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Microelectric Treatment by Transcutaneous Electrical Nerve Stimulation in a Rat Model of Acute Spinal Cord Injury

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Study Design: Animal study.

Objectives: To investigate the effects of microelectric treatment by transcutaneous electrical nerve stimulation (TENS) on functional recovery and histological changes in a rat model of spinal cord injury (SCI).

Summary of Literature Review: The effects of TENS on spasticity and its underlying mechanisms remain unclear.

Materials and Methods: SCI was induced by a 1.5-mm impactor with 200,000–260,000 dyne after laminectomy. Rats were divided into the following groups: group I (normal control), group II (microelectric treatment of 0 A), group III (microelectric treatment of 100 μ A for 1 hr/day), group IV (microelectric treatment of 400 μ A for 1 hr/day), and group V (microelectric treatment of 400 μ A for 24 hr/day). After inducing SCI, rats were assessed by a sensory test with von Frey filaments and the locomotor recovery test (BBB rating scale) at 1, 4, 7, 14, 21, and 28 days. To evaluate spinal cord damage, histopathological studies were performed with hematoxylin and eosin. Brain-derived neurotrophic factor (BDNF) and TrkB immunohistochemistry studies were performed at 28 days.

Results: In groups IV and V, the BBB score had significantly improved on days 21 and 28 after SCI, and the TENS-treated groups showed significant neuronal recovery. After SCI, groups IV and V showed a significant recovery of locomotor function and the motor sensory response of the withdrawal threshold to 3.5 g. In addition, necrotic tissue and cystic spaces in the spinal cord were significantly reduced and BDNF/TrkB-positive cells were highly expressed in groups III, IV, and V.

Conclusions: Microelectric treatment can play a role in facilitating the recovery of locomotion following SCI.

Key Words: Spinal cord injury, Microelectric treatment, Transcutaneous electric nerve stimulation, Histologic finding, Functional recovery

Introduction

Traumatic spinal cord injury (SCI) is one of the foremost reason for neurological disability globally, with an annual prevalence of 15–52.5 cases per million.¹⁾ The most common causes of SCI are traumatic traffic accidents, falls, and violence.²⁾ In SCI, the force of the injury damages neural tissue, which causes a sudden loss of neurologic function. SCI affects a patient's mental and physical condition and has a subsequent economic impact to society.³⁾ SCI induces secondary cellular and molecular changes that lead to neuronal death, microglial activation, inflammation, and reactive astrogliosis.^{4,5)} Increasing evidence has suggested that apoptotic neuronal cell death is a critical determinant of direct tissue damage and associated

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neurological dysfunction after SCI.^{6–8)} Apoptosis of neurons, oligodendroglia, and microglia also occurs after SCI.^{9,10)}

Treatment with transcutaneous electrical nerve stimulation (TENS) has been proposed as an alternative tool for peripheral neuropathic pain and disease,¹¹⁾ e.g., patients with diabetic neuropathy,¹²⁾ nerve regeneration,¹³⁾ and patients with pain of differing origin. In activity-based restorative therapy, TENS sensory settings are used to achieve sensory input to the nervous system and as a tone and spasticity management technique. Nerve regeneration is achieved when TENS generates a strong non-painful electrical sensation beneath the electrodes. The treatment is usually rapid in onset and stops shortly after TENS is turned off. For this reason, patients are encouraged to receive TENS for as long as needed, which may be for hours at a time and throughout the day.¹⁴⁾ The large variation in results, also seen in patients with SCI or other etiologies, may be a direct consequence of not only our limited knowledge of the chosen parameters—such as stimulation frequency, intensity, duration, and electrode sites—but also the outcome measures used to evaluate improvement.

A recent review on electrostimulation for nerve regeneration concluded that making conclusive recommendations for treating neuropathic disease with TENS is still difficult because of the sparse evidence;¹³⁾ however, those reports stated that TENS could be better than placebo. We still have very little support for TENS duration or electric current, even though basic research has provided us with more information on the mechanisms involved during the last few years, with support for the hypothesis that TENS acts via different physiological mechanisms. Therefore, the aim of present study was to investigate the effects of TENS by various treatment time and electric current on acute SCI in a rat model. Motor sensory assessment, behavior assay, and histologic assessment were carried out to determine the underlying therapy mechanism of TENS following SCI. We hope that our findings informs translational opportunities to improve nerve recovery outcomes following TENS treatment.

Materials and Methods

1. Animals

A total of 48 male Wistar rats of specific pathogen-free grade, aged 10 to 12 weeks, weighing 230–250 g were

provided by the animal experimental center of Chosun University, Gwangju, Korea (CIACUC 2016–S0001). The animal research ethic committee of Chosun University approved the study. Rats were maintained in a ventilated, humidity (50–60%) and temperature-controlled room (21±2°C) on a 12-hour light/dark cycle. The animals were housed with sawdust and received food pellets and water ad libitum. Rats were acclimatized to their environment for 1 week before any experimental procedure. All behavioral experiments were performed during the light phase of the cycle, i.e., between 8:00 am and 3:00 pm.

2. Surgical procedure of SCI

Rats were anaesthetized by ketamine hydrochloride injection (72 mg/kg of body weight) intraperitoneally. The dorsal aspect of the thoracic region was shaved and washed with povidone iodine solution. Under aseptic precautions and draping, a dorsal longitudinal mid-line incision was made over the vertebral region from T9 to T11. Fascia and paravertebral muscles were gently dissected until the lamina and transverse processes were exposed. Hemostasis was strictly maintained. A T10 laminectomy was performed, and the spinal cord was exposed; a modified Ohio impactor (1.5 mm displacement, 300m/s) was then triggered to deliver a mechanical injury with 200,000–260,000 dyne (Fig. 1A) according to previous study^{15,16)}. The lesion was confirmed by micro-CT (Fig. 1B). CT imaging was performed using a Quantum GX μ CT imaging system (PerkinElmer, Hopkinton, MA) located at the Korea Basic Science Institute. Radiography parameters were set at 90 kV and 88 mA with a field of view of 45 mm

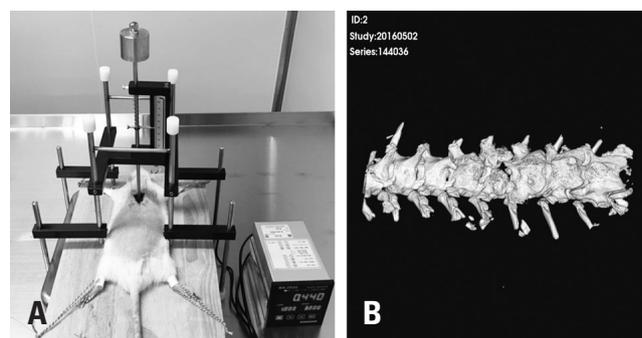


Fig. 1. Establishment of the spinal cord injury (SCI) model in the rats and confirmation of T9-10 laminectomy. **(A)** Establishment of the SCI model in the rats using the Modified Ohio Impactor. **(B)** Confirmation of T9-10 lesions using micro-computed tomography.

(voxel size, 90 μ m; scanning time, 14 min). CT images were represented in 3D Viewer (supplied with the Quantum GX system). Paravertebral muscles and skin were sutured in layers. A milliliter of NS with crystalline penicillin (80000 units diluted in 5 mL NS) was injected subcutaneously following surgery to replace the body fluid loss during surgery and as prophylaxis for any post-operative infection. During recovery from anesthesia, rats were placed on a warm pad and covered with a warm towel until re-establishment of thermoregulation and righting reflex. Following the disappearance of a tail-wagging reflex, paralysis of both hind limbs was considered successful. Antibiotics (crystalline penicillin 80,000 units diluted in 5 mL NS) were injected subcutaneously for 7 days to prevent any infection. Rats were nursed and housed in a cage with water and softened rodent chow was provided ad libitum. Rats demonstrated spontaneous bladder and bowel emptying 2 days after surgery.

3. Experimental animals

Rats were randomly allocated into the following groups according as previous study¹⁷⁾: Group I (normal control), Group II (Microelectric treatment of 0 A after SCI), Group III (Microelectric treatment of 100 μ A for 1 hr/day after SCI), Group IV (Microelectric treatment of 400 μ A for 1 hr/day after SCI), and Group V (Microelectric treatment of 400 μ A for 24 hr/day after SCI). The electrodes of the TENS instrument (SungJin Global Co., Gwangju, Korea) were located at both ends of the incision and then connected to the

TENS instrument (Fig. 2). Rats from each group were used in the following experiments 1 day after SCI.

4. Locomotor recovery

Rats were tested for locomotor function prior to SCI and then at days 1, 4, 7, 14, 21 and 28 after SCI. The rats were allowed to move for 4 mins in an open field and hind limb functions were assessed by 4 observers blinded to the rat experimental procedures; they scored the rats based on the Basso, Beattie and Bresnahan (BBB) locomotor rating scale developed by Basso et al.¹⁸⁾ The BBB scale is a 21-point system based on operationally defined behavioral features that follow the process of recovery from complete paralysis to normal locomotion; a score of 0 indicates complete hind limb paralysis and a score of 21 indicates complete normal locomotion function.

5. Motor sensory response

Rats were tested for motor sensory prior to SCI and then at days 1, 4, 7, 14, 21 and 28 subsequent to SCI. Graded von Frey hair (vFH) monofilaments were applied to the plantar surface of the foot approximately 1 cm posterior to the footpad of the middle phalange with the up-down method using procedures adapted from Chaplan et al.¹⁹⁾ Rats were placed in an inverted Plexiglas cage (20 cm \times 9 cm \times 10 cm) with a wire mesh bottom (0.635 cm grid size) allowing access to the plantar surface of the hind paws. Rats were given a food reward throughout testing to prevent visual recognition of application of the monofilament. The 50% withdrawal threshold was determined. A series of 11 von Frey filaments with approximately equal logarithmic incremental (0.19) von Frey values (3.38, 3.56, 3.75, 3.93, 4.12, 4.31, 4.49, 4.68, 4.86, 5.05 and 5.24) were used to determine the threshold stiffness required for 50% paw withdrawal. Since von Frey values are logarithmically related to gram (g) values [$VF = \log(10,000 * g)$], the chosen von Frey numbers are equivalent to 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10 and 12 in gram value, respectively. Starting with filament 4.31, the von Frey filaments were applied perpendicularly to the ventral surface of the paw. Interpolation of the 50% threshold was carried out according to the method of Dixon.²⁰⁾

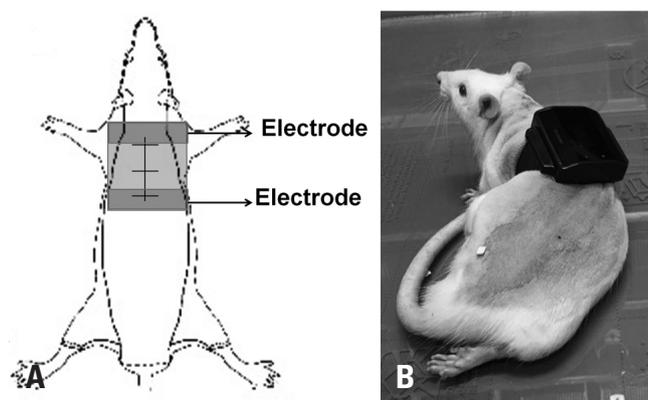


Fig. 2. Transcutaneous electrical nerve stimulation (TENS) treatment in the rat model of spinal cord injury (SCI). **(A)** Schematic representation of the rat model system for TENS treatment through 2 coated electrodes located below the instrument. **(B)** Constant current stimuli were applied to the dorsal surface of the SCI rats.

6. Tissue processing, H&E staining and Immunohistochemistry

On day 28 following SCI, the tissues were fixed in cold 4% paraformaldehyde. Bone tissue was first decalcified in a sodium citrate solution before processing and mounting on histologic slides. Decalcified spines were cut at the midpoint and embedded in paraffin blocks. Serial paraffin sections were stained with hematoxylin and eosin (H&E). Images of stained tissue were captured using a microscope slide scanner (3D-HISTECH Ltd., Budapest, Hungary).

In addition, the expression of some important neuronal factors was analyzed in the tissue to understand the effects of TENS on SCI. For this purpose, we focused on BDNF and Trk- β , which primarily support axonal outgrowth and neuronal cell survival, respectively. The spine sections (3 μ m thick) were deparaffinized by soaking in three changes of xylene and rehydrated in a graded series of ethanol/distilled water solutions. For antigen retrieval, slides were placed in 0.01 M citrate-buffer (pH 6.0) and heated in a steamer for 30 min. Endogenous peroxidases were stained by incubation in 3% hydrogen peroxide for 20 min at room temperature. Sections were incubated overnight at 4°C with the following primary antibodies (1:50 dilution): anti-BDNF (Santa-Cruz Biotechnology) and anti Trk-b (Santa-Cruz Biotechnology). Subsequently, sections were incubated with a biotinylated secondary antibody (LSAB; Dako Cytomation, Glostrup, Denmark, K0675) for 30 min, washed in PBS, and incubated with a streptavidin-peroxidase conjugate (LSAB, Dako Cytomation, K0609) for 30 min. The reaction was performed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, S-1141) for 5 min. Slides were briefly counterstained in hematoxylin and dehydrated; a cover slip was then placed. Negative and positive controls were run simultaneously. Mammary tissue served as a positive control. Images were captured using a microscope slide scanner (3D-HISTECH Ltd).

Quantification of immunohistochemical study was performed by examining at least 1,000 cells to obtain the percentage of positive cells. Each field was examined at $\times 100$ (10 objective \times 10 ocular) magnification. The immunohistochemical findings were scored independently by three observers who were blinded to the clinicopathological details. All discrepancies were resolved by consensus.

7. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). A one way analysis of variance followed by Bonferroni's post hoc test was utilized to determine significant differences among multiple groups. The independent sample t-test was used to compare significant mean differences between two groups. Image Pro-Plus 6.0 was used for histopathologic analysis of the images captured. All values were expressed as the mean \pm standard deviation; $p < 0.05$ was considered to indicate statistical significance.

Results

1. Locomotor recovery

The BBB locomotor rating scale was used to analyze the recovery of locomotor function after SCI. All rats had normal

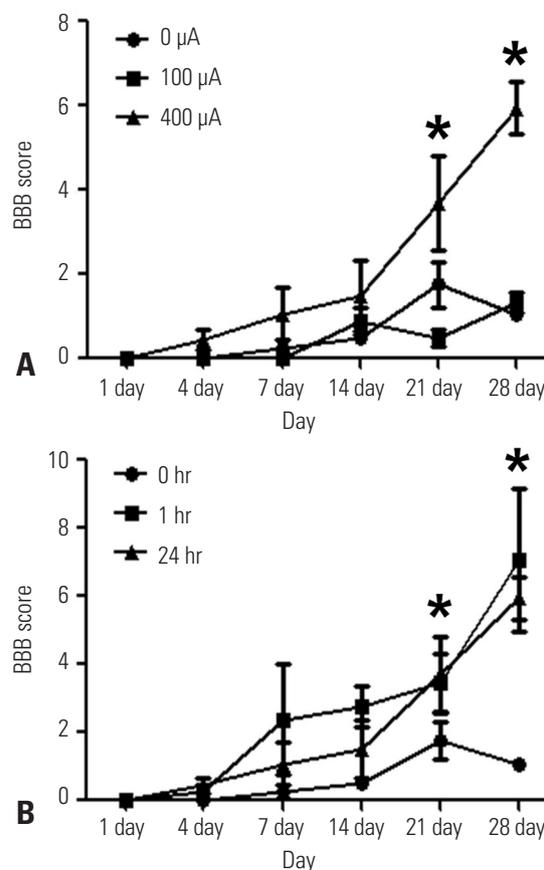


Fig. 3. Locomotor recovery test using BBB scoring. **(A)** Transcutaneous electrical nerve stimulation (TENS)-treated animals (400 μ A) showed better BBB locomotor scores than the animals treated with 0 A ($*p < 0.05$). **(B)** TENS treatment for 1 hr/day and 24 hr/day improved BBB locomotor scores more than 0 hr/day of treatment ($*p < 0.05$).

limb function and obtained a score of 21 before SCI. Following SCI, all rats had a BBB score of 0 indicating a total injury. TENS at 400 μ A resulted in significant improvement in BBB score at day 21 and 28 ($p < 0.05$ vs. the control) (Fig. 3A). Additionally, TENS for 1 hr or 24 hrs improved BBB score at day 21 and 28 ($p < 0.05$ vs. the control) (Fig. 3B).

2. Motor sensory response

The motor sensory response test was applied to determine the effect of TENS after SCI. A 50% withdrawal threshold to von Frey filament application to the plantar surface of the hind limb was determined. Fig. 4A shows the results of the withdrawal threshold at 0, 100, and 400 μ A in the TENS-treated rats after SCI. At 1 day after SCI, rats were seldom responsive to even the highest TENS strength. However, TENS with 400 μ A led to recovery of a response to 3.5 g at 28 days. Additionally, both of 1 hr and 24 hrs of treatment with TENS led to significant recovery of the withdrawal threshold to 3.5 g, meaning that both intermittent and continuous stimulation could improve the recovery of the motor sensory response (Fig. 4B).

3. Histopathology findings

H&E staining was performed on spinal cord tissues at 28 days after SCI to elucidate the morphologic changes at the

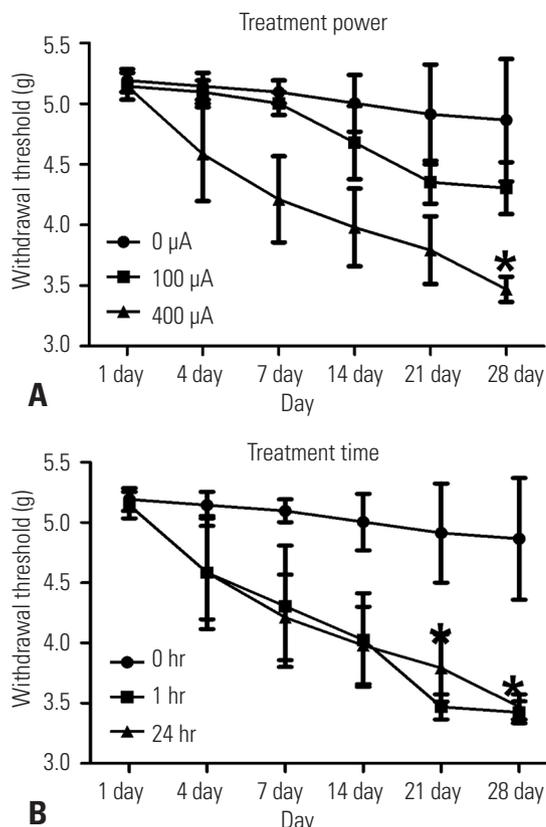


Fig. 4. Motor sensory response test by the 50% withdrawal threshold through von Frey filament application. **(A)** Transcutaneous electrical nerve stimulation (TENS)-treated animals (400 μ A) had a lower 50% withdrawal threshold than the animals treated with 0 A ($*p < 0.05$). **(B)** TENS treatment for 1 hr/day and 24 hr/day led to a lower 50% withdrawal threshold than 0 hr/day of treatment ($*p < 0.05$).

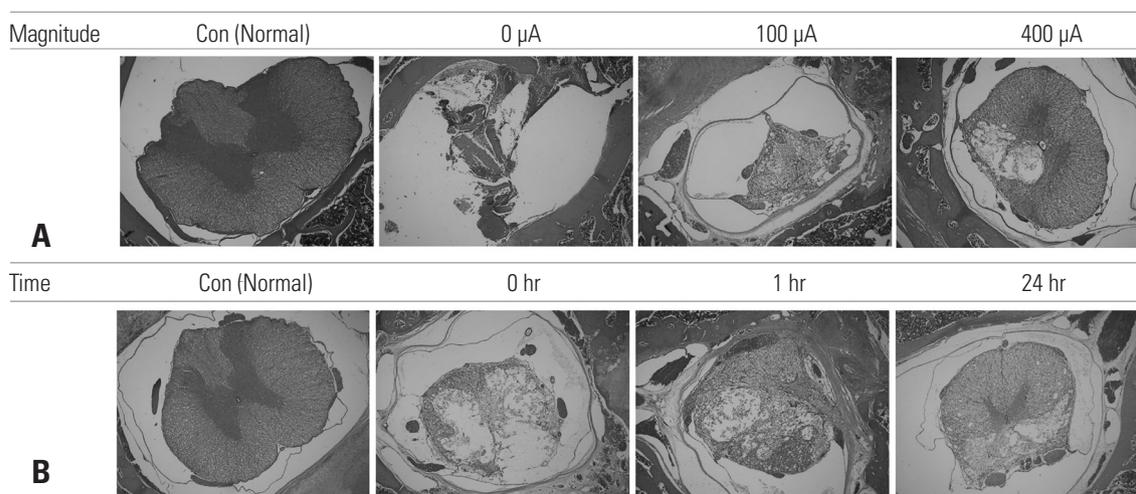


Fig. 5. Photographs taken from the site of a spinal cord injury, excised 28 days after the injury. The spinal cord segments were cut into 8- μ m axial sections. Lesion swelling is seen on low-power magnification ($\times 40$) with hematoxylin and eosin staining. **(A)** Transcutaneous electrical nerve stimulation (TENS)-treated animals (400 μ A) showed better spinal cord recovery than the animals treated with 0 A. **(B)** TENS treatment for 1 hr/day and 24 hrs/day favored spinal cord recovery in comparison with 0 hr/day of treatment.

injury site and the extent of neural damage using the modified Ohio impactor to create an SCI model (Fig. 5A, B). Using sections from the control group rats, well-defined butterfly shaped areas of gray matter and white matter containing myelin could be observed. However, at the injury site in the SCI group, the structure of the spinal cord was destroyed and the formation of cystic spaces was observed. The nerve fibers were disorganized with liquefaction of spinal tissue. Extradural hemorrhage was observed at the injury site with neutrophil infiltration and necrotic tissue. Neuronal apoptosis and the number of gliocytes increased. However, extent of histopathologic changes in the 400 μ A of TENS treated group was evidently recovered the structure of the spinal cord in the injury site (Fig. 5A). In addition, 1 hr or 24 hrs of TENS treatment led to a significant decrease in necrotic tissue and cystic spaces in the spinal cord structure (Fig. 5B). Although some cystic lesions remained, many neurons recovered and showed a nearly normal morphology, which indicated that TENS (400 μ A) for 1 hr or 24 hrs have successfully recovered the lesion site.

4. Immunohistochemistry results

In all groups, detectable levels of BDNF were expressed in the lesion sites (Fig. 6). Especially, the number of BDNF-positive cells and the intensity of BDNF staining were

significantly higher in the TENS treatment groups. The reduced size of the cavities (cell-free regions) in the spinal cord in the groups receiving TENS treatment may be explained by the expression levels of BDNF during TENS treatment. However, Trk- β was expressed only in the TENS-treated rat tissues (Fig. 7). Together with BDNF, Trk- β may regulate the regeneration process without scar tissue formation. Regarding 400 μ A of TENS, it is unclear whether the intermittent or continuous stimulation led to the significant improvement of neuronal recovery.

Discussion

TENS has long been used in orthopedic and neurological rehabilitation. Its efficacy and application are well-documented from knee osteoarthritis to stroke. TENS has no known drug interactions and thus can be used in combination with pharmacotherapy to reduce medication dosage, medication-related side effects, and associated costs. TENS has a rapid onset of action, unlike medication, and there is no potential for toxicity or overdose.¹⁴⁾ However, treatment by TENS in SCI is only supported by studies of small sample size; therefore, there is insufficient evidence to determine whether its use is clinically indicated and necessary. Rats have been chosen to study traumatic SCI not only because they are readily

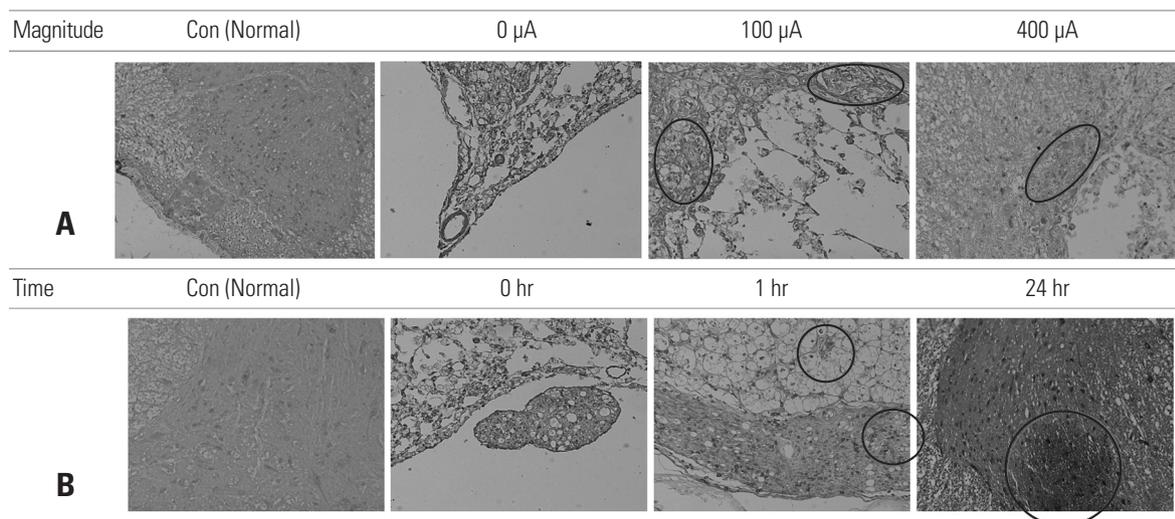


Fig. 6. Immunohistochemical study of brain-derived neurotrophic factor (BDNF) expression at the site of a spinal cord injury, excised 28 days after the injury. Each field was examined at $\times 100$ (10 objective \times 10 ocular) magnification. **(A)** Transcutaneous electrical nerve stimulation (TENS)-treated animals (400 μ A) showed higher BDNF expression than the animals treated with 0 A. **(B)** TENS treatment for 1 hr/day and 24 hr/day led to higher BDNF expression than 0 hr/day of treatment.

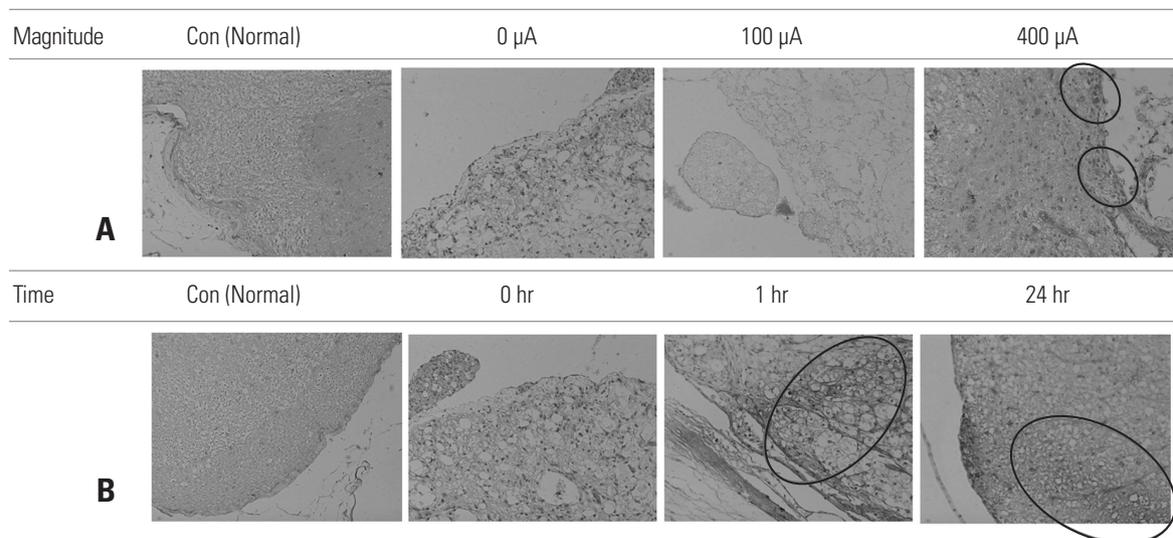


Fig. 7. Immunohistochemical study of TrkB expression at the site of a spinal cord injury, excised 28 days after the injury. Each field was examined at $\times 100$ (10 objective \times 10 ocular) magnification. **(A)** Transcutaneous electrical nerve stimulation (TENS)-treated animals (400 μ A) showed higher TrkB expression than the animals treated with 0 A. **(B)** TENS treatment for 1 hr/day and 24 hr/day led to higher TrkB expression than 0 hr/day of treatment.

that TENS with intermittent or continuous treatment recover the behavioral and sensory responses and TENS could be helpful for recovery from SCI. In the previous study, Solak et al. compared continuous TENS (CTENS) with intermittent TENS (ITENS) and found both to be equal in reducing pain; however, CTENS led to greater reduction in consumption of analgesics compared to ITENS²³. Although the quantity of these results cannot be compared because methodology and assays vary in each study, our results imply that both of continuous and intermittent TENS exerts an exceptionally recovered behavioral and sensory response in SCI rat model.

To analyze the effect of TENS on neuronal regeneration, histologic assessment and immunohistochemical study of BDNF and Trk- β were carried out on the injured cord following dissection. TENS treatment significantly recovered the structure of the spinal cord at the injury site and led to a decrease in necrotic tissue and cystic spaces. In addition, the high expression of BDNF and Trk- β was observed in the TENS-treated SCI rat. BDNF and Trk- β primarily support axonal outgrowth and neuronal cell survival and BDNF expression is particularly important in the axonal outgrowth of neurons.²⁴ In another previous study, high-frequency of TENS significantly reduced the proportion of activated microglia observed after SCI.²⁵ BDNF has been shown to play a role in protecting neuronal injured tissue from toxic events,

for neuronal morphogenesis and synaptic plasticity.²⁶ In this study, especially, the Trk- β -positive cells were only observed in the TENS-treated groups, which indicates a significant improvement in neuronal recovery by TENS.

Our present study had several limitations. First of all, the sample size of the subjects was not sufficient for generalization of the results. Second, we did not compare the efficacy of low frequency TENS with that of high frequency TENS or any other electrotherapy because of the limited number of subjects.

Conclusion

Taken together, our results have demonstrated the therapeutic effects of TENS with a single application on neuronal regeneration in SCI. Our results also suggest that continuous and intermittent TENS is effective for the recovery of motor function and sensory response. Especially, the positive effects of TENS on neuronal recovery have been found. However, further studies are needed to demonstrate the effects of repetitive TENS on the recovery of motor function following SCI and establish the adequate parameters for TENS application.

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척수 손상 백서에서 경피신경전기자극을 통한 미세전류 치료 효과

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연구 계획: 동물 실험

목적: 척수 손상 백서 동물 모델에서 경피신경자극을 통한 미세전류 치료가 기능적 회복과 조직학적 변화에 미치는 영향을 알아보고자 하였다.

선행 연구문헌의 요약: 경피신경자극의 척수 기능 향상에 대한 보고는 드물다.

대상 및 방법: 실험 쥐의 후궁 절제 후 1.5 mm impactor를 이용하여 200,000-260,000 dyne의 외력으로 척수 손상을 시켰다. 실험군은 그룹 I; 비수술 군, 그룹 II; 미세전류치료(0 A) 군, 그룹 III; 미세전류치료(100 μ A, 하루 한시간) 군, 그룹 IV; 미세전류치료(400 μ A, 하루 한시간) 군, 그룹 V; 미세전류치료 (400 μ A, 하루 24시간) 군으로 나누어 실험을 진행하였다. 척수 손상 후 1, 4, 7, 14, 21일과 28일에 BBB척도를 이용한 운동 기능 검사와 von-Frey monofilament를 이용한 감각 검사를 시행하였다. 또한 손상 부위의 조직학적 변화 관찰을 위한 H&E 염색 및 신경성장인자의 발현 분석을 위한 BDNF, Trk-b의 면역조직화학염색을 손상 후 28일에 시행하였다.

결과: 그룹 IV, V군에서 척수 손상 후, 21일과 28일에서 BBB 점수가 상당히 향상하였으며, 유의한 신경원 회복을 보였다. 또한 척수 손상 후 그룹 IV, V군에서 유의한 운동기능 향상과 감각기능 회복을 보였으며, 척수의 괴사조직이 상당히 감소됨을 확인할 수 있었다. 또한 그룹 III, IV, V군에서는 BDNF 및 Trk- β 의 발현이 증가하였다.

결론: 척수 손상 후 경피신경전기자극 치료는 운동 기능 회복의 향상에 도움이 되리라 사료된다.

색인 단어: 척수 손상, 미세전류 치료, 경피신경자극, 조직학적 소견, 기능 회복

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