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Cornea & External Disease

Corneal Neuro-Regenerative Effect of Transcutaneous Electrical Stimulation in Rabbit Lamellar Keratectomy Model

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Purpose: This study aimed to evaluate the effect of transcutaneous electrical stimulation (TES) on corneal nerve regeneration in rabbits injured from superficial lamellar keratectomy (SLK).

Methods: New Zealand White rabbits were used in this experimental study. To induce corneal nerve damage, SLK was performed using a 7.0-mm trephine. TES was applied for 28 days after the corneal nerve injury. Corneal sensitivity, Western blotting, real-time polymerase chain reaction (PCR), and immunofluorescence were performed to observe changes in the corneal tissue.

Results: In the 2-Hz and 20-Hz electrical stimulation groups, the degree of corneal wound healing increased by more than 10% compared to the control group, but no significant difference was observed. Conversely, the electrical stimulation (2-Hz or 20-Hz) group showed significantly increased corneal sensitivity compared to the control group. Western blot analysis revealed that small proline-rich protein 1A (SPRR1a), a regeneration-associated protein was significantly increased in the 2-Hz group on days 1 and 7 compared to that in the other groups. Once again, nerve regeneration in the 2-Hz group was supported by the results of PCR, in which a significant increase in the nerve growth factor (NGF) on day 1 was observed compared with the other groups. Moreover, immunofluorescence after 28 days of electrical stimulation showed significant nerve regeneration in the 2-Hz group.

Conclusions: TES promoted corneal nerve regeneration in rabbit SLK model. The application of electrical stimulation of 2-Hz frequency was more effective than the 20-Hz frequency, showing potential clinical applications for corneal diseases.

Translational Relevance: This study shows how application of TES to the eyes that exhibit corneal nerve damage can improve corneal nerve regeneration examined by histologic analysis.

Introduction

The corneal nerve plays a significant role in maintaining tear film stability, secreting tear, blinking reaction, and corneal wound healing.[1,2] The corneal epithelium is the most densely innervated tissue of the body, which is composed of approximately 500 times more nerve fibers than most typical epithelial tissue. Moreover, corneal nerves are important to maintain corneal homeostasis, structure, and function.[3–5]

Dry eye and corneal pain after laser surgery, such as laser-assisted in situ keratomileusis (LASIK) or laser epithelial keratomileusis (LASEK), are clinical manifestations of corneal nerve damages.[6–8] Although there are signs of improvement over time, a significant portion of corneal nerve damage remains unrecovered for a long period of time ultimately inducing

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eye dryness and pain.[7–11] Otherwise, central sensitization caused by an amplification of neural signaling within the central nervous system may develop and induce pain hypersensitivity when the damaged corneal nerve was recovered.[12–14] Damage to the corneal nerve fibers are due to various reasons, including laser surgery, and the accompanying dry eye disease (DED) is suggested to be resolved by targeting both structural and functional recovery of corneal nerves.[15]

The revised Tear Film and Ocular Surface Society Dry Eye Workshop (TFOS DEWS) II, in 2017, recognized neurotrophic conditions, such as abnormality in sensation, as one of the causes of DED.[16–18] DED related to corneal nerve damage presents with reduced corneal sensitivity and signs of ocular surface disease that results from tear insufficiency and/or tear film instability. Managing such chronic corneal nerve damage, however, is not considered in the existing treatment options.[18] Although neurotrophic conditions with impaired corneal and conjunctival sensitivity are associated with DED, only attempts have been made to relieve subjective symptoms of dryness and to improve the condition of the damaged ocular surface.[18,19] To address corneal nerve damage, the sole treatment options available are topical artificial tears and surface lubricants.[19] Some reports have demonstrated improvements in corneal nerve parameters with the use of platelet-rich plasma in neurotrophic keratitis.[20,21] The pathogenetic roles of corneal nerve fiber damages in DED and corneal pain are not quite clear or definite. However, it is suspected that damages on the eye surface and corneal nerve structure degeneration are suspected to play a role.[20,22,23]

Various reports suggest that electrical stimulation helps with wound healing and nerve regeneration.[24–26] Electrical stimulation can promote cell migration and proliferation, and accelerate nerve regeneration.[25,27] It has been suggested that the promotion of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), through an increase calcium influx into the neurons can support with nerve and tissue regeneration.[28,29] In particular, Ghaffariyeh et al. reported that transcutaneous electrical stimulation (TES) improved recovery of corneal nerve sensitivity at 3 months after surgery.[30] It could be suggested that the corneal nerve regeneration was accelerated by electrical stimulation. Although the functional recovery of the corneal nerves by electrical stimulation has been revealed, the structural recovery of the corneal nerves and the physiological mechanism by which electrical stimulation acts on corneal nerve recovery have not yet been revealed. Furthermore, optimal parameters of electrical stimulation for corneal nerve recovery have not yet been clearly elucidated.

In this study, we developed a protocol that uses electrical stimulation to treat DED-related corneal nerve damage, which was defined as eyes exhibiting a reduction in corneal sensitivity and signs of ocular surface disease, such as defects of the corneal epithelial layer. Using an animal model of damaged corneal nerves, we selected the parameters of electrical stimulation and investigated their mode of action through biochemical analysis.

Methods

Subjects

New Zealand White (NZW) rabbits were purchased from Doo Yeol Biotech and acclimated under stable conditions (12 hours light/dark cycle) for 1 week before the experiment. All animals were provided with free access to a standard diet (PMI LabDiet) and water. To induce superficial lamellar keratectomy in NZW rabbits, using a 7.0 mm trephine, the cornea was penetrated the paracentral area to a depth 100 μm. After the surgery, rabbits had been treated with levofloxacin (Cravit, Santen, Japan) eye drops and 0.1% fluorometholone eye drops (Flumetholon, Santen, Japan) twice daily. All rabbits were anesthetized by intramuscular injection of 40 mg/kg ketamine and 12 mg/kg xylazine before any procedure.

All animal care and experimental procedures were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Samsung Medical Center (No. 20180521001) and were handled according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines and regulations of the Laboratory Animal Research Center at Samsung Medical Center, Sungkyunkwan University School of Medicine.

Superficial Lamellar Keratectomy in NZW Rabbits

To investigate the effect of TES in NZW rabbits, superficial lamellar keratectomy (SLK) was performed on 2.0 to 2.5 kg NZW rabbits ($n = 9$). SLK was performed in the central area of the cornea of the right eye of each rabbit. After marking with a corneal trephine of 7.0-mm diameter, a diamond knife (AE-8122, ASICO, Westmont, IL, USA) was used to cut the cornea along the peripheral cornea marked with

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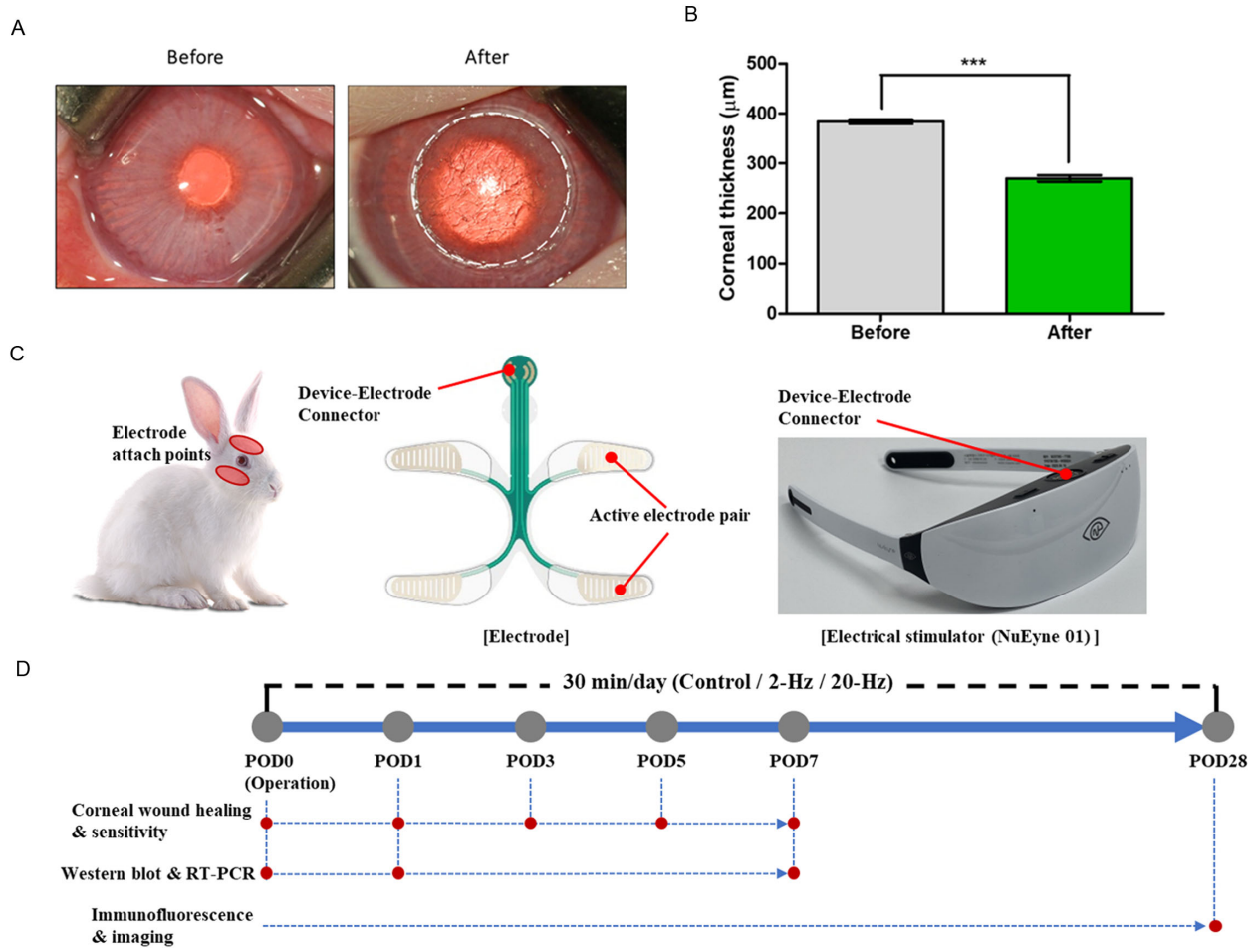

Figure 1. Transcutaneous electrical stimulation (TES) after superficial lamellar keratectomy (SLK) in rabbit model. (**A**) The corneal images before and after SLK. (**B**) Corneal thickness was measured using pachymetry before and after SLK ($n = 9$). Error bars indicate ± standard error of the mean (SEM). Data were analyzed by *t*-test. ***$P < 0.001$. (**C**) Representative pictures of performing TES in a rabbit model and device for TES. (**D**) Schematic diagram of TES.

trephine with 100 μm of depth. Then, blunt keratectomy was performed to dissect the stromal layer with spatula (Sarnicola Big Bubble Spatula; ASICO). All the rabbits' epithelium and superficial lamellae of the stroma were cut to aim a depth of 100 μm (Fig. 1A).

Transcutaneous Electrical Stimulation

The fur of the subjects in the experimental group were shaven above and below the ocular midline to place the electrodes in close proximity and generate TES. The TES was applied using a customized electrical stimulator for clinical trials (NuEyne 01; Nu Eyne Inc., Seoul, South Korea). To compare the nerve regeneration effects of 2-Hz[31] and 20-Hz[32] electrical stimulation, biphasic pulses of 2 Hz and 20 Hz were used in this study. The phase duration was 250 μs and the interphase interval was 5 μs. The pulse was 7 mA. TES was applied for 30 minutes each day from the postoperative day 0 (POD0) to POD28.

Corneal Epithelial Wound Healing and Corneal Sensitivity

Corneal epithelial defects were stained with 0.25% fluorescein sodium and photographed on the following postoperative days: POD0, POD1, POD3, POD5, and POD7. Differences in the epithelial defect areas were measured using the ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

Corneal sensitivity was measured laterally using an esthesiometer (Luneau Ophthalmolgie, Chartres, France) in the central cornea without anesthesia.[31,32] Briefly, the length of the monofilament varied from 6.0 to 0.5 cm with 0.5-cm fractions until the corneal touch

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threshold was determined. The cornea was tested thrice for each filament length. When no blink was elicited by the monofilament touch, the response was considered negative. On the contrary, when the animal blinked in equal or more than 50% of the times tested, the response was considered positive. No blink response at a monofilament length of 0.5 cm was recorded as 0 for corneal sensitivity.

Protein Extraction and Western Blot

Proteins that are associated with corneal nerve regeneration and corneal wound healing were selected for Western blot analysis.[33,34] Corneal tissues were homogenized and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium dodecyl sulfate, protease inhibitor cocktail; Cell Signalling, USA). The homogenate was centrifuged (12,000 rpm for 20 minutes at 4°C), and the supernatant was analyzed via Western blotting. After determining the protein concentration by using the BCA assay, the protein sample lysates were loaded and separated using 10% to 12% SDS-PAGE gels. Proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes in a transfer buffer. After blocking of the PVDF membranes, they were incubated overnight with primary antibodies, including anti-β3 tubulin (1:1000; Abcam, Cambridge, MA, USA), anti-vascular endothelial growth factor A (VEGFA; 1:500; Abcam), anti-SPRR1a (1:500; Abcam), and β-actin (1:2000; Abcam). Subsequently, the membranes were washed in phosphate-buffered saline (PBS)-T buffer and incubated with peroxidase-conjugated anti-rabbit (AbFrontier, Seoul, Korea) or anti-mouse IgG (AbFrontier) antibodies. Proteins were visualized using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, USA), and the fluorescence intensity were quantified using Image J software (NIH).

RNA Extraction and Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction

The mRNA expression of neurotropic and growth factors was measured using real-time polymerase chain reaction (PCR) analysis. Total RNA from corneal tissues was extracted using a Hybrid-R kit (GeneAll, Korea), according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using a reverse transcription kit (Thermo Fisher Scientific). Then, quantitative real-time PCR was performed using the SYBR green PCR master Mix (Thermo Fisher Scientific) and the QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with the indicated primers (epidermal growth factor [EGF; Forward, 5′-GTG TGC TGG CAG TGT TTC TG -3′; Reverse, 5′-GGG CAT CCC GGG AAA CTA TT-3′], NGF [Forward, 5′-ACG GAC ATC AAG GGC AAT GA-3′; Reverse, 5′-TGC AGT ACG AGT TCC AGT GC-3′], glial cell-derived neurotrophic factor [GDNF; Forward, 5′-GGA ATC CAC TGG CGT CTT CA-3′; Reverse, 5′-CAC GCC CAT CAC AAA CAT GG-3′], neurotrophin-3 [NT3; Forward, 5′-CCG GGA TAA TGA TGA GGC AGA-3′ ; Reverse, 5′-TGG ATG CCA CGG AGA TAA GC-3′], and GAPDH [Forward, 5′-GGA ATC CAC TGG CGT CTT CA-3′; Reverse, 5′-CAC GCC CAT CAC AAA CAT GG-3′]). Real-time PCR data were analyzed with QuantStudio real-time PCR software using the delta-delta Ct method, as previously described. GAPDH was used as a reference gene, and the results are presented as relative expressions to the control.

Immunofluorescence and Imaging

At POD28, corneas were fixed with 2% paraformaldehyde for 2 hours and 1% paraformaldehyde for 30 minutes at 4°C and room temperature, respectively. Moreover, corneas were dehydrated using 20% and 10% sucrose solutions, followed by a permeation step consisting of 3 washes with PBS for 15 minutes. Samples were then blocked for 2 hours at room temperature with 5% normal donkey serum in PBS, followed by an overnight incubation with primary antibodies (β3 tubulin, 1:500; Abcam). Upon overnight incubation, the samples were washed 3 times with PBS for 30 minutes and incubated with fluorescently labeled secondary antibodies for 1 hour (1:1000 dilution). Images were captured using a confocal microscope (LSM780; Carl Zeiss Microscopy Ltd., Germany).

Statistical Analyses

Animals and samples were not randomized during the experiments and were not excluded from the analyses. The minimum number for the statistical analysis was performed in the present study. All parameters of the rabbits were compared with those of the littermate controls. Values are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined by the *t*-test or two-way analysis of variance (ANOVA). Statistical analysis was performed with GraphPad Prism 5 (GraphPad

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software, Inc.) for windows. Statistical significance was set at $P < 0.05$.

Results

SLK in NZW Rabbits

The corneal thickness was removed by 113.8 ± 17.3 µm compared to before (383.8 ± 14.5 µm) the SLK. Corneal thickness was 270 ± 20.0 µm (Fig. 1B).

Effect of Transcutaneous Electrical Stimulation on Epithelial Wound Healing and Corneal Sensitivity

Images of epithelial defects stained with fluorescent dye were obtained on POD0, POD1, POD3, POD5, and POD7 days after SLK (Figs. 2A, 2B). No complications were associated with the wound creation procedure. Following image analysis, the healed areas (percentages) of the epithelial defects in the control (n = 3), 2-Hz group (n = 3), and 20-Hz group (n = 3) were compared at each time point. The mean values of epithelial wound healing area on POD1, POD3, POD5, and POD7 were evaluated. Although the mean values of epithelial wound healing area in the 2-Hz group (POD1 [1.3 ± 2.3%], POD3 [46.4 ± 13.0%], POD5 [66.2 ± 6.7%] and POD7 [87.5 ± 7.7%]) and 20-Hz group (POD1 [2.7 ± 4.7%], POD3 [42.5 ± 4.5%], POD5 [62.1 ± 10.2%], and POD7 [87.3 ± 3.0%]) increased compared to those in the control group (POD1 [0.0 ± 0.0%], POD3 [34.5 ± 4.5%], POD5 [54.8 ± 19.3%], and POD7 [74.1 ± 15.2%]), there were no significant differences in epithelial wound healing area among the 3 groups at each time point (Fig. 2C).

At POD1, there were no significant differences in the corneal sensitivity between three groups. The corneal sensitivity in the 2-Hz group (POD3 [0.8 ± 0.3 cm]

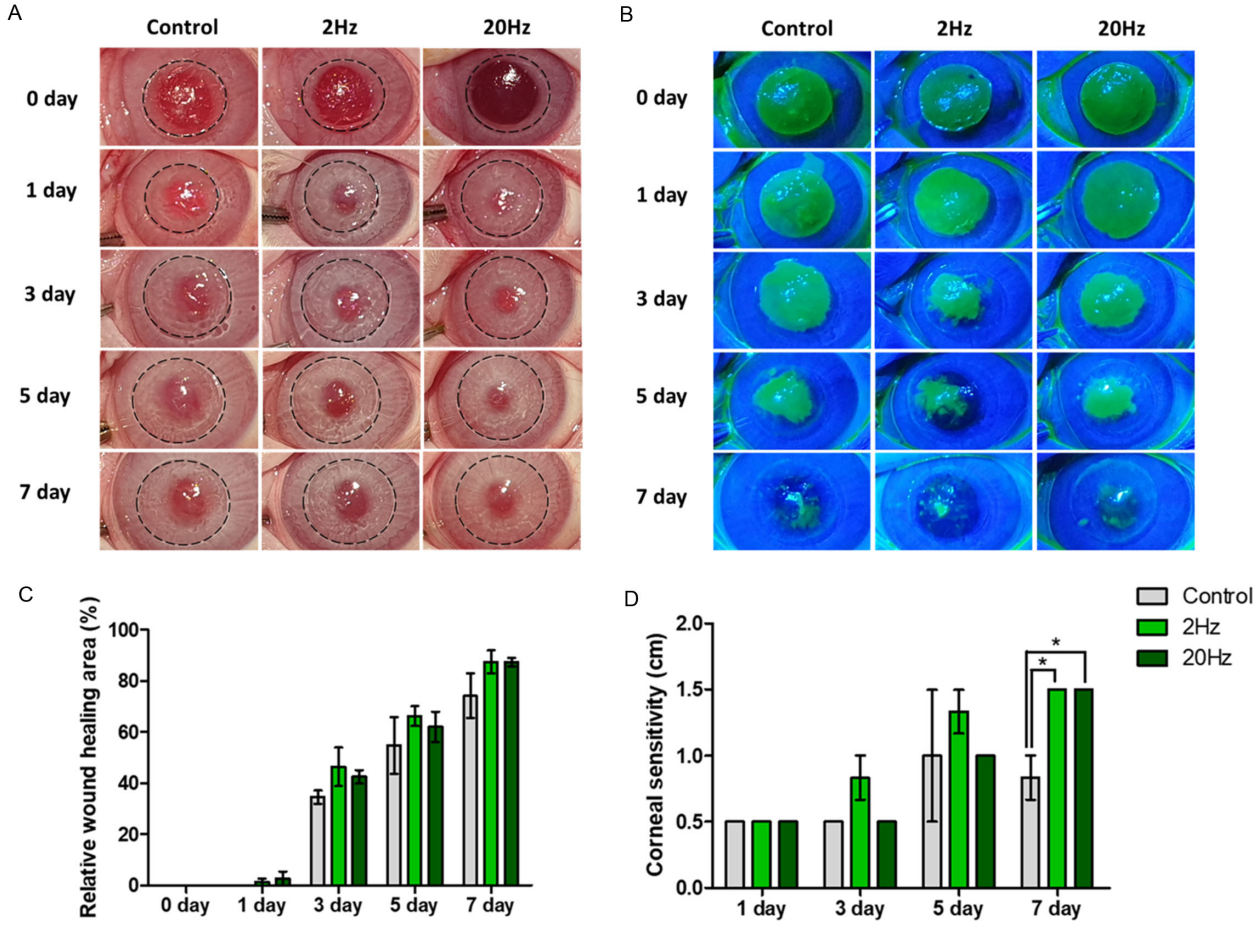

Figure 2. The temporal comparison of corneal epithelial wound healing and corneal sensitivity in the control group and the electrical stimulation group after SLK. (**A**) Representative microscopic images after SLK. (**B**) Images of the fluorescein staining after SLK. (**C**) Wound healing area were measured using Image J. (**D**) Corneal sensitivity were measured using esthesiometer (n = 3). Error bars indicate means ± SEM. Data were analyzed by 2-way analysis of variance (ANOVA). * $P < 0.05$.

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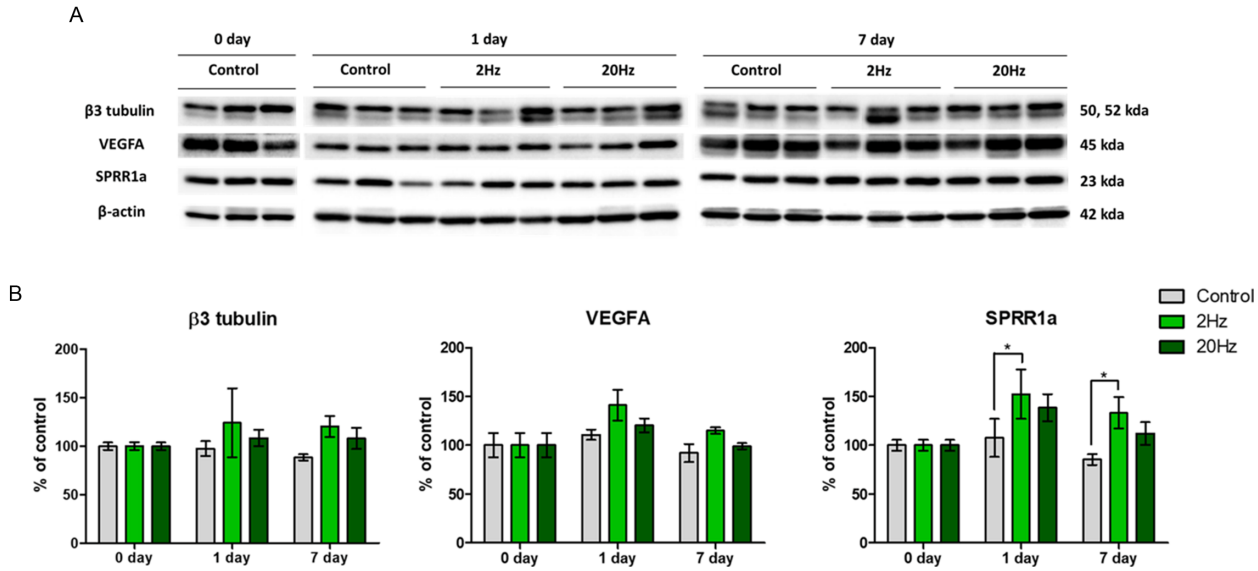

Figure 3. The effect of transcutaneous electrical stimulation on β3 tubulin, vascular endothelial growth factor A (VEGFA), and SPRR1a. (**A**) The expression intensities of b3 tubulin, VEGFA, and SPRR1a. (**B**) β3 tubulin, VEGFA, and SPRR1a expression were normalized to day 0 in the control group ($n = 3$). Error bars indicate ± SEM. Data were analyzed by 2-way ANOVA. *$P < 0.05$.

and POD5 [1.3 ± 0.3 cm]) increased compared to the control group (POD3 [0.5 ± 0.0 cm] and POD5 [1.0 ± 0.9 cm]) and the 20-Hz group (POD3 [0.5 ± 0.0 cm] and POD5 [1.0 ± 0.0 cm]) on POD3 and POD5, but there were no significant differences. In contrast, the corneal sensitivity of the 2-Hz and 20-Hz groups were significantly increased (1.5 ± 0.0 cm) in comparison with that of the control group (0.8 ± 0.3 cm) at POD7. Although there were no differences in corneal sensitivity between the 2-Hz and 20-Hz groups on POD7, we found that the TES groups (2 Hz and 20 Hz) had increased corneal sensitivity compared to the control group (Fig. 2D).

Increased Protein Levels in the Electrical Stimulation Groups in NZW Rabbits After SLK

To determine the effect of TES on the protein levels related to neuronal growth, we euthanized rabbits on POD0, POD1, and POD7 and observed protein levels of vascular endothelial growth factor A (VEGFA), neuronal factor β3 tubulin, and small proline rich protein 1a (SPRR1a; Fig. 3A). On POD1 and POD7, VEGFA, an angiogenesis factor, slightly increased in the electrical stimulation groups (2-Hz group, POD1 [141.0 ± 27.3%] and POD7 [114.9 ± 5.8%]) and the 20-Hz group, POD1 [120.4 ± 12.3%] and POD7 [99.1 ± 5.5%]) compared to the control group (POD1 [110.8 ± 9.1%], and POD7 [92.0 ± 16.0%]), but there were no significant differences. The neuronal factor β3 tubulin also increased on POD1 and POD7 in the electrical stimulation groups (2-Hz group, POD1 [124.0 ± 61.7%] and POD7 [120.3 ± 18.6%]) and the 20-Hz group, POD1 [108.4 ± 14.6%] and POD7 [108.1 ± 18.8%]) compared to the control group (POD1 [97.5 ± 13.2%] and POD7 [88.5 ± 5.4%]), but there were no significant differences. SPRR1a, a regeneration-associated gene, also increased in the electrical stimulation groups (2-Hz group, POD1 [152.4 ± 43.7%] and POD7 [133.1 ± 27.8%]) and the 20-Hz group, POD1 [138.6 ± 24.1%] and POD7 [112.0 ± 20.1%]) when compared to the control group (POD1, [107.6 ± 33.8%] and POD7 [85.4 ± 9.6%]) on POD1 and POD7. In particular, the 2-Hz electrical stimulation group (POD1 [152.4 ± 43.7%] and POD7 [133.1 ± 27.8 %]) significantly increased when compared to the control group (POD1 [107.6 ± 33.8%] and POD7 [85.4 ± 9.6%]; Fig. 3B). The expressions of SPRR1a in the damaged cornea were accelerated by electrical stimulation from POD1 to POD7, and this effect was higher in the 2-Hz electrical stimulation group than in the 20-Hz electrical stimulation group.

Effect of Transcutaneous Electrical Stimulation on mRNA Levels in NZW Rabbits After SLK

To determine the effect of TES on the mRNA levels of proteins related to wound healing, such as EGF, GDNF, NGF, and neutrophin-3 (NT-3). We

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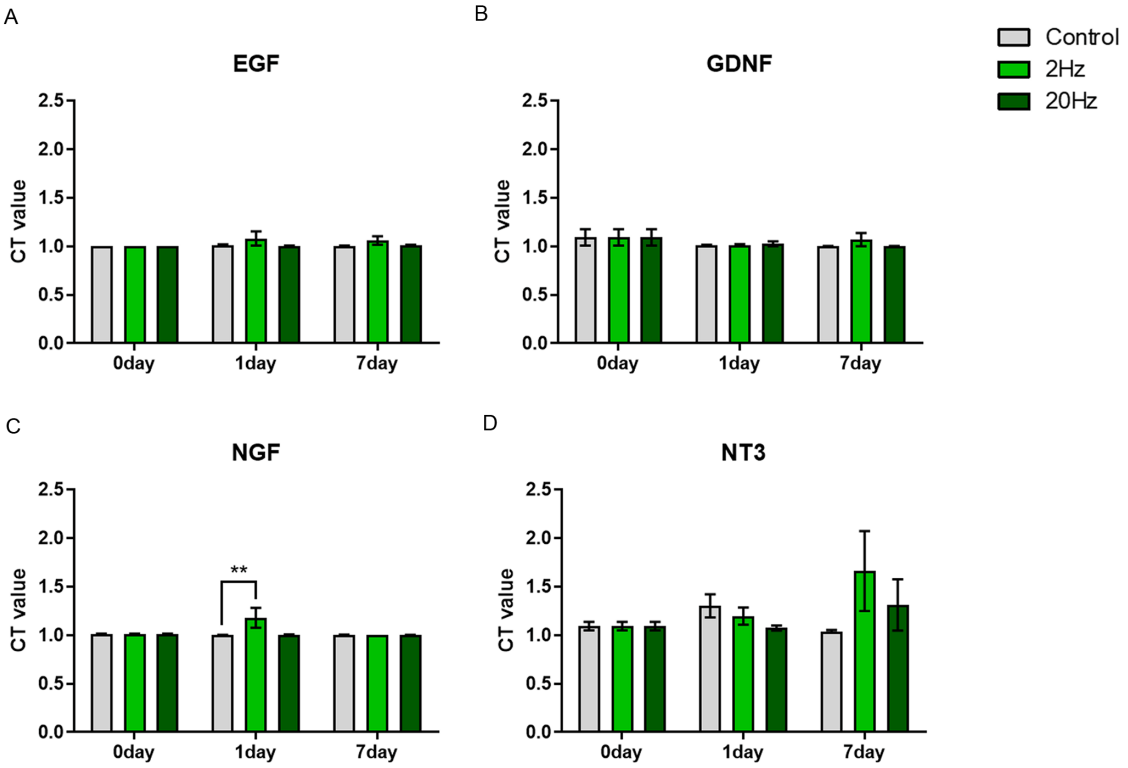

Figure 4. Real time polymerase chain reaction (PCR) analysis of neurotrophic and growth factors mRNA expression. Real time PCR analysis comparing the expression of epidermal growth factor (EGF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and neurotrophin-3 (NT3). As a result, there was a significant change in the NGF 1 day after the stimulus ($n = 3$). Error bars indicate means ± SEM. Data were analyzed by 2-way ANOVA. $^{**}P < 0.01$.

euthanized rabbits on POD0, POD1, and POD7 and observed the mRNA levels (Fig. 4). The mRNA levels of EGF in the 2-Hz electrical stimulation group (POD1 [108.0 ± 14.5%] and POD7 [105.9 ± 8.7%]) increased on POD1 and POD7 in comparison with that in the control group (POD1 ([01.0 ± 1.8%] and POD7 [100.5 ± 0.4%]) or the 20-Hz electrical stimulation group (POD1 [100.4 ± 0.6%] and POD7 [100.8 ± 1.4%]; Fig. 4A); however, there were no significant differences among the groups. Similarly, mRNA levels of GDNF in the 2-Hz electrical stimulation group (97.9 ± 12.5%) increased at POD7 in comparison to that in the control group (91.7 ± 0.2%) or the 20-Hz electrical stimulation group (91.8 ± 0.3%; Fig. 4B); however, no significant differences were observed. The mRNA levels of NGF in the 2-Hz electrical stimulation group (116.5 ± 20.4%) significantly increased at POD1 compared to that in the control group (99.0 ± 0.2%) or the 20-Hz electrical stimulation group (99.2 ± 0.3%; Fig. 4C). Finally, mRNA levels of NT3 of the 2-Hz (105.5 ± 21.3%) and the 20-Hz (93.8 ± 2.3%) electrical stimulation groups increased at POD7 in comparison to that in the control group (98.4 ± 8.0%; Fig. 4D); however, there were no significant differences among the groups. The 2-Hz electrical stimulation promoted expression of NGF in the damaged cornea at POD1.

Increased Corneal Innervation in the Electrical Stimulation Groups in NZW Rabbits After SLK

On POD28, the rabbits were euthanized and the corneal innervation was observed. The densities of nerve terminals and sub-basal nerve plexuses in the control and electrical stimulation groups were compared (Figs. 5A, 5B). In the control group, nerve terminals were rarely distributed and loss of fibers in the sub-basal nerve plexuses was observed. The nerve terminal and sub-basal plexus in the electrical stimulation groups (2 Hz and 20 Hz) were denser than those of the control group. Especially, the 2-Hz electrical stimulation group showed significant increase in nerve terminal density (368.3 ± 228.2%) as compared to the control group (100 ± 45.0%) or the 20-Hz electrical stimulation group (153.5 ± 41.3%; Fig. 5C). There was no difference in the nerve terminal densities between the control and the 20-Hz electrical stimulation groups.

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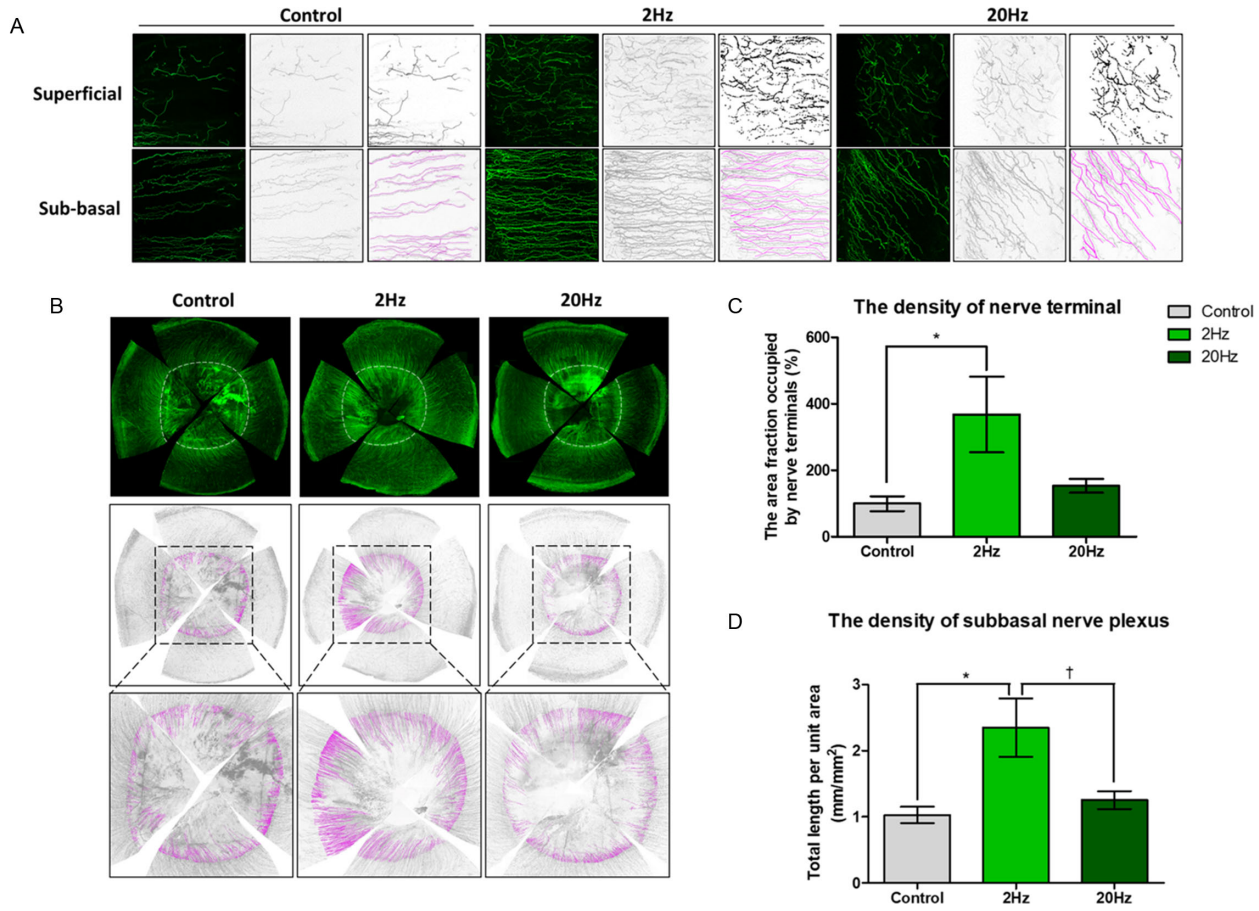

Figure 5. The effect of transcutaneous electrical stimulation on corneal innervation. (**A, B**) Representative images of superficial layer and sub-basal layer in the control, 2-Hz, and 20-Hz groups at POD28. Approximate depth of superficial layer and sub-basal layer were 22, 24, and 16 µm, and 30, 36, and 32 µm in the control, the 2-Hz, and the 20-Hz groups, respectively. (**C, D**) Nerve terminal density was measured using Image J. Error bars indicate ± SEM. Data were analyzed by *t*-test. $^{*,\dagger}P < 0.05$.

Likewise, the sub-basal nerve plexus densities of the 2-Hz electrical stimulation group (2.3 ± 0.9 mm/mm^2) were significantly increased than that of the control (1.0 ± 0.2 mm/mm^2) or the 20-Hz electrical stimulation groups (1.3 ± 0.3 mm/mm^2; Fig. 5D). Overall, the corneal innervation was significantly increased in the 2-Hz electrical stimulation group than in the other groups.

Discussion

In this study, we observed that electrical stimulation had effects on corneal nerve regeneration based on the histologic analysis with visualization in a rabbit model of corneal damage. Overall, electrical stimulation at 2 Hz and 20 Hz led to higher corneal sensitivity 7 days after injury compared to the control group that received no simulation. In addition, the 2-Hz group demonstrated superiority over the 20-Hz and control groups with respect to a regeneration-associated marker SPRR1a and NGF, an increase in corneal nerve density. Despite these findings, no differences were observed in epithelial wound healing among the three groups. Our results show that electrical stimulation induces the expression of mRNA related to the nerve growth factor first followed by the expression of the protein that subsequently leads to corneal nerve regeneration and increase in corneal sensitivity.

There have been several studies to improve corneal nerve regeneration. Topical pergolide in a mouse model of corneal wound injury,[35] pigment epithelium-derived factor peptide 44-mer in a rabbit corneal nerve injury model,[36] and VEGFA 188 protein in a mouse corneal injury model[37] are some of the suggested candidate materials for corneal nerve regeneration. In addition, human platelet lysates in corneal wounds in a rat model,[38] autologous plasma treatment in neurotrophic keratopathy,[39] and surgical procedures such as corneal neurotization[40] have also been suggested. However, a

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previous investigation by Paik et al. concluded that there are limitations in attempts to resolving symptoms that arise from corneal nerve damage solely with pharmaceuticals.[41]

There have been attempts to electrically stimulate the trigeminal nerves to improve DED symptoms, along with promising results reported recently in randomized controlled clinical trials. TES combined with artificial tears showed improvements in OSDI, TBUT, Schirmer's I test, and corneal fluorescein scores.[42] Similarly, another study also demonstrated improvements in the OSDI, TBUT, fluorescein staining of the cornea, and Schirmer's I test.[43] In addition, intranasal stimulation of the nasolacrimal neural pathway increases acute tear production and relieves DED symptoms.[44,45] The intensities of ocular pain and light sensitivity were reportedly reduced by TES of the trigeminal nerve.[46] Although corneal nerve regeneration was not mentioned, it should be noted that the electrical stimulation utilized in these clinical investigations was associated with nerve stimulation. In a study by Gaffariyeh et al., electrical stimulation in patients who undergo LASIK had improved recovery from corneal hypoesthesia. They applied electrical stimulation of continuous 20-Hz rectangular, biphasic wave pulses for 60 minutes.[30] Corneal sensitivity was significantly improved in comparison with the control group at 3 months after surgery (32 ± 13 mm improvement).[30] This is consistent with the findings of improved corneal sensitivity in the groups applied with 2-Hz and 20-Hz electrical stimulations in this study.

Although several parameters of electrical stimulation for nerve recovery have been explored,[31,47–50] the optimal parameters in peripheral nerves, including the corneal nerve, have not yet been confirmed. Studies of electrical stimulation with a 20-Hz frequency on the damaged facial and sciatic nerves of rats demonstrated increased BDNF expression and improved nerve structure.[50–52] Compared to the control group, sciatic function index and axonal density were increased by 26% and 19%, respectively, after 3 weeks of 20-Hz electrical stimulation.[52] Facial function recovered more rapidly in the 20- and 40-Hz electrical stimulation groups.[50] As for the muscle action potentials, the regenerated sciatic nerves treated with 2-Hz of electrical stimulation had a significantly shorter latency, longer duration, and faster nerve conductive velocity compared to the other groups (the control, 1-Hz, 20-Hz, and 200-Hz groups).[31] A 2-Hz burst pulse showed better nerve regeneration capacity than 100-Hz pulse electrical stimulation in the study by Cavalcante Miranda de Assis et al.,[47] and NGF expression was maximized (4.1-fold increase) with 1-Hz electrical stimulation on cultured Schwann cells in the study by Huang et al.[48] Morimoto et al. reported the neuroprotective effect of 1-, 5-, and 20-Hz electrical stimulations observed from retinal ganglion cells and suggested that other parameters, such as current intensity, pulse duration, and stimulation time, have potential for different neuroprotective effects.[49] At 1-, 5-, and 20-Hz, the mean retinal ganglion cells density was significantly higher than that in the sham stimulated retinas. The neuroprotective and regenerative effects of electrical stimulation are high at low frequencies (1–5 Hz) and at 20 Hz.[47,49] It has also been confirmed that the lower the frequency of electrical stimulation, the higher the NGF expression.[48] Based on previous data, in this study, we compared the effects of 2-Hz and 20-Hz electrical stimulation on corneal nerve regeneration, and 2-Hz frequency was more effective in increasing both SPRR1a and NGF expression in corneal tissue. Moreover, 2-Hz electrical stimulation with a relatively small number of stimulates may have an advantage over 20-Hz electrical stimulation in user compliance related to electrical stimuli-associated pain.

The NGF is critical for the survival and maintenance of sensory neurons. The promoted expression of NGF by electrical stimulation could help regenerate injured corneal nerves.[53] Many studies have suggested that NGF is responsible for not only wound healing and improved sensitivity of the eye surface but also increases conjunctival goblet cell density and tear production.[54–57] Sacchetti et al. clinically investigated its utility in DED treatment with NGF and demonstrated its promotion of wound healing and improvement of corneal sensitivity.[58] Additionally, both subjective (OSDI, SANDE frequency) and objective (TBUT, tear film osmolarity) scores suggested its efficacy.[58] Through this finding, the increase in NGF expression and corneal nerve recovery with electrical stimulation demonstrated its potential for DED treatment.

In this animal study, rabbit corneas were used to confirm the effect of TES on the recovery of corneal nerves. Electrical current was applied to the nerves around the eyeball through an electrode patch in clinical trials. The corneal size of rabbits is similar to that of humans; therefore we assumed using rabbits for this study can mimic the same conditions as performed during clinical trials. However, there were limitations in selecting genes and proteins relating to tissue or nerve growth to confirm the recovery of corneal nerves since the antibodies for the corneal tissue of rabbits are not as diverse as those of rats or mice. In addition, there was other limitation in identifying types of recovered nerves. Although we observed significantly increased corneal sensitivity and corneal nerve density in the 2-Hz electrical stimulation group than in the control

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group, types of recovered nerves were not identified in this study. As types of recovered nerves are crucial for the functional recovery of cornea, further studies are needed to evaluate types of recovered nerves by electrical stimulation.

TES promoted corneal nerve regeneration in the rabbit SLK model. Our study showed that 2-Hz electrical stimulation was more effective than 20-Hz electrical stimulation, showing potential clinical applications. A large-scale clinical study based on the results of this study is required to determine whether electrical stimulation can be used as a treatment for DED-related corneal nerve damage.

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