerve grafting; however, these are delicate procedures and sometimes ineffective in cases of severe injury (2). For promoting regeneration of an injured peripheral nerve, benefits of cell therapy (3) and electromagnetic stimulation (4) have been reported.

Schwann Cells (SCs) produce a large variety of neurotrophic factors and cytokines. They also express cell adhesion molecules and extracellular molecules that are known to support axonal regeneration (5-10). Following transection, SCs provide structural support as well as neurotrophic guidance to regenerating axons (11, 12). When SCs are injected into an injured peripheral nerve, they assist nerve regeneration using these characteristics (13, 14). Despite the advantages, there is limitation in the application of SCs because it is difficult to culture a large amount of cells, culture time is lengthy, and the technique is technically complex. Moreover, this technique requires sacrifice of another peripheral nerve to culture SCs for auto-transplantation (10, 15, 16). Considering the problems listed above, the ideal ‘transplantable cell’ should be easily accessible, capable of rapid expansion in culture, immunologically tolerable, capable of long-term survival, and capable of integration in the host tissue (17). Mesenchymal Stem Cells (MSCs) have great translational potential in regenerative medicine given their availability and potential for multi-lineage differentiation into bone, cartilage, muscle, fat, and tendon (18, 19). Due to these attractive characteristics of MSCs, they are currently being attempted as a treatment option in injured nerve regeneration studies (20-22).

Many studies have shown the effect of an electromagnetic field with low frequency on cells. Pulsed Electromagnetic Field (PEMF) has been reported to help MSC proliferation (23) and to differentiate bone marrow MSCs into neuron-like cells in some induced conditions (24). Low-frequency electromagnetic fields affect actions in intracellular and membrane proteins, including ion channels (25). PEMF of low frequency application can induce neural differentiation of MSCs without nerve growth factors (26). Hence, nerve regeneration might be promoted even more when PEMF is used with MSCs rather than using MSCs alone.

The aim of the present study was to evaluate the effects of PEMF on the proliferation and growth factor release of MSCs and to evaluate the effect of PEMF pre-treated MSCs (PMSCs) on peripheral nerve regeneration.

Ⅱ. Materials & Methods

1. Isolation of MSCs and culture

MSCs were isolated as described previously (27, 28). Shortly, five-week-old Sprague Dawley (SD) male rats (220 - 250g) were sacrificed with carbon dioxide (CO_2). Their hind limbs were harvested and washed in 70 % ethanol and 1 X phosphate-buffered saline (PBS). The bones without soft tissue were then stored in pure Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Co., Grand Island, NY, USA) on ice. Using a 10-mL syringe with a 26-gauge needle, the DMEM solution was introduced in the spongy bone, and the leakage was collected in a 50 mL conical tube. The collected culture medium was filtered to remove bone and muscle using a 70-μm nylon mesh (Falcon, Franklin Lake, NJ, USA). The cells were centrifuged at 800 rpm for 5 minutes, and the supernatant liquid was removed through aspiration. The centrifuged cells were resuspended in MSC growth media, a low glucose DMEM containing 10 % MSC fetal bovine serum (Life Technologies Co.), and 10 mg / mL of gentamicin (Life Technologies Co.). The suspended cells were seeded into a 100-mm cell culture dish and were incubated at 37 °C, 95 % humidity, and 5 % CO_2 until adhesion. After the adhered MSCs were grown to 85 % confluency, subculture was performed using 0.05 % trypsin (assigned as Passage 1), and the MSCs of the rat were further cultivated for the main experiment.

1-1. Characterization of MSCs

The cultivated MSCs were used in this experiment until passage 5 and were

placed in culture slides. For the negative control, immunocytochemical analysis was performed using PC12 cells. The cells were fixed for 30 minutes using 4 % paraformaldehyde (PFA; Merck, Daramstadt, Germany) and then washed three times with PBS. They were processed with Proteinase K (DAKO, Carpinteria, CA, USA) for 10 minutes and washed three times with PBS. They were then treated with peroxidase block (DAKO) for 30 minutes and washed three times with PBS. The cells were treated with 5 % horse serum (Vector, Burlingame, CA, USA) for 30 minutes. Then, without washing, the primary antibodies CD 29 (Biolegend, San Diego, CA, USA) and CD 105 (Abcam Inc., Cambridge, UK), diluted 1:100, were applied, and the cells were stored at 4 °C overnight. A secondary antibody, FITC, was diluted 1:200. The culture slides were mounted with DAPI for observation under a fluorescent microscope (CLSM, LSM700, Carl Zeiss, Oberkochen, Germany).

2. Pulsed Electromagnetic Field (PEMF) treatment

The PEMF device used was made with the help of Professor Soochan Kim's Signal Processing Lab (Graduate School of Bio & Information Technology, Hankyong National University, Korea). The device consisted of Helmholtz coils that were coiled with 1,000 turns of enamel copper wire (AWG #25, 0.5 mm diameter). The coil's inner diameter was 30 cm, with a width of 7 cm and a 15-cm distance between two coils. The PEMF conditions were 50 Hz, 1 mT, 1 hour / day (29-33). Cell culture dishes were placed in the center between the coils (Figure 1).

2-1. Cell proliferation assay

To determine the effect of PEMF pre-treatment on cell proliferation, a quantitative DNA assay was performed with the PEMF pre-treated MSCs (PMSCs) and the MSCs. MSCs were seeded into plates at a density of $1x10^3$ cells / well. Culture medium was removed on the 5^{th}, 7^{th}, and 10^{th} days. After washing with PBS, 10 μL of EZ - Cytox solution (Daeil Lab Service Co. Std, Seoul, Korea) was added to each well, and the plate was incubated for 4 hours in a CO_2 incubator. DNA was quantified with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) by reading the absorbance at a wavelength of 450 nm.

2-2. mRNA expression level by RT-PCR

To decide the duration of PEMF for the PEMF pre-treated MSCs (PMSCs), MSCs were seeded, and cells were harvested at days 5, 7, and 10 to compare the gene expression (six dishes each group, a total of 18 dishes). On the day of the experiment, the cell was washed twice with PBS, and 1 mL of TRIzol was added to the dishes. The cells were harvested into 1.5-mL centrifuge tubes. Next, 200 μL of chloroform was added, the well was mixed by shaking, and the tubes were incubated for 12 minutes. The mixture was centrifuged for 10 minutes at 12,000 *g* and 4 °C. During centrifugation, a new tube containing 500 μL isopropyl alcohol was prepared. When the centrifuge stopped, the tube was handled carefull, and the top layer of liquid was moved to the new tube and incubated for 10 minutes. Then, the tube was centrifuged for another 10 minutes at 12,000 *g* at 4 °C. The supernatant was removed, and the mixture was washed with 1 mL of 75 % ethanol. Then, the mixture was centrifuged again for 5 minutes at 7,500 *g* and then air-dried at room temperature. It was subsequently dissolved into diethylpyrocarbonate-treated water and incubated for 20 minutes at 50 °C. RNA was quantified with an

ultraviolet spectrophotometer at 260 and 280 nm. Reverse transcription (RT) polymerase chain reaction (PCR) was performed essentially as described previously (34). Using an RT reagent kit, the total RNA was synthesized into cDNA. PCR was performed using primers for S100, GFAP, NGF, and BDNF (33-35). The PCR products were analyzed using 2 % agarose gel electrophoresis.

3. The effect of PMSCs on the regeneration of the mental nerve from a crush injury in rats

3-1. Animal crush injury model

Five-week-old male SD rats (200 – 250 g) were used in the present experiment. SD rats were anesthetized with Chloropent (1 cc / 100 g). The mental nerve on the left was exposed, and crush injury was induced using a needle holder (Fine Science Tools Inc., No. 12503-15, North Vancouver, British Columbia, Canada) with a 3-mm width that was clipped up to the second ratchet (Figure 2A). All animal experimental procedures were carried out in accordance with the guidance of the Laboratory of Animal Resources of Seoul National University, Korea (SNU-130201-2).

3-2. Cell tracking with DiI-labeled MSCs and PMSCs

According to the manufacturer's instructions, MSCs and PEMF pre-treated MSCs (PMSCs) were labeled using 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI solution, Molecular Probes Inc., Eugene, OR, USA) in order to confirm cell viability after injection. MSCs and PEMF pre-treated MSCs (PMSCs) suspension ($1x10^6$ cells) with 5 μL DiI solution

was mixed by gentle pipetting and incubated for 15 minutes at 37 °C in CO_2 incubator. DiI-labeled MSCs and PEMF pre-treated MSCs (PMSCs) were injected into the injured nerve using a 26-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA) 3 mm proximal to the injury site. The mental nerves were harvested at 1 and 2 weeks after injection, and 18 μm-thick nerve sections were obtained with a cryocut microtome (CM3050 S, Leica, Nussloch, Germany). Sections were observed under a fluorescence microscope (CLSM, LSM700, Carl Zeiss, Oberkochen, Germany).

3-3. Evaluation of PMSCs in the animal model

Each group was subdivided into four groups with six rats in each group: the sham group; the group that was injected with PBS after mental nerve crush injury; the group that had MSCs injected in the nerve; and the group that had PEMF pre-treated MSCs (PMSCs) injected in the nerve (Figure 2B). The site of injury was marked with a 9/0 nylon suture (Ethicon, Ethicon Inc., Somerville, NJ, USA) on the outer part of the nerve for recognition. MSCs and PEMF pre-treated MSCs (PMSCs) ($1x10^6$ cells/5 μL PBS) were injected immediately after injury using a 26-gauge Hamilton syringe 3 mm proximal to the injury site. Immediately after the injection, the injured site was sutured with a 4/0 nylon suture (Dafilon, B-Braun VetCare SA, Barcelona, Spain).

3-3-1. Sensory function

A sensory test was performed, and scores were calculated using the method of Seino et al. (36). The result was used to assess the behavioral response 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 was 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 was calculated as the value of the ipsilateral lip area (a) minus the value in the vicinity of the mental foramen (b). By definition, a higher score means poorer recovery. As the damaged nerve recovers functionally, the scores approach "score zero." Series of von Frey filaments were used to determine pain sensitivity to mechanical stimulation. A sensory test was performed using von Frey filaments (Semmes-Weinstein Monofilaments, North Coast Medical, Inc., Arcata, CA, USA). 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 applied from above to the center of the whisker pad. Head withdrawal, touching, or scratching the facial region upon von Frey filament application was considered a 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 1 week and 2 weeks within each group were recorded.

3-3-2. Histomorphometric analysis

After the two-week experimental period, six rats from each group were anesthetized under the same conditions of animal surgery. The 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 one day. The nerve segment was then post-fixed with 2 % osmium tetroxide for two days. It was subsequently washed

with PBS (pH 7.4) solution and then routinely processed and embedded in Epon. Serial transverse micro-thin sections of 0.45 μm thickness were cut with an ultra-microtome (RMC Boeckeler, Tucson, AZ, USA) and stained with 1 % toluidine blue for light microscopy examination. Images were captured using a specialized system, SPOT RTTM-KE color mosaic, and digitized by SPOT software (Ver. 4.6). For the sake of simplifying axon counting, the total cross-sectional area of the nerve was measured at 40 X magnification, and three sampling fields were then randomly selected at 200 X magnification using a protocol previously reported. Mean fiber density was calculated by dividing the total number of nerve fibers within the sampling field by its area (N / mm^2). Myelinated fibers counts were 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.

3-3-3. TG retrograde labeling

Trigeminal ganglion (TG) neurons were retrograde-labeled with a fluorescent dye: DiI (Molecular Probes, Eugene, OR, USA). Two weeks after crush injury, the mental nerves were re-exposed, and the surfaces of the exposed nerves were soaked in a generous amount of dil. Five days later, the animals were anesthetized and perfused with 4 % PFA solution. After craniotomy, TG neurons were harvested and post-fixed overnight with the same solution of 4 % PFA. The TG neurons were immersed in a 20 % sucrose solution for four days, then embedded in Tissue Tek (Sakura Finetek USA Inc., Torrance, CA, USA) and frozen in liquid nitrogen. Serial 18 μm longitudinal sections were made at -20 °C in a cryostat microtome

(Leica CM 3050, Milano, Italy). Sections were observed under a fluorescence microscope (CLSM, LSM700, Carl Zeiss) for the number of axons labeled with DiI, and these data were compared between the control group and the experimental groups.

4. Statistical analysis

An ANOVA was performed using StatView software (version 5.0.1, SAS Institute, Cary, NC, USA). Proliferation assay and mRNA expression data were analyzed using a one-group t-test, and the sensory test, histomorphometric analysis, and retrograde labeling data were analyzed with ANOVA. The Mann-Whitney *U* test was used for nonparametric analysis. The test results are expressed as mean ± standard deviation, and statistical significance was set as $p < 0.05$.

Ⅲ. Results

1. MSC Characterization

From the study of fluorescent dye using CD 29 and CD 105 antibodies, expression was not observed in the negative control; however, it was observed in the passage of primary cultured MSCs (Figure 3).

2. PEMF treatment

2-1. The effect of PEMF on cell proliferation

The absorbance value at a wavelength of 450 nm showed that PEMF pre-treated MSCs (PMSCs) had a higher tendency than untreated MSCs during all 5, 7, and 10 days. However, there was no statistical significance (Figure 4).

2-2. The effect of PEMF on mRNA expression level

mRNA expression levels were compared between MSCs and PEMF pre-treated MSCs (PMSCs) on days 5, 7, and 10 (Figure 5) and were graphically presented using Image J software (NIH, Bethesda, MD, USA) (Figure 6). S100, GFAP, and NGF expression levels were higher in PEMF pre-treated MSCs (PMSCs) pre-treated for 5, 7 and 10 days than in the MSCs. Specifically, the BDNF expression level of PEMF pre-treated MSCs (PMSCs) on the 10^{th} day was statistically significantly higher (*** $p < 0.001$; PMSCs vs. MSCs). Accordingly, an additional experiment was performed in which MSC cells were pre-treated with PEMF for 10 days. As a result, S100, GFAP, NGF, and BDNF expression (Figure 7) was again

observed to be statistically significantly higher for PEMF pre-treated MSCs (PMSCs) compared to MSCs (Figure 8; * $p < 0.05$, ** $p < 0.01$; PMSCs vs. MSCs).

3. The effect of PEMF on the regeneration of the nerve after a rat mental crush injury

3-1. The viability of MSCs in the mental nerve

DiI labeled MSCs and PEMF pre-treated MSCs (PMSCs) were observed. (Figure 9A). When PBS was injected in vivo as a negative control, DiI labeling was not observed within the nerve (Figure 9B a, b). DiI-labeled MSCs and PEMF pre-treated MSCs (PMSCs) were observed in the mental nerve at first and second week. Second week showed decreased labeling in both MSCs and PEMF pre-treated MSCs (PMSCs) compared to first week (Figure 9B).

3-2. Recovery of sensory function after cell therapy

All of the groups exhibited a decreased gap score at week 1 after the crush injury except the sham group. All groups seemed to recover at two weeks, and the recovery speed of the PEMF pre-treated MSCs (PMSCs) group tended to be faster than the PBS and MSC groups. Also, the difference score result was similar to the gap score result. The difference score for the PEMF pre-treated MSCs (PMSCs) group tended to increase less than that in the PBS and MSC groups. All groups tended to recover at different speeds as well as in regard to the difference score at two weeks; the PEMF pre-treated MSCs (PMSCs) group recovered fastest. However, statistical significance was not observed in either gap or difference score (Figure 10).

3-3. Histomorphometric analysis of axonal regeneration

Histomorphological sections of all groups (Figure 11) are graphically presented as myelinated axon counts and axon density (Figure 12). In myelinated axon counting, the only shape that was counted was the circle type. In the graph of myelinated axon count by group, the PEMF pre-treated MSCs (PMSCs) group was higher at a statistically significant level compared to the PBS group (+++ $p < 0.001$; PMSCs vs. PBS) and the MSC group (** $p < 0.01$; PMSCs vs. MSCs). The MSC group showed a higher value than the PBS group, and this difference was also statistically significant ($$ $p < 0.01$; MSCs vs. PBS). The axon density of the PEMF pre-treated MSCs (PMSCs) group was also statistically significantly higher than that of the PBS group (+++ $p < 0.001$; PMSCs vs. PBS, ** $p < 0.01$; PMSCs vs. PBS).

3-4. TG retrograde labeling

As for the myelinated axon, only circle-shaped DiI-labeled neurons were counted for TG retrograde labeling. DiI-positive neurons in TG of all groups (Figure 13) are graphically presented (Figure 14). The PEMF pre-treated MSCs (PMSCs) group showed a statistically significantly greater number of TG neurons compared to the PBS and MSC groups (++ $p < 0.01$; PMSCs vs. PBS, * $p < 0.05$; PMSCs vs. MSCs).

Ⅳ. Discussion

Many studies have reported the ability of MSCs to improve injury recovery and improve regeneration via growth factors, cytokines, and adhesion molecules (37, 38). In the present study, in the sensory function test, statistical significance was not observed; however, the group injected with MSCs tended to recover faster than those injected with PBS. As a result, using a histomorphometric assay, the MSC group exhibited a statistically significantly greater myelinated axon count compared to the PBS group. Statistical significance was not observed in axon density, but a higher tendency was observed. These in vivo results show that MSCs promoted the regeneration of injured nerves.

PEMF pre-treatment on cells leads to a significant increase in cell number (23); induces secretion of RA (39), FSK (40), b-FGF (41), and PDGF (42); increases nerve growth factor (NGF) secretion (43); improves MSC proliferation (23, 44); and promotes neurite outgrowth (45, 46). S100 is a calcium-binding protein that stimulates cell proliferation, and PEMF affects calcium ion channels in cell membranes (47-49). Also, PEMF enhances BDNF expression through an L-type voltage-gated calcium channel and Erk-dependent signaling pathways in neonatal rat DRG neurons (50). Based on these findings, the present study attempted to investigate the effect of PEMF application on MSC proliferation and growth factor release. Moreover, the present study aimed to evaluate the effect of nerve regeneration when PEMF pre-treated MSCs (PMSCs) were injected into an injured mental nerve. In our previous study, we showed that PEMF statistically significantly increased cell proliferation, S100, and BDNF release (29). Also, from other studies concerning MSCs, cell proliferation and growth factor release have

increased when PEMF was applied on the cell at 50 Hz (30-33). In the present study, when PEMF was applied for 5, 7, or 10 days, MSC proliferation and mRNA expression showed the highest increase after 10 days of PEMF, so 10 days was set as the duration for subsequent experiments. Also, the in vitro PEMF condition used in the present study was set at 50 Hz for 1 hour. The in vitro results of the present study confirmed that PEMF pre-treated MSCs (PMSCs) not only promoted cell proliferation, S100 levels, and GFAP levels, but also increased the release of NGF and BDNF growth factors compared to MSCs. Some papers reported that PEMF affects cell survival, propagation, and peripheral nerve regeneration in regard to the intensity, time interval, and frequency (23, 24). To summarize these results, PEMF application on MSCs increases cell proliferation, which induces more growth factor release and conclusively helps regeneration of injured nerves. The PEMF device used in the present study could be set at various frequencies (50, 60, 100, or 150 Hz) and times (1 or 12 hours); however, the intensity could only be set to 1 mT. Therefore, to further optimize PEMF for peripheral nerve regeneration, a PEMF device is needed that can be set to various frequencies, intensities, and times. To confirm a positive effect of 10-day PEMF on MSCs, additional evaluations such as Western blot and neurite length measurement should be performed in future studies.

After injecting PEMF pre-treated MSCs (PMSCs) in vivo for 10 days into the injured rat mental nerve, a sensory function test and an axonal regeneration assessment were performed. A mental nerve sensory test of crush-injured rats was performed using the method by Seino et al (36). Several studies have reported that injured nerve recovery is observed between one to two weeks (29, 51-53). To assess the sensory nerve function objectively in this study, a blind test was performed with the rats in all groups. A pre-test of sensory function was performed before crush injury, and the test was performed one and two weeks post-surgery.

When the ipsilateral lip area (a), ipsilateral mental area (b), and contralateral area (d) were stimulated with the filament, the act of only raising the forefoot was set as positive reaction, and this was recorded numerically. Regarding both gap score and difference score, all of the experimental groups except the sham group showed damage in the sensory function of the mental nerve one week post-surgery. All of the experiment groups seemed to have started to recover at two weeks. The present results agree with the literature mentioned above. Among the experimental groups, the sensory function recovery rate of the PEMF pre-treated MSCs (PMSCs) group was faster than that of the MSC and PBS groups. However, a statistically significant difference was not seen. In the sensory function test using filaments, subjectivity could not be entirely eliminated as the reaction of the animal is determined by the experimenter's subjective judgement. Moreover, because the test was carried out while the experimenter was holding filaments with their hands, there could have been variations in the uniformity of the pressure that was delivered. So, there is a need for alternative methods to confirm sensory nerve function recovery.

According to histological analysis of published studies, total axon number and axon density are crucial in observing axonal nerve regeneration (54, 55). Retrograde labeling with dye is an important method by which to distinguish motor neurons from sensory neurons and to explore the relationship between the spinal cord and peripheral nerves (56). To obtain functional recovery after injury, injured peripheral nerve fibers must grow into the correct target organ (56, 57). In regard to myelinated axon count and density, all experiment groups in the present study showed lower values compared to the sham group. Both PEMF pre-treated MSCs (PMSCs) and MSC groups showed a tendency for higher myelinated axons and a greater density compared to the PBS group; among the experimental groups, the

PEMF pre-treated MSCs (PMSCs) group was observed to be statistically significantly higher than the other groups. However, other studies reported that nerve fiber diameter is the most reliable parameter to compare experimental groups and individuals within the same group (58, 59). Another study determined that axonal diameter and the width and length of the myelin sheath are the most relevant factors regarding histologic nerve recovery (60). Hence, measurement of axon diameter, width, and length of the myelin sheath are required in further study. In retrograde labeling results, all experimental groups showed lower values compared to the sham group. The PEMF pre-treated MSCs (PMSCs) group exhibited significantly higher retrograde tracing compared to the MSC and PBS groups. Although statistical significance was not observed, the MSC group showed a higher tendency compared to the PBS group. In conclusion, PEMF pre-treatment of MSCs promoted nerve regeneration, as observed in a proliferation assay, via mRNA expression level, and in a sensory test.

Ⅴ. Conclusion

PEMF application in vitro increased not only the growth rate of MSCs, but also the expression of nerve growth factors. Moreover, when these PEMF pre-treated MSCs (PMSCs) were injected into an injured nerve, they were observed to be more effective at promoting nerve regeneration than untreated MSCs. This suggests PEMF pre-treatment of MSCs as an enhanced strategic tool in cell therapy for recovery of injured nerves.

References

1. Li BH, Kim SM, Yoo SB, Kim MJ, Jahng JW, Lee JH. Recombinant human nerve growth factor (rhNGF-beta) gene transfer promotes regeneration of crush-injured mental nerve in rats. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012;113(3):e26-34.
2. Lee SK, Wolfe SW. Peripheral nerve injury and repair. J Am Acad Orthop Surg. 2000;8(4):243-52.
3. Kilmer SL, Carlsen RC. Chronic infusion of agents that increase cyclic AMP concentration enhances the regeneration of mammalian peripheral nerves in vivo. Exp Neurol. 1987;95(2):357-67.
4. Aebischer P, Valentini RF, Dario P, Domenici C, Galletti PM. Piezoelectric guidance channels enhance regeneration in the mouse sciatic nerve after axotomy. Brain Res. 1987;436(1):165-8.
5. Chernousov MA, Carey DJ. Schwann cell extracellular matrix molecules and their receptors. Histol Histopathol. 2000;15(2):593-601.
6. Fu SY, Gordon T. The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol. 1997;14(1-2):67-116.
7. Goldberg JL, Barres BA. The relationship between neuronal survival and regeneration. Annu Rev Neurosci. 2000;23:579-612.
8. Ide C. Peripheral nerve regeneration. Neurosci Res. 1996;25(2):101-21.
9. Lonze BE, Riccio A, Cohen S, Ginty DD. Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron. 2002;34(3):371-85.
10. Mimura T, Dezawa M, Kanno H, Sawada H, Yamamoto I. Peripheral nerve regeneration by transplantation of bone marrow stromal cell-derived Schwann

cells in adult rats. J Neurosurg. 2004;101(5):806-12.

11. Bunge RP. The role of the Schwann cell in trophic support and regeneration. J Neurol. 1994;242(1 Suppl 1):S19-21.
12. Scherer SS. The biology and pathobiology of Schwann cells. Curr Opin Neurol. 1997;10(5):386-97.
13. Mosahebi A, Woodward B, Wiberg M, Martin R, Terenghi G. Retroviral labeling of Schwann cells: in vitro characterization and in vivo transplantation to improve peripheral nerve regeneration. Glia. 2001;34(1):8-17.
14. Kobayashi M, Ishibashi S, Tomimitsu H, Yokota T, Mizusawa H. Proliferating immature Schwann cells contribute to nerve regeneration after ischemic peripheral nerve injury. J Neuropathol Exp Neurol. 2012;71(6):511-9.
15. Mosahebi A, Fuller P, Wiberg M, Terenghi G. Effect of allogeneic Schwann cell transplantation on peripheral nerve regeneration. Exp Neurol. 2002;173(2):213-23.
16. Dai LG, Huang GS, Hsu SH. Sciatic nerve regeneration by cocultured Schwann cells and stem cells on microporous nerve conduits. Cell Transplant. 2013;22(11):2029-39.
17. Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts. Proc Natl Acad Sci U S A. 1998;95(7):3908-13.
18. Koh KS, Oh TS, Kim H, Chung IW, Lee KW, Lee HB, Park EJ, Jung JS, Shin IS, Ra JC, Choi JW. Clinical application of human adipose tissue-derived mesenchymal stem cells in progressive hemifacial atrophy (Parry-Romberg disease) with microfat grafting techniques using 3-dimensional computed tomography and 3-dimensional camera. Ann Plast Surg. 2012;69(3):331-7.

19. Parekkadan B, Milwid JM. Mesenchymal stem cells as therapeutics. Annu Rev Biomed Eng. 2010;12:87-117.

20. Sung MA, Jung HJ, Lee JW, Lee JY, Pang KM, Yoo SB, Alrashdan MS, Kim SM, Jahng JW, Lee JH. Human umbilical cord blood-derived mesenchymal stem cells promote regeneration of crush-injured rat sciatic nerves. Neural Regen Res. 2012;7(26):2018-27.

21. Cooney DS, Wimmers EG, Ibrahim Z, Grahammer J, Christensen JM, Wu LW, Sarhane KA, Lopez J, Wallner C, Furtmüller GJ, Yuan N, Pang J, Sarkar K, Lee WP, Brandacher G. Mesenchymal Stem Cells Enhance Nerve Regeneration in a Rat Sciatic Nerve Repair and Hindlimb Transplant Model. Sci Rep. 2016;6:31306.

22. Tohill M, Mantovani C, Wiberg M, Terenghi G. Rat bone marrow mesenchymal stem cells express glial markers and stimulate nerve regeneration. Neurosci Lett. 2004;362(3):200-3.

23. Sun LY, Hsieh DK, Yu TC, Chiu HT, Lu SF, Luo GH, Kuo TK, Lee OK, Chiou TW. Effect of pulsed electromagnetic field on the proliferation and differentiation potential of human bone marrow mesenchymal stem cells. Bioelectromagnetics. 2009;30(4):251-60.

24. Kim BC, Bae H, Kwon IK, Lee EJ, Park JH, Khademhosseini A, Hwang YS. Osteoblastic/cementoblastic and neural differentiation of dental stem cells and their applications to tissue engineering and regenerative medicine. Tissue Eng Part B Rev. 2012;18(3):235-44.

25. Lacy-Hulbert A, Metcalfe JC, Hesketh R. Biological responses to electromagnetic fields. FASEB J. 1998;12(6):395-420.

26. Cho H, Seo YK, Yoon HH, Kim SC, Kim SM, Song KY, Park JK. Neural stimulation on human bone marrow-derived mesenchymal stem cells by

extremely low frequency electromagnetic fields. Biotechnol Prog. 2012;28(5):1329-35.

27. Deng W, Bivalacqua TJ, Chattergoon NN, Hyman AL, Jeter JR, Jr., Kadowitz PJ. Adenoviral gene transfer of eNOS: high-level expression in ex vivo expanded marrow stromal cells. Am J Physiol Cell Physiol. 2003;285(5):C1322-9.
28. Deng W, Bivalacqua TJ, Chattergoon NN, Jeter JR, Jr., Kadowitz PJ. Engineering ex vivo-expanded marrow stromal cells to secrete calcitonin gene-related peptide using adenoviral vector. Stem Cells. 2004;22(7):1279-91.
29. Hei WH, Byun SH, Kim JS, Kim S, Seo YK, Park JC, Kim SM, Jahng JW, Lee JH. Effects of electromagnetic field (PEMF) exposure at different frequency and duration on the peripheral nerve regeneration: in vitro and in vivo study. Int J Neurosci. 2016;126(8):739-48.
30. Li X, Zhang M, Bai L, Bai W, Xu W, Zhu H. Effects of 50 Hz pulsed electromagnetic fields on the growth and cell cycle arrest of mesenchymal stem cells: an in vitro study. Electromagn Biol Med. 2012;31(4):356-64.
31. Sert C, Mustafa D, Duz MZ, Aksen F, Kaya A. The preventive effect on bone loss of 50-Hz, 1-mT electromagnetic field in ovariectomized rats. J Bone Miner Metab. 2002;20(6):345-9.
32. Feng X, He X, Li K, Wu W, Liu X, Li L. The effects of pulsed electromagnetic fields on the induction of rat bone marrow mesenchymal stem cells to differentiate into cardiomyocytes-like cells in vitro.
33. Bai WF, Xu WC, Feng Y, Huang H, Li XP, Deng CY, Zhang MS. Fifty-Hertz electromagnetic fields facilitate the induction of rat bone mesenchymal stromal cells to differentiate into functional neurons. Cytotherapy. 2013;15(8):961-70.
34. Kashani IR, Golipoor Z, Akbari M, Mahmoudi R, Azari S, Shirazi R, Bayat M, Ghasemi S. Schwann-like cell differentiation from rat bone marrow stem cells.

Arch Med Sci. 2011;7(1):45-52.

35. Jiang H, Qu W, Han F, Liu D, Zhang W. Establishment of immortalized Schwann cells derived from rat embryo dorsal root ganglia. Int J Mol Med. 2012;30(3):480-6.
36. Seino H, Seo K, Maeda T, Someya G. Behavioural and histological observations of sensory impairment caused by tight ligation of the trigeminal nerve in mice. J Neurosci Methods. 2009;181(1):67-72.
37. Bhagavati S, Xu W. Isolation and enrichment of skeletal muscle progenitor cells from mouse bone marrow. Biochem Biophys Res Commun. 2004;318(1):119-24.
38. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simontetti Dw, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143-7.
39. Pirozzoli MC, Marino C, Lovisolo GA, Laconi C, Mosiello L, Negroni A. Effects of 50 Hz electromagnetic field exposure on apoptosis and differentiation in a neuroblastoma cell line. Bioelectromagnetics. 2003;24(7):510-6.
40. Cain CD, Adey WR, Luben RA. Evidence that pulsed electromagnetic fields inhibit coupling of adenylate cyclase by parathyroid hormone in bone cells. J Bone Miner Res. 1987;2(5):437-41.
41. Hopper RA, VerHalen JP, Tepper O, Mehrara BJ, Detch R, Chang EI, Baharestani S, Simon BJ, Gurtner GC. Osteoblasts stimulated with pulsed electromagnetic fields increase HUVEC proliferation via a VEGF-A independent mechanism. Bioelectromagnetics. 2009;30(3):189-97.
42. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Med. 2011;9:66.
43. Longo FM, Yang T, Hamilton S, Hyde JF, Walker J, Jennes L, Stach R, Sisken

BF. Electromagnetic fields influence NGF activity and levels following sciatic nerve transection. J Neurosci Res. 1999;55(2):230-7.

44. Luo F, Hou T, Zhang Z, Xie Z, Wu X, Xu J. Effects of pulsed electromagnetic field frequencies on the osteogenic differentiation of human mesenchymal stem cells. Orthopedics. 2012;35(4):e526-31.
45. Zhang Y, Ding J, Duan W. A study of the effects of flux density and frequency of pulsed electromagnetic field on neurite outgrowth in PC12 cells. J Biol Phys. 2006;32(1):1-9.
46. Lekhraj R, Cynamon DE, DeLuca SE, Taub ES, Pilla AA, Casper D. Pulsed electromagnetic fields potentiate neurite outgrowth in the dopaminergic MN9D cell line. J Neurosci Res. 2014;92(6):761-71.
47. Shah J, Midkiff P, Brandt P, Sisken B. Growth and differentiation of PC6 cells: the effects of pulsed electromagnetic fields (PEMF). Bioelectromagnetics. 2001;22(4):267-71.
48. Zienowicz RJ, Thomas BA, Kurtz WH, Orgel MG. A multivariate approach to the treatment of peripheral nerve transection injury: the role of electromagnetic field therapy. Plastic and reconstructive surgery. 1991;87(1):122-9.
49. Grant G, Cadossi R, Steinberg G. Protection against focal cerebral ischemia following exposure to a pulsed electromagnetic field. Bioelectromagnetics. 1994;15(3):205-16.
50. Li Y, Yan X, Liu J, Li L, Hu X, Sun H, Tian J. Pulsed electromagnetic field enhances brain-derived neurotrophic factor expression through L-type voltage-gated calcium channel-and Erk-dependent signaling pathways in neonatal rat dorsal root ganglion neurons. Neurochemistry international. 2014;75:96-104.
51. Kim YT, Hei WH, Kim S, Seo YK, Kim SM, Jahng JW, Lee JH. Co-treatment effect of pulsed electromagnetic field (PEMF) with human dental pulp stromal

cells and FK506 on the regeneration of crush injured rat sciatic nerve. Int J Neurosci. 2015;125(10):774-83.

52. Hildebrand C, Fried K, Tuisku F, Johansson CS. Teeth and tooth nerves. Prog Neurobiol. 1995;45(3):165-222.
53. Naftel JP, Richards LP, Pan M, Bernanke JM. Course and composition of the nerves that supply the mandibular teeth of the rat. Anat Rec. 1999;256(4):433-47.
54. Hubbard JH. The quality of nerve regeneration. Factors independent of the most skillful repair. Surg Clin North Am. 1972;52(5):1099-108.
55. Vasconcelos BC, Gay-Escoda C. Facial nerve repair with expanded polytetrafluoroethylene and collagen conduits: an experimental study in the rabbit. J Oral Maxillofac Surg. 2000;58(11):1257-62.
56. Yu Yl, Li Hy, Zhang Px, Yin Xf, Han N, Kou Yh, Jiang Bg. Comparison of commonly used retrograde tracers in rat spinal motor neurons. Neural regeneration research. 2015;10(10):1700.
57. Hayashi A, Moradzadeh A, Hunter DA, Kawamura DH, Puppala VK, Tung TH, mackinnon SE, Myckatyn TM. Retrograde labeling in peripheral nerve research: it is not all black and white. J Reconstr Microsurg. 2007;23(7):381-9.
58. Fernand V, Young J. The sizes of the nerve fibres of muscle nerves. Proceedings of the Royal Society of London B: Biological Sciences. 1951;139(894):38-58.
59. Donovan A. The nerve fibre composition of the cat optic nerve. J Anat. 1967;101(Pt 1):1-11.
60. May M, Schaitkin BM. The facial nerve: May's second edition: George Thieme Verlag; 2000.

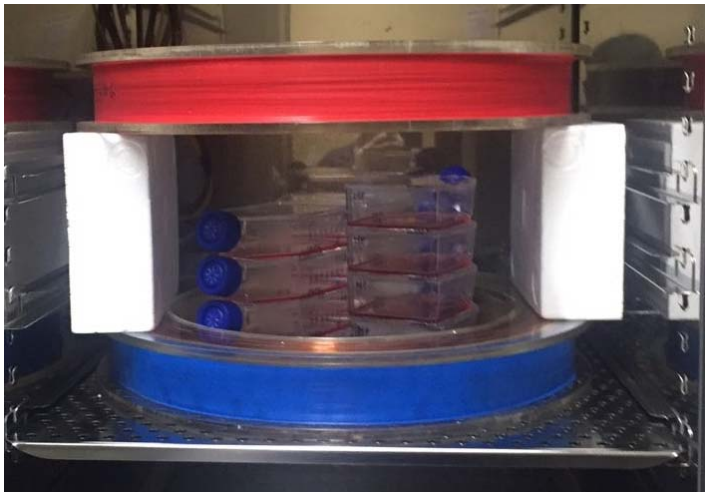

Figure 1. Pulsed Electromagnetic Field (PEMF) device. Two Helmholtz coils of 30-cm diameter, 7-cm width, and 15-cm distance apart; the PEMF device was placed in a CO_2 incubator. Cell culture dishes were placed at the center between the coils for 5, 7, or 10 days.

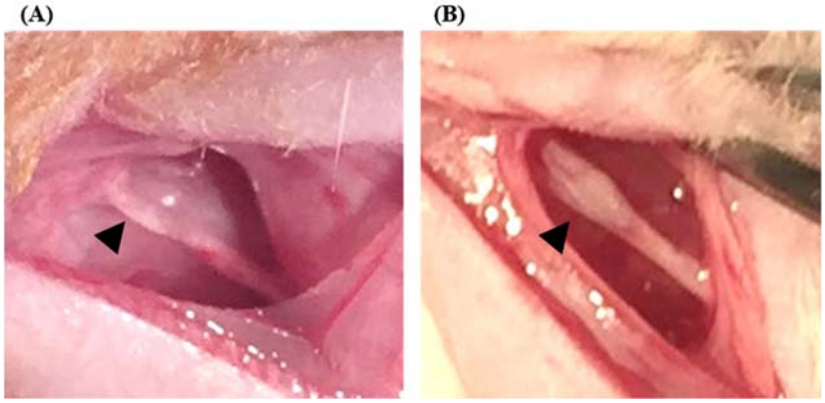

Figure 2. Mental nerve crush injury model. (A) The mental nerve with a 3-mm crush injury using a needle holder. The black arrow indicates the site of PEMF pre-treated MSCs (PMSCs) or MSC injection using a Hamilton syringe after the mental nerve crush injury.

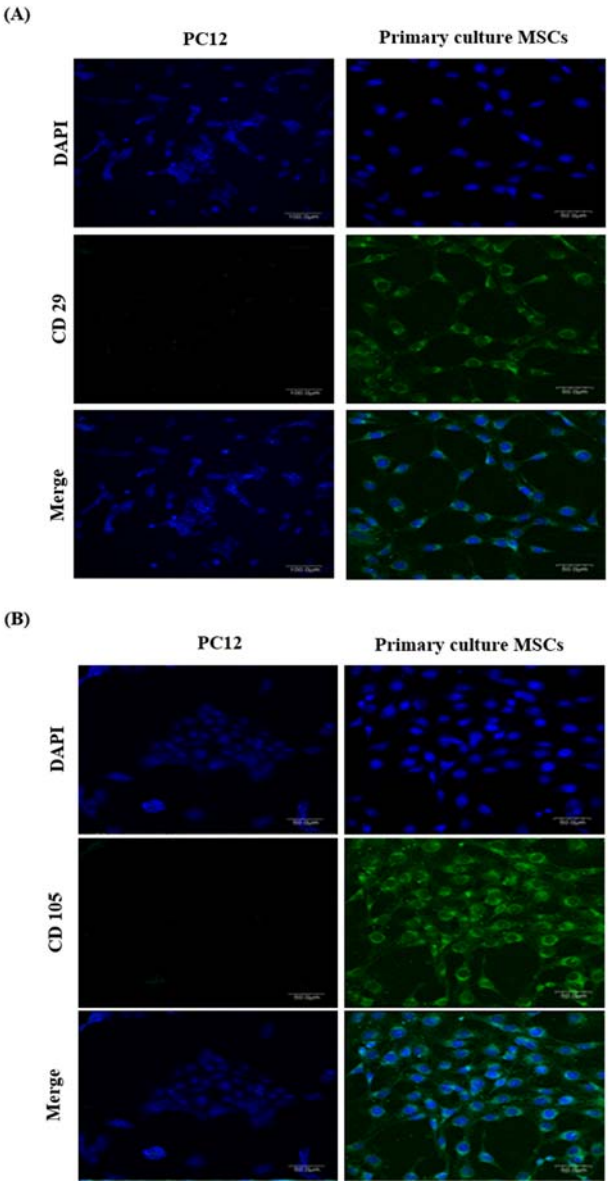

Figure 3. Immunostaining of primary cultured MSCs with CD 29 and CD 105. Primary cultured passage 5 MSCs expressed positive (A) CD 29 and (B) CD 105. However, PC12 cells did not. Nuclei were stained with DAPI (blue). Scale bars = 50 μm.

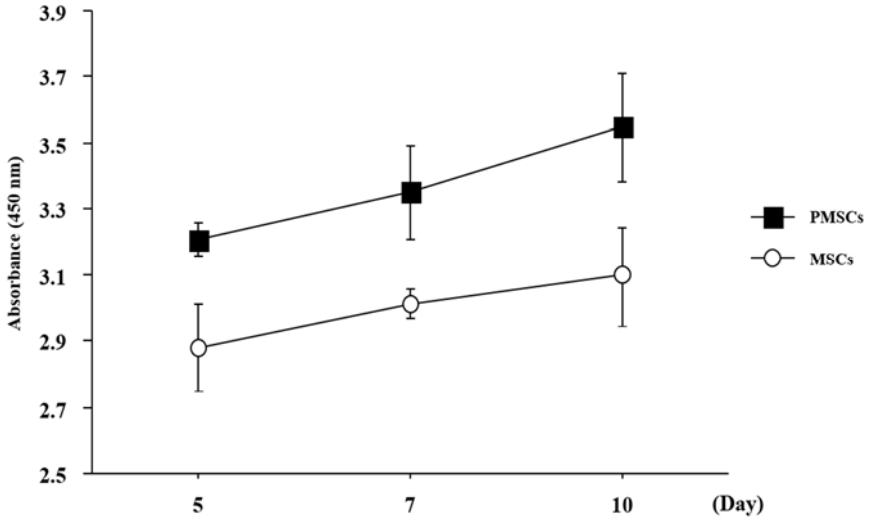

Figure 4. The effect of PEMF pre-treatment on MSC proliferation. Cell proliferation was measured at a wavelength of 450 nm. PEMF pre-treated MSCs (PMSCs) were higher than MSCs on the 5th, 7th, and 10th days. (n=6 / group. Data are presented as mean ± SEM.)

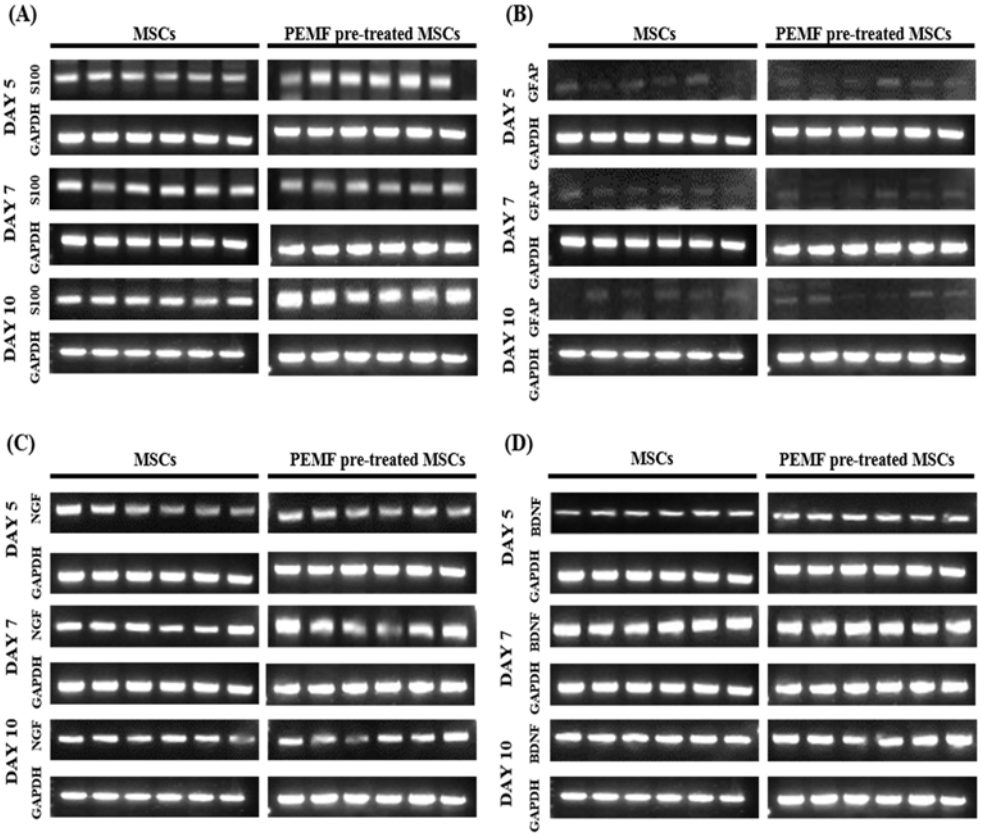

Figure 5. mRNA expression. mRNA levels of (A) S100, (B) GFAP, (C) NGF, and (D) BDNF were higher for PEMF pre-treated MSCs (PMSCs) than MSCs on the 5^{th}, 7^{th}, and 10^{th} days.

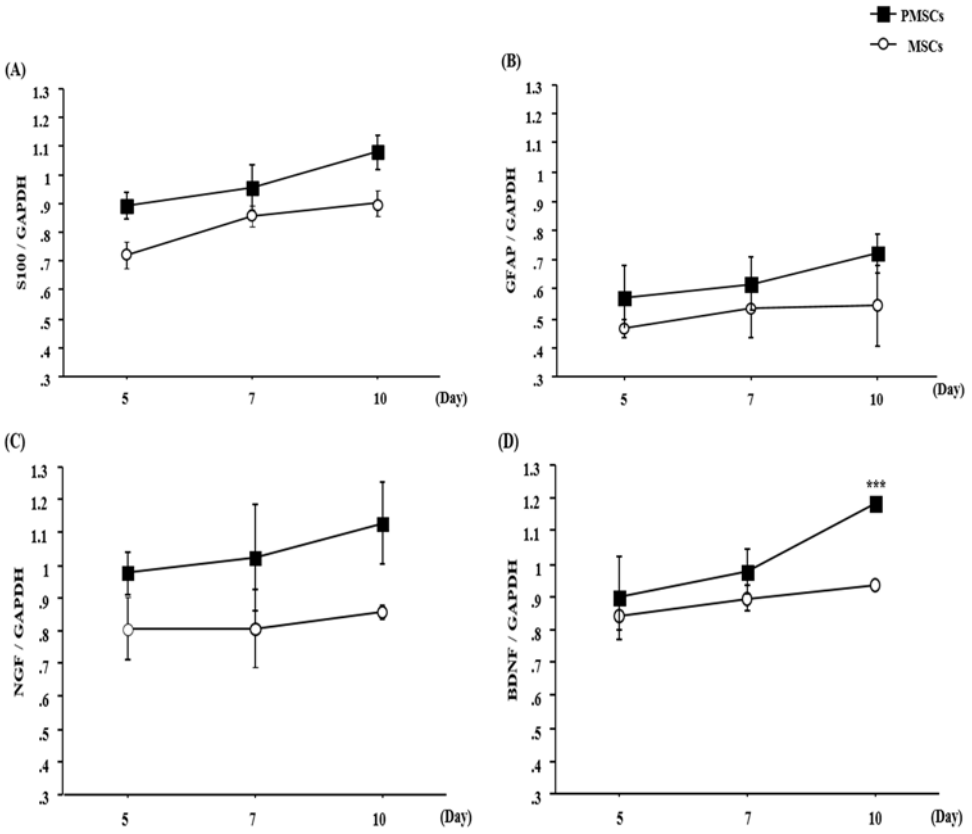

Figure 6. Graphs of each primer numerically expressed using ImageJ. mRNA expression levels of (A) S100, (B) GFAP, and (C) NGF were higher in PEMF pre-treated MSCs (PMSCs) than MSCs. mRNA expression level of (D) BDNF was significantly higher in the PEMF pre-treated MSCs (PMSCs) than in the MSCs (*** $p < 0.001$). PEMF pre-treated MSCs (PMSCs) was highest on the 10^{th} day. (n=6 / group. Data are presented as mean ± SEM.)

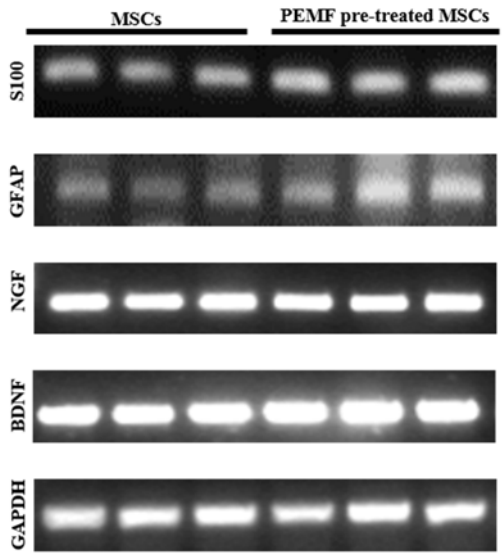

Figure 7. mRNA expression in MSCs pre-treated with PEMF for 10 days. mRNA expression levels of (A) S100, (B) GFAP, (C) NGF, and (D) BDNF were higher in PEMF pre-treated MSCs (PMSCs) than untreated MSCs.

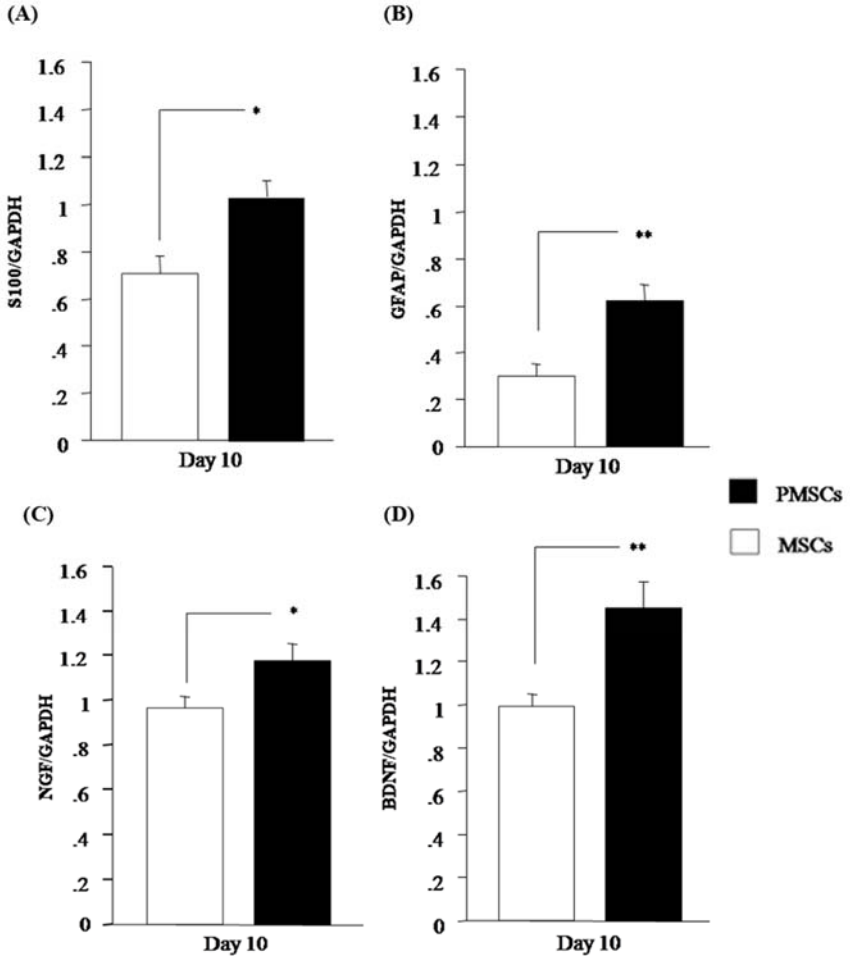

Figure 8. Graphs of each primer numerically expressed using ImageJ. (A) S100, (B) GFAP, (C) NGF, and (D) BDNF expression levels were higher for PEMF pre-treated MSCs (PMSCs) than MSCs at a statistically significant level. * $p < 0.05$; vs. MSCs, ** $p < 0.01$; vs. MSCs. (n=6 / group. Data are presented as mean ± SEM.)

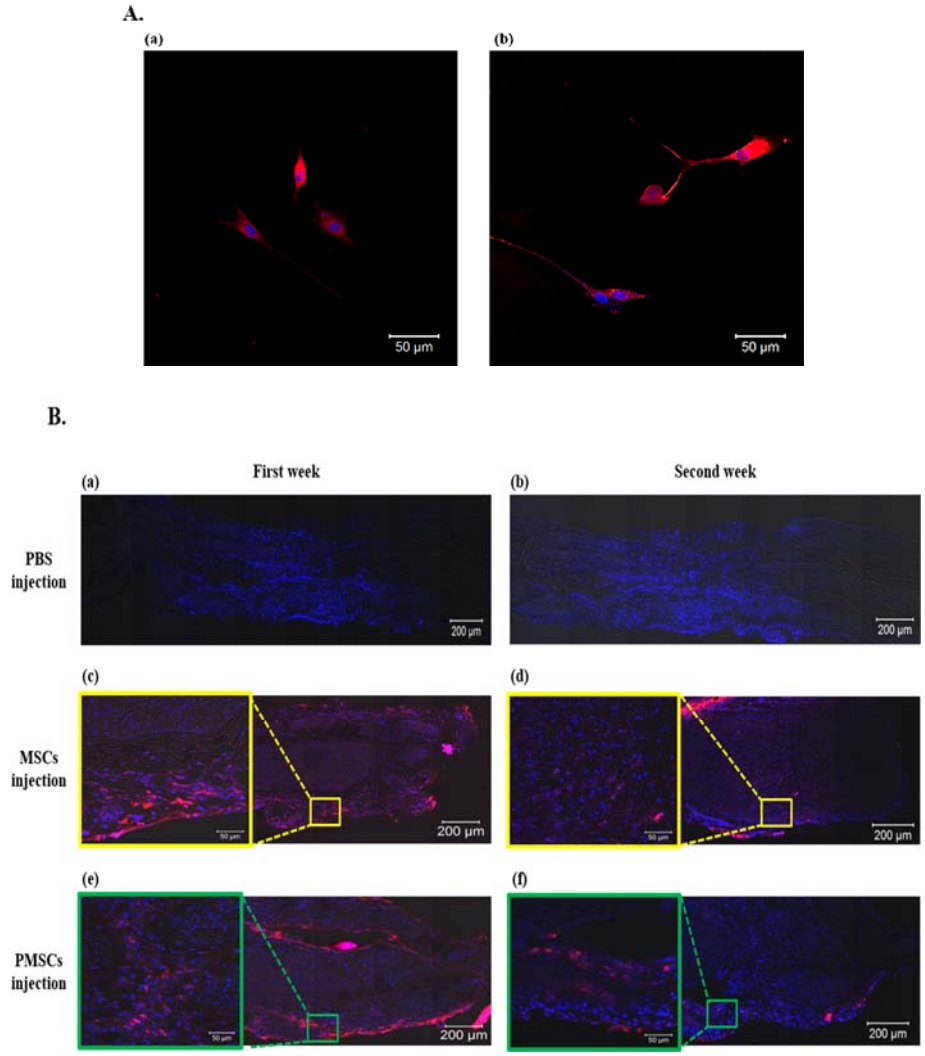

Figure 9. Cell tracking with DiI-labeled MSCs, PMSCs. A. DiI-labeled (a) MSCs and (b) PEMF pre-treated MSCs (PMSCs) were observed, Scale bars = 50 μm. B. DiI-labeled MSCs (c, d) and PMSCs (e, f) in vivo with negative control PBS injection (a, b) harvested after one week after injection (a, c, e) and two weeks after injection (b, d, f). DiI labeling; red, DAPI; blue. Scale bars = 200 μm. Boxed area in right image were enlarged, scale bars = 50 μm.

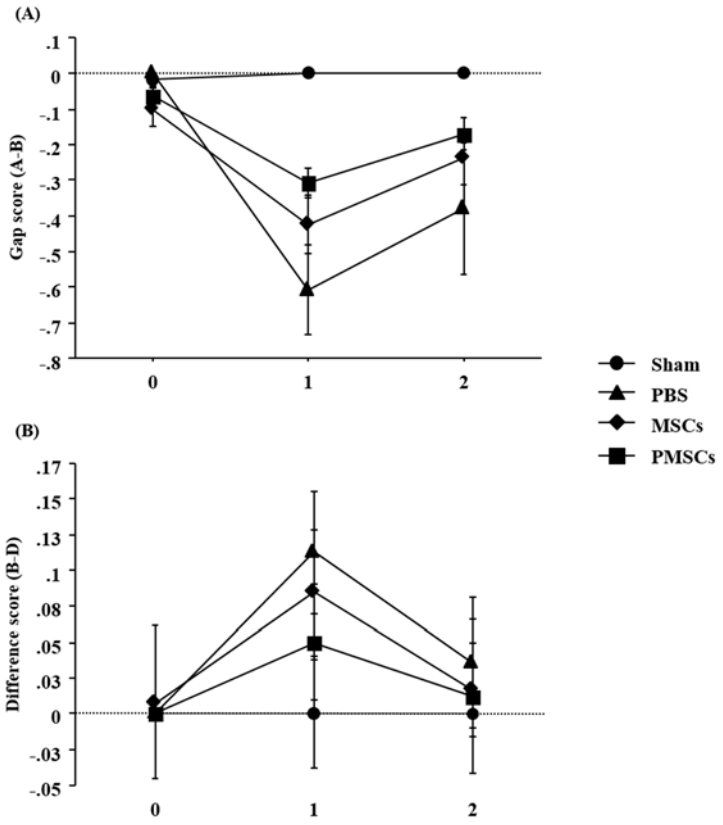

Figure 10. Results of the mental nerve sensory test for all groups. (A) The gap score values for all groups decreased in response to the crush injury at 1 week and began to recover at 2 weeks. (B) The difference scores of all groups increased due to the crush injury at 1 week and subsequently decreased at 2 weeks. The recovery rate was highest for the PEMF pre-treated MSC (PMSC) group. (n=6 / group. Data are presented as mean ± SEM.)

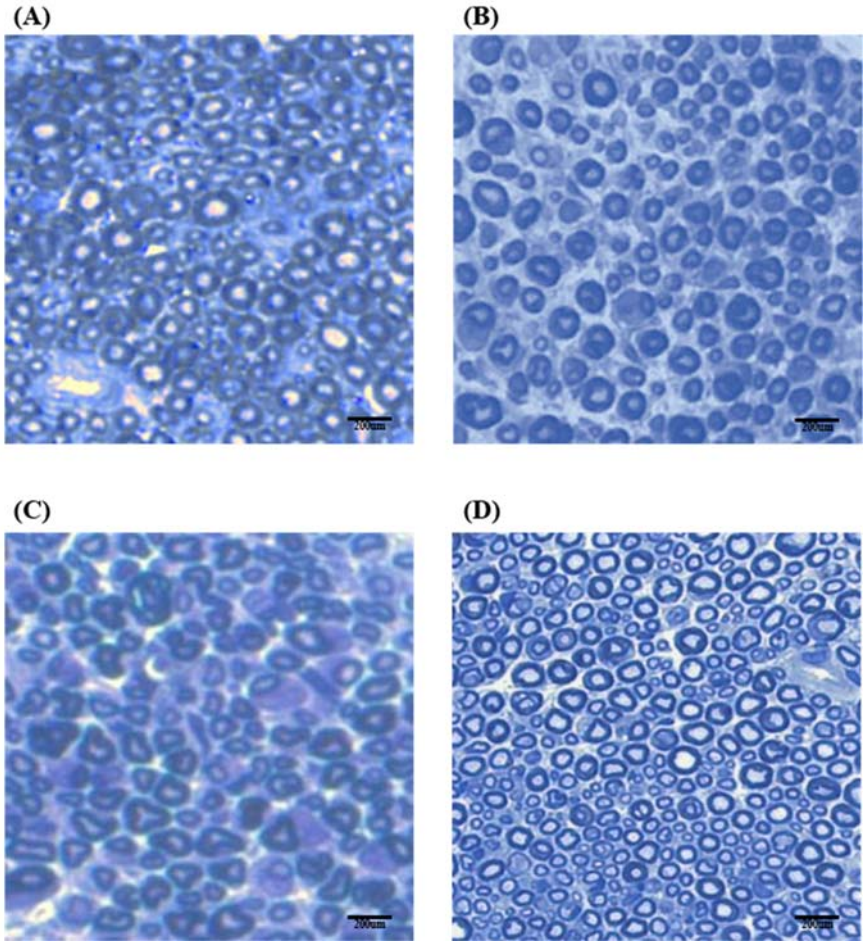

Figure 11. Photomicrographs of histologic features in semi-thin sections of the nerve. The mental nerve of the (A) Sham, (B) PBS, (C) MSCs, and (D) PEMF pre-treated MSCs (PMSCs) groups was harvested at two weeks post-injury. Scale bars = 200 μm.

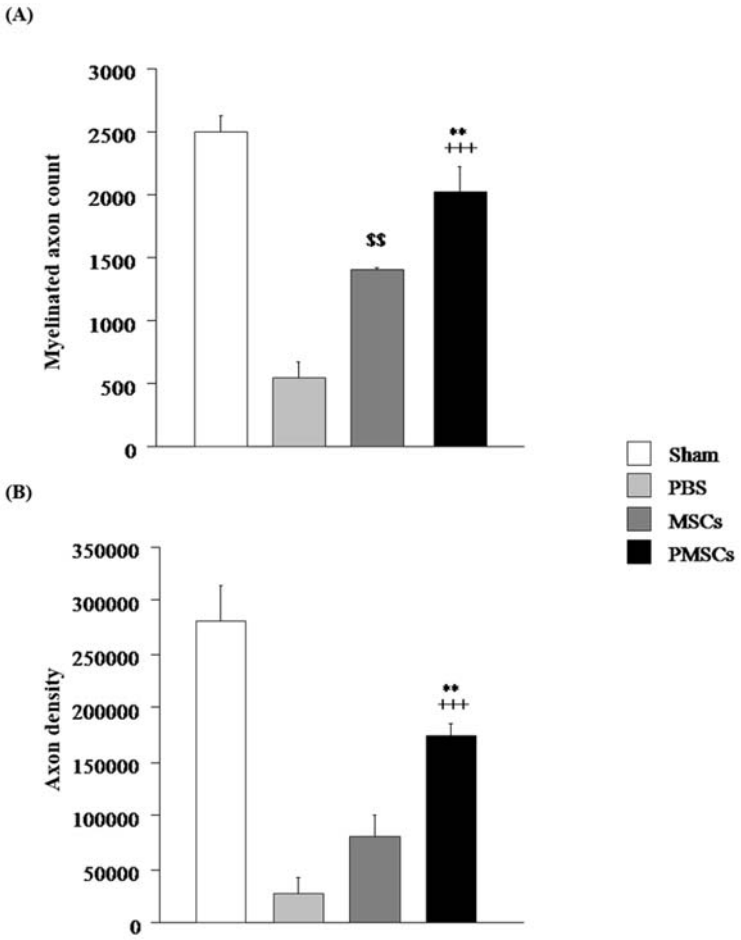

Figure 12. Comparison of axon regeneration in all groups. (A) Myelinated axon count (+++ $p < 0.001$; PMSCs vs. PBS, ** $p < 0.01$; PMSCs vs. MSCs, $$ $p < 0.01$; MSCs vs. PBS) and (B) axon density (+++ $p < 0.001$; PMSCs vs. PBS, ** $p < 0.05$; PMSCs vs. MSCs) were significantly higher for PEMF pre-treated MSCs (PMSCs) than for MSCs and PBS. (n=6 / group. Data are presented as mean ± SEM.)

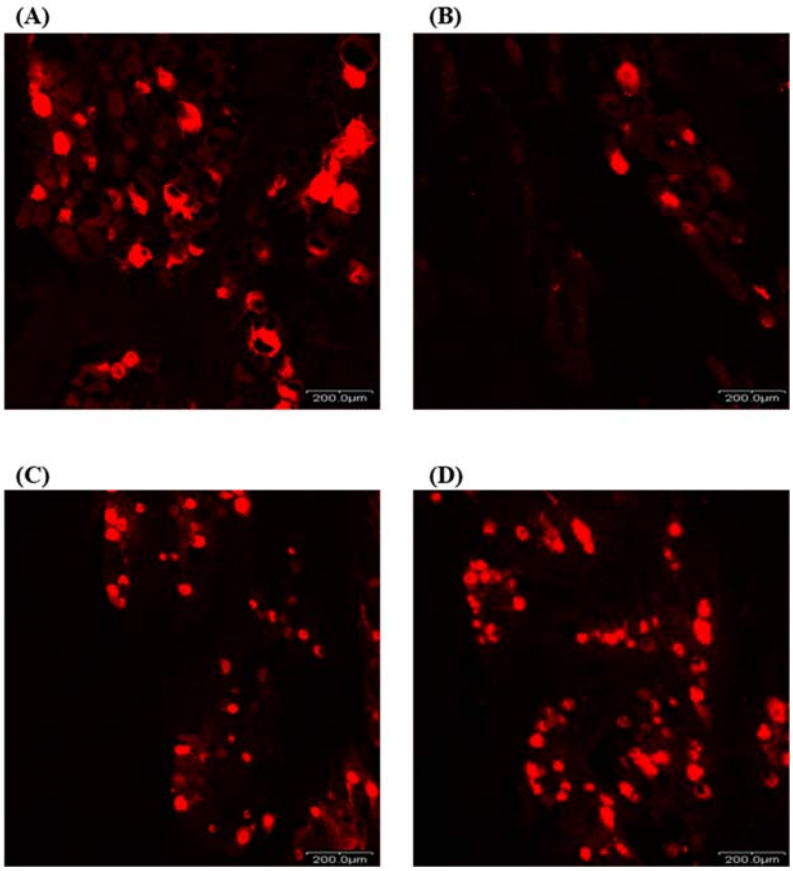

Figure 13. Retrograde TG labeling with DiI. (A) Sham, (B) PBS, (C) MSCs, and (D) PEMF pre-treated MSCs (PMSCs). PMSCs showed more positive TG neurons than those in the MSC and PBS groups. Scale bars = 200 μm.

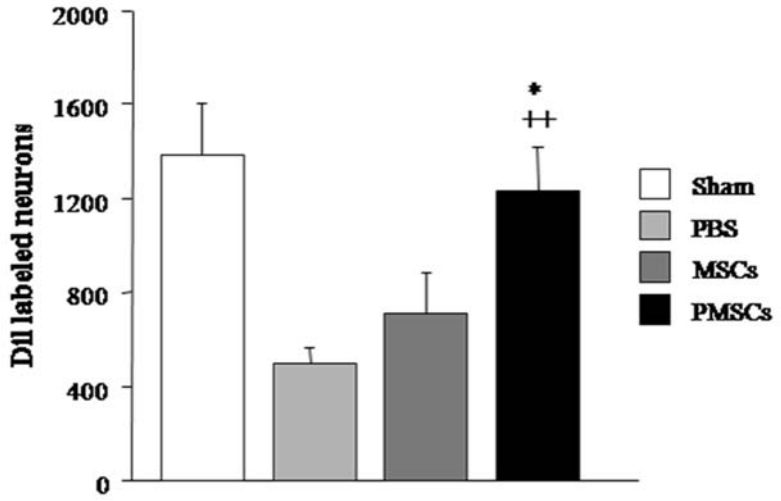

Figure 14. Comparison of DiI-labeled neurons between groups. There were significantly more DiI-labeled neurons in the PEMF pre-treated MSCs (PMSCs) group compared to the MSC and PBS groups (++ $p < 0.01$; PMSCs vs. PBS, * $p < 0.05$; PMSCs vs. MSCs). (n=6 / group. Data are presented as mean ± SEM.)

국문초록

전자기장 전처리
중간엽줄기세포를 이용한
백서 아랫턱신경 압박 손상 재생 연구

서울대학교 대학원 치의과학과 신경생물학 전공

(지도교수: 이 종 호)

서 나 리

1. 목 적

말초신경손상은 치과임상에서 종종 접하게 된다. 손상된 말초신경을 재생시키기 위한 많은 방법 중 세포치료법이 시도되고 있는데, 이러한 목적으로 사용되는 세포는 채취와 중식이 용이하고 그리고 면역학적으로 안정적이어야 한다. 중간엽줄기세포는 이러한 면에서 손상된 말초신경의 재생을 도모하는 새로운 방법으로서 제시되고 있다. 전자기장 또한 손상된 신경을 재생에 도움이 된다고 알려져 있다. 전자기장의 체외실험에서의 적용은 세포의 증식 속도와 신경성장인자의 배출을 증가시켜 주며 특히 Schwann like cell 로서의 분화를 유도함이 보고되었다. 체내실험에서 전자기장은 손상된 말초신경, 운동, 감각 뉴런의 재생을 향상 시켜준다.

본 연구에서는 체외실험에서 전자기장의 적용이 중간엽줄기세포의 증식과 신경성장인자 발현 정도에서 어떠한 차이를 보이는지 관찰하고자 하였으며, 체내실험으로 아랫턱신경 압박 손상을 가한 백서에 전자기장 전처리 중간엽줄기세포를 주입하였을 때 나타나는 말초 신경의 재생 효과를 관찰하고자 하였다.

2. 방 법

본 연구에서는 5 주령 백서의 (200-250g) 정강이, 넙다리 뼈에서 유래된 중간엽줄기세포를 사용하였으며, CD29 와 CD105 항체로 면역 형광 염색을 실시하였다. 전자기장이 (50 Hz, 1 hour/day, 1 mT) 중간엽줄기세포에 미치는 영향을 관찰 하기 위하여 중간엽줄기세포의 증식 반응과, S100, GFAP, BDNF 와 NGF 의 mRNA 발현을 관찰하였다. 체내 실험은 Sham, PBS, 중간엽줄기세포 주입군, 전자기장 전처리 중간엽줄기세포 주입군 등 총 4 그룹으로 진행하였다. 5 주령 백서를 복강 마취하여 좌측 턱 신경을 노출 시킨 후 지침기를 이용하여 3 mm 폭의 압박 손상을 1 분간 가하였다. 손상 유발된 신경에 중간엽줄기세포와 전자기장 전처리 중간엽줄기세포 ($1x10^6$ 개)는 5 μl 의 PBS 에 현탁 한 뒤, 해밀턴실린지를 이용하여 주입하였다. 주입된 중간엽줄기세포의 생존을 확인하기 위하여 GFP 표지 한 뒤, 2 주간 관찰하였다. 아랫턱신경 기능 변화를 관찰 하기 위하여 술 전과 술 후 1 주, 2 주에 필라멘트를 이용하여 감각 검사를 실시하였다. 축삭 재생을 평가하기 위하여 술 후 2 주에 신경 손상 정중부를 횡절단하고 두께 0.45 ㎛ 시편을 만들어 toluidine blue 염색을 한 후, 축삭 수와 밀도를

측정하였다. 2 주 후 턱 좌측 신경을 노출시켜 DiI 를 흡수 시키고 5 일 후, 삼차신경절을 채취하여 역행성 축삭 수송기능을 관찰하였다.

3. 결 과

전자기장 전처리 중간엽줄기세포 군은 전처리 하지 않은 군에 비하여 세포 증식 속도뿐만 아니라, 신경성장인자 발현 정도 또한 더 높은 것을 관찰되었다. 턱 신경 감각검사에서는 전자기장 전처리 중간엽줄기세포 주입군이 전처리 하지 않은 중간엽줄기세포 주입군보다 더 빠른 회복을 보이는 것을 관찰하였다. 전체 축삭 수 및 밀도에서는 전자기장 전처리 중간엽줄기세포 주입군이 다른 군과 비교하였을 때 축삭 밀도가 더 높은 것으로 보아, 축삭의 재생과 기능적 회복이 촉진되었음을 관찰하였다. 또한 역행성 축삭 수송기능 평가에서 전자기장 전처리 중간엽줄기세포 주입군이 다른 군에 비하여 염색된 뉴런 계수가 더 높음을 관찰되었다.

4. 결론

체외실험에서 전자기장의 적용은 세포의 증식 속도는 물론 신경성장인자 발현도 증가시켰다. 또한 전자기장 전처리 중간엽줄기세포를 손상된 신경에 주입하였을 때, 전처리 하지 않은 중간엽줄기세포보다 재생에 더 효과적임을 관찰 할 수 있었다. 이러한 결과는 전자기장 전처리 중간엽줄기세포가 손상된 신경의 회복을 위한 세포실험법에서 보다 향상된 치료기법으로서 가능성을 보여주었다.

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주요어 : 중간엽줄기세포, 전자기장, 말초신경재생, 턱(이)신경, 압박신경손상
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