Dry eye disease (DED) is a multifactorial disease characterized by unstable tear film and ocular surface damage that results in a visual disturbance with ocular discomfort. While the pathogenesis of DED has not been fully established, chronic ocular surface inflammation was reported to play a prominent role. When the pathogenesis of DED has not been fully established, chronic ocular surface and to regulate the homeostasis of the immune function of the corneal nerve is essential to maintain a healthy ocular surface and to prevent a visual disturbance with ocular discomfort. While the cornea is exposed to desiccating stress, tear film is in a hyperosmolar state and the inflammatory cytokines are released from the ocular surface tissues. Th17 cells are known as a key player in the pathogenesis of DED. Not only the desiccating stress but also the various conditions such as aging, contact lens wear, laser in situ keratomileusis (LASIK) surgeries, and eyelid disorders are able to initiate the immune cascade responses. Inflammation is suppressed under neural control, allowing the corneal surface as an immune-privileged site. A recent study reported that circular corneal incision in one eye could abolish the immune privilege of the bilateral ocular surface, leading to a high rate of corneal allograft rejection. Additionally, it was reported that some corneal disease in the unilateral eye could alter the immune cells, nerve density, and cytokine levels in bilateral eyes. Based on the previous studies, we evaluated whether the corneal nerve cut in a single eye would have a bilateral effect on (1) immune reaction in ocular surface, (2) lacrimal secretory function, and (3) morphologic changes of corneal nerve fibers in a murine model.

**METHODS**

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute (IACUC no. 16-0107-S1A0 and 17-0091-S1A0). Animal experiments were performed in accordance with the principles of laboratory animal care and scientific procedures.
dance with the ARVO Statement for Use of Animals in Ophthalmic Vision and Research.

Animals
Sixty 7-week-old BALB/c female mice were purchased (Orient Bio Inc., Seongnam, Gyeonggi-do, Korea). The mice were randomly divided into two groups (control and nerve-cutting [NC] groups, \( n = 30 \) per group). The experimental scheme is summarized in Figure 1A.

Corneal NC
To sever the subbasal nerve plexus and the stromal nerves of the cornea, the corneal surface of the left eye in the NC group was incised using a circular punch (Kai Europe GmbH, Solingen, Germany) with a 2.0-mm diameter.\(^{14}\) The punch was applied to the corneal surface and then twisted five to seven times with slight pressure until the epithelium and the half-depth of the stroma were incised (Fig. 1B), a technique that was adopted from a previous study.\(^{14}\) At the end of the procedure, a drop of 0.5% levofloxacin eyedrops was instilled into the trephined eye. As the NC was performed for only the left eye, the right and left eyes of the NC group were defined as contralateral and trephined eyes, respectively, for statistical analysis.

Corneal Epithelial Damage Staining
The amount of corneal epithelial damage was evaluated using lissamine green dye (3% Lissamine Green B; Sigma-Aldrich Corp., St. Louis, MO, USA). Under anesthesia, one drop of the dye was applied to the ocular surface. The extent of the staining was assessed by a single observer (L.H.K) in a blinded manner using a grading scale as follows: 0 for no punctuate staining, 1 when less than one-third of the corneal surface was stained, 2 when from one-third to two-thirds were stained, 3 when more than two-thirds were stained.\(^{18}\) The staining evaluation was performed before the trephination and every week for a total of 4 weeks of the observation period.
FIGURE 2. Time-dependent change in the distribution of subbasal and stromal nerve bundles following unilateral corneal trephination. Representative corneal whole-mount images of immunostaining for class III β-tubulin and epithelial nuclei before (A), immediately after (B), 1 (C) and 2 weeks (D) from the trephination. Full (×40 magnification; scale bar: 500 μm) and detailed (×100 magnification; scale bar: 200 μm) images were taken in each cornea tissue. (A) In the normal, healthy cornea, dense and regular subbasal nerve bundles with central whorl-like appearance (arrows) and deeper stromal nerves (arrowheads) were observed. (B) Trephined line (arrows) was marked in the trephined eye. Note the presence of patchy-like focal dropout of subbasal nerve bundles (arrowheads) in the contralateral eye. (C) At 1 week after the unilateral trephination,
subbasal and stromal nerve density was markedly decreased in the trephined eye. Broad denervation with the patchy-like loss (arrows) was observed in the central and peripheral cornea. In the contralateral eye, subbasal and stromal nerve density was also decreased compared with those of normal cornea. Notably, the whorl-like appearance of nerve bundles was not observed in the central area. (D) At 2 weeks after the unilateral trephination, the loss of nerve fiber bundles and morphologic changes were more extensive. In the trephined eye, subbasal and stromal nerve fiber was not observed in the central and midperipheral area. In the contralateral eye, central denervation (arrows) was more marked compared with those at 1 week.

Tear Volume Measurement

Aqueous tear production was measured by the phenol red thread test. Under anesthesia, a phenol red-impregnated cotton thread (FCI Ophthalmics, Pembroke, MA, USA) was placed at the lower lateral canthus. After 60 seconds, the thread was gently removed, and the length of the wet portion (denoted by the red color change) was measured in millimeters. The tear volume evaluation was performed before the NC and every week for 4 weeks.

Corneal Whole-Mount Staining

The mice were euthanized by cervical dislocation under deep anesthesia with Zoletil, according to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2013 edition). After enucleation, the corneas were freed from the anterior segments. The corneas were fixed for 10 minutes in ice-cold acetone mixed with methanol (1:1) and then rinsed in PBS. The preparations were stored in 2% BSA at 4°C for 24 hours. The tissues were blocked (Protein Block Serum-Free; Dako, Carpinteria, CA, USA) for 10 minutes at room temperature. To identify the corneal nerve fibers, we used neuronal class III β-tubulin antibody (AB15708A4, Alexa Fluor 488-conjugated, 1:400; Millipore, Billerica, MA, USA). At 4 hours after the antibody mixture was applied, the cornea tissues were rinsed with PBS. Four radial incisions were made toward the center of the cornea, and the flattened tissues were observed using mounting medium containing 4',6-diamidino-2-phenylindole (Vectorshied; Vector Laboratories, Burlingam, CA, USA).

Flow Cytometry

At day 14 and 28 after the corneal NC, the cornea/conjunctiva, draining lymph nodes, and spleens were extracted and collected. The proportion of mature dendritic cells (DCs) was determined by measuring the expression of the costimulatory molecule (CD86) or major histocompatibility complex (MHC) class II on CD11c- or CD11b-expressing cells, using flow cytometry. To determine the subset of the effector T cells, the proportion of IFN-γ, IL-17A, CD103, and the CD69 T cells were assessed. For regulatory T cells, simultaneous expression of CD25 and Forkhead box protein 3 (Foxp3) on CD4⁺ or CD8⁺ T cells were measured.

To get the cell suspensions, the collected lymph nodes and spleens were minced between the frosted ends of two glass slides in RPMI media (WelGENE, Daegu, Korea). The media contained 10% fetal bovine serum and 1% penicillin-streptomycin. The extracted cornea and conjunctiva tissues were cut into small pieces by microscissors and lysed in the RPMI media.

Enzyme-Linked Immunosorbent Assay

ELISA was performed to quantify the level of neuropeptides, using specific commercially available kits. For peptide extraction, extraorbital lacrimal glands were minced into small pieces and sonicated in protein extraction solution (PRO-PREP; Intron Biotechnology, Seongnam, Korea) on ice. The supernatant was collected after centrifugation at 13,000 × g for 20 minutes, and assayed for the concentration of substance P (SP; detection limit: 43.8 pg/mL; R&D Systems, Minneapolis, MN, USA), calcitonin gene-related peptide (CGRP; detection limit: 0.16 ng/mL; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA), vasoactive intestinal peptide (VIP; detection limit: 0.12 ng/mL; Phoenix Pharmaceuticals Inc.), and neuropeptide Y (NPY; detection limit: 0.09 ng/mL; Phoenix Pharmaceuticals Inc.) by ELISA according to the manufacturer’s protocol.

Statistical Analysis

Statistical analysis software (GraphPad Prism, ver. 5.01; GraphPad Software Inc., La Jolla, CA, USA) was used for statistical tests. Homogeneity of variance was assessed by a Levene test. To compare three groups (control, contralateral, trephined groups), data were analyzed by the Kruskal-Wallis test. When the null hypothesis was rejected (P < 0.05), pairwise comparisons of the groups were performed with the Mann-Whitney test. Comparison of data from two groups (control and NC groups) was performed with the Mann-Whitney test. Data were presented as the mean ± standard error. Differences were considered significant when P < 0.05.

RESULTS

Severing Corneal Nerves in a Single Eye Induced Bilateral DED in a Murine Model

We first evaluated whether severing the corneal nerve in one eye would induce clinical manifestations of DED in bilateral eyes. In the NC group, corneal staining grades were significantly increased in bilateral eyes compared with those in the control group for all 4 weeks of the observation (Fig. 1C, D; Supplementary Fig. S1). The difference of the staining volume was significantly decreased in bilateral eyes in the NC group during the study period (Fig. 1E). The maximal statistical significance of the tear volume measurements was shown on day 7. We concluded that, taken together, unilateral corneal nerve damage would lead to bilateral DED that would persist for at least 28 days.
**FIGURE 3.** The result of flow cytometry analysis for DCs in cornea/conjunctiva. (A) For DCs, CD11b⁺ or CD11c⁺ cells were counted. For mature form, CD86 or MHC class II expression on CD11b⁺ or CD11c⁺ cells were gated. (B) At day 14, the number of CD11b⁺ or CD11c⁺ cells and the mature forms were increased in bilateral cornea/conjunctiva in the NC group. In particular, CD11b⁺CD86hi or CD11b⁺MHCIIhi cells were significantly increased. (*P < 0.05 by Kruskal-Wallis test.) (C) At day 28, except for CD11c⁺ cells, the number of DCs didn’t show statistical difference among the groups. (*P < 0.05 by Kruskal-Wallis test.)
Severing Corneal Nerves in a Single Eye Induced Loss of Corneal Subbasal and Stromal Nerve in Bilateral Eyes

We assessed the state of corneal innervation by double-labeling of nerves and cell nuclei. In the normal cornea, two layers of innervation—stromal nerve network and dense subbasal nerve plexus—were observed (Fig. 2A). The subbasal nerve bundles ran centripetally and formed a whorl-like structure at the central area. Immediately after unilateral trephination, focal areas of subbasal nerve dropout began to be observed in the contralateral eyes (Fig. 2B). At day 7, in the trephined eyes, subbasal and stromal nerve fiber loss with dropouts of overlying epithelium was observed in the broad area (Fig. 2C). In the contralateral eyes, the architecture of the nerve bundles was partially disrupted, especially in the corneal center, and the whorl-like structure was not observed. At day 14, the area of nerve loss in the trephined cornea became much enlarged (Fig. 2D). In the contralateral eyes, central denervation was more prominent than were those at day 7.

Severing Corneal Nerves in a Single Eye Enhanced Accumulation and Maturation of DCs in Bilateral Cornea/Conjunctiva and Draining Lymph Nodes

Next, we explored the DC population in cornea/conjunctiva and draining lymph nodes using flow cytometry. First, we gated CD11b+ or CD11c+ cell population. The expression of CD86 or MHC class II was then assessed for mature DCs (Fig. 3A). At day 14, the proportion of CD4+CD69hi cells increased significantly in bilateral eyes of the NC group compared with those of the control group, with significant and marginal differences, respectively (P = 0.0249, 0.0548, respectively) (Fig. 3B). Also, mature DCs expressing CD86 or MHC class II were significantly accumulated more in bilateral eyes of NC group compared with controls. However, at day 28, DC population in cornea/conjunctiva didn’t show any significant difference among the groups, except the CD11c+ cells (Fig. 3C). Meanwhile, in draining lymph nodes at day 28, mature DCs such as CD11c+CD86hi and CD11c+MHCIIhi cells notably increased bilaterally with moderate and significant differences, respectively (P = 0.0562, P = 0.0010, respectively) (Fig. 4).

Unilateral Corneal NC Activated IFN-γ–producing CD8+ T Cells in Draining Lymph Nodes

We then evaluated the subset of resident memory or effector T-cell population in bilateral cornea/conjunctiva and draining lymph nodes. First, we gated CD103- and CD69-expressing T cells in cornea/conjunctiva tissue (Fig. 5A). At day 14, the proportion of CD4+CD69hi cells increased significantly in bilateral eyes of the NC group (P = 0.0249) (Fig. 5B). However, at day 28, CD69 expression didn’t show significant differences among the groups (Fig. 5C). In draining lymph nodes, we measured IL-17A or IFN-γ expression on CD4+ or CD8+ T cells (Fig. 6A). At day 28, the proportion of Th17 cells significantly increased in the NC group (P = 0.0247) (Fig. 6B). Notably, IL-17A expression on CD8+ T cells decreased in the NC group, while IFN-γ expression increased with statistical significances (P = 0.0102, 0.0169, respectively).
FIGURE 5. The result of flow cytometry analysis for effector T cells in cornea/conjunctiva. (A) For gating resident and activated effector T cells in cornea/conjunctiva, the expression of CD103 and CD69 on CD4⁺ or CD8⁺ T cells was assessed, respectively. (B) At day 14, CD4⁺ T cell and its activated form expressing CD69 increased in cornea/conjunctiva in the NC group. (P = 0.0388, P = 0.0249, respectively.) (C) At day 28, the proportion of effector T cells in cornea/conjunctiva didn’t increase in the NC group. (*P < 0.05 by Kruskal-Wallis test.)
Severing Corneal Nerves in a Single Eye Enhanced the Generation of Regulatory T Cells in the Spleen

Along with the assessment of the immune cell subsets in cornea/conjunctiva and draining lymph nodes, we analyzed splenic cell population at day 14 and 28. To assess the regulatory T-cell population, we gated the concomitant expression of CD25 and Foxp3 on CD4$^+$ or CD8$^+$ T cells (Fig. 7A). At day 14, the proportion of regulatory T cells was not significantly different between control and NC groups (Fig. 7B). However, at day 28, the proportion of splenic CD4$^+$CD25$^{hi}$Foxp3$^{hi}$ and CD8$^+$CD25$^{hi}$Foxp3$^{hi}$ cells increased in the NC group compared with controls ($P = 0.0231$, $P = 0.1231$, respectively) (Fig. 7C).

Severing Corneal Nerves in a Single Eye Would Alter the Neuropeptide Level of Extraorbital Lacrimal Glands

The level of SP, NPY, CGRP, and VIP in extraorbital lacrimal glands was assessed by ELISA. Immediately after the corneal trephination, the VIP level was significantly increased only in trephined eyes ($P = 0.0276$) (Fig. 8A). At day 7, CGRP level was significantly increased in contralateral and trephined eyes compared with those of the control group ($P = 0.0078$) (Fig. 8B). At day 14, the CGRP level significantly decreased in the contralateral eyes ($P = 0.0144$), and VIP level was suppressed in contralateral and trephined eyes ($P = 0.0089$) (Fig. 8C).

DISCUSSION

Our study shows that unilateral circumferential corneal nerve severing induces (1) bilateral ocular surface inflammation mediated by anterograde nerve degeneration and (2) bilateral hypofunction of lacrimal glands by retrograde neural pathway, as presumptively illustrated in Figure 9.

Since the subbasal nerve bundles run from the periphery to the center in a perpendicular direction, we assumed that the subbasal nerve fibers were completely severed by the midperipheral circular trephination. When the trephination was restricted to the corneal epithelium, the subbasal nerve...
fibers would rapidly recover to their original density within a few days. However, the recovery of the nerve fibers was delayed in this study, as the subbasal and stromal nerves had been cut together by the trephination through the upper half of the stromal layer. As expected, the subbasal and stromal nerve density gradually decreased during the 14 days of the observation period, rather than recovered. Surprisingly, the corneal nerve density in the opposite eye, which was not directly injured, also decreased.

Antidromically evoked depolarization enables the release of neuropeptides contained in the corneal nerve endings not directly exposed to the stimulus, leading to extended inflammation to neighboring areas, called "neurogenic inflammation." This study didn’t prove local neuroinflammation. On the contrary, this study showed some of the immune-activated cells traveled to the draining lymph nodes and reached out to the contralateral ocular surface. The bilateral nerve density reduction may be explained by the activation of the immune response mediated by Wallerian nerve degeneration. During this process, degenerated axons are removed by Schwann cells, macrophages, monocytes, or neutrophils. In the inflammatory environment, the DCs become mature, expressing MHC class II and costimulatory molecules. The mature DCs egress from the cornea to the draining lymph nodes and activate effector lymphocytes. The mature DCs and effector lymphocytes may migrate to the bilateral ocular surface through the systemic blood circulation. The effector cells that arrive at the bilateral cornea may degenerate corneal nerves further. This hypothesis is supported by the accumulation of mature DCs in the bilateral ocular surface and draining lymph nodes and an increase of effector T cells in bilateral draining lymph nodes, shown in this study. Meanwhile, as the inflammatory tone increased in the immune-privileged site, it was expected that the efforts to return immune homeostasis would be accompanied as well.

Accordingly, the regulatory T cells that increased in the spleen at day 28 can be considered as a possible compensatory reaction.

**FIGURE 7**. The result of flow cytometry for regulatory T cells in the spleen. (A) For regulatory T cells, concomitant expression of CD25 and Foxp3 on CD4+ or CD8+ T cells was assessed. (B) At day 14, regulatory T cells in the spleen didn’t show a significant difference between the control and the NC groups. (C) At day 28, splenic regulatory T cells increased in the NC group compared with controls. Especially, CD4+CD25hiFoxp3hi cells showed significant differences (P = 0.0251). (*P < 0.05 by Kruskal-Wallis test.)
In addition to the immune response triggered by the anterograde nerve degeneration, the retrograde signals transmitted to the contralateral eye through the centripetal neural interconnection may contribute to the bilateral efferent nerve alteration. Given that the trigeminal nuclei may project the nerve fibers to both sides, with the axons crossing between bilateral dorsal horns through the dorsal commissure, 16,28 retrograde nerve signals from the corneal afferent nerve injury may mediate bilateral inflammatory responses, leading corneal nerve degeneration as well as the altered secretory function of the lacrimal glands in both eyes. This mechanism may also explain bilateral corneal nerve alteration observed in the unilateral ocular disorder, such as herpes zoster ophthalmicus and herpes simplex keratitis, described in the previous studies.16,17 Unilateral corneal trephination reduced the bilateral tear volume significantly for more than 28 days. This tear reduction may be explained by the altered parasympathetic tone innervating lacrimal glands as well as the altered secretory function of the lacrimal glands in both eyes. This mechanism may also explain bilateral corneal nerve alteration observed in the unilateral ocular disorder, such as herpes zoster ophthalmicus and herpes simplex keratitis, described in the previous studies.16,17 Unilateral corneal trephination reduced the bilateral tear volume significantly for more than 28 days. This tear reduction may be explained by the altered parasympathetic tone innervating lacrimal glands in response to the afferent nerve injury. Having the possible existence of bilateral projecting trigeminal nerves, the afferent nerve impulses propagated to the centripetal direction may stimulate the crossing nerve fibers, resulting in changes of CGRP and VIP releases from bilateral efferent autonomic fibers. Stimulation of either parasympathetic or sympathetic nerves release neurotransmitters that regulate secretion of proteins, electrolytes, or water in lacrimal glands.29,30 Accordingly, both VIP, as a parasympathetic neurotransmitter, and NPY, as a sympathetic neurotransmitter, contribute to the secretion of lacrimal glands.29,30 In this study, a statistically significant decrease of VIP in both lacrimal glands seems to be involved in reduction of bilateral tear secretion. NPY tended to decrease at 14 days in the contralateral lacrimal gland, which was statistically insignificant. The alteration of the sympathetic pathway is possible, but it is still inconclusive. The second explanation for the bilateral tear volume reduction may be related to the diminished afferent inputs resulting from the extensive bilateral sensory neural loss. The lacrimal functional unit consisting of the ocular surface, lacrimal gland, and interconnecting innervation finely controls the tear secretion.31,32 If any part of this unit is compromised, tear production is impeded.33 Taken together, both centripetal stimulation of trigeminal nerves that project bilaterally to trigeminal brainstem nuclei and systemic activation of the immune system seem to act on ocular and neural structures of both sides. Further studies should be undertaken to reveal the role of each neuropeptide following the corneal nerve injury in the future.

**FIGURE 8.** The result of ELISA for neuropeptide level in the extraorbital lacrimal glands. The level of neuropeptides was compared among the control, contralateral, and trephined eyes. (A) Immediately after the unilateral corneal trephination, VIP level significantly increased in the trephined eye ($P = 0.0276$), while the level of SP, NPY, and CGRP was not different among the groups. (B) At day 7, CGRP level significantly increased in the contralateral and trephined eyes compared with that of the control group ($P = 0.0078$), while the other neuropeptides were not different among the groups. (C) At day 14, CGRP level significantly decreased in the contralateral eyes ($P = 0.0144$), and VIP level significantly decreased in the contralateral and trephined eyes ($P = 0.0089$), while the level of SP and NPY didn’t show significant changes. (*$P < 0.05$ by ANOVA.)
The hypothetical illustration showing scenario of how the unilateral corneal nerve cut may affect bilateral ocular surface through the immunologic and neural pathway. This schematic illustration summarizes the effect of the unilateral corneal cutting on the bilateral ocular surface and lacrimal glands. When the corneal subbasal and stromal nerves are cut by trephination in one eye, the signals formed from the injured stimuli transmitted to the bidirectional way. The anterograde nerve degeneration may increase the immune response of corneal tissue, thereby generating mature DCs. The mature DCs migrating to the draining lymph nodes can activate effector lymphocytes, and the effector cells arrive ipsilateral and contralateral ocular surface through the blood vessels. Meanwhile, the retrograde centripetal nerve signals to the brain stem nucleus may cross over and alter the bilateral lacrimal gland secretory function.

Because of the short experimental period, we do not know when the sensory nerve hypofunction would recover back to normal. In the previous LASIK studies, the corneal sensation was reduced for 3 weeks to 9 months or nerve density didn’t recover during 2 years. Therefore, the nerve attenuation may persist for a long time in this study.

In the previous studies, several animal models have been introduced to evaluate the morphologic alteration of the corneal nerves following the trigeminal nerve injury by transecting of ciliary nerves or electrolysis of the trigeminal nerve. However, we intended to evaluate the effect of corneal nerve damage presumably occurring in LASIK, cataract surgery, or DED, and thus corneal trephination was adopted. Although the injured branches were a more distal part of trigeminal nerves in our study, bilateral reduction of nerve density corresponds well with the previous study. Notably, similar to the previous study, the nerve degeneration in the contralateral eyes was restricted in the central cornea. The disparity of nerve degeneration between the central and peripheral cornea may be originated from the differences of the nerve fiber structure. Given that the main nerve fibers are unmyelinated nerves in the central cornea and the unmyelinated nerves degenerate faster from the distal part than myelinated nerves, it explains that the neural attenuation was marked in the central cornea.

This study was limited because (1) it is an observational study and (2) structural changes of the gland atrophy or neural alteration in the lacrimal gland were not evaluated. Nevertheless, we believe it is worthy of notice by revealing bilateral substantial changes in lacrimal glands and immune cell profiles in the conjunctiva/cornea through the circumferential unilateral nerve cut.

In summary, the unilateral corneal nerve cut may have an effect on both bilateral ocular surface and bilateral lacrimal glands through the bidirectional nerve stimulation. Anterograde Wallerian degeneration may activate the adaptive immune response, and retrograde central stimulation may alter the secretory function of lacrimal function, leading to bilateral tear volume reduction and ocular surface inflammation. Therefore, the unilateral corneal nerve injury may mechanistically participate in the development of an immune inflammatory disorder such as DED.

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