The prevalence of dry eye was reported to be 33% in patients older than 50 years of age in a broad epidemiologic study. The 2017 Dry Eye WorkShop (DEWS) report defined dry eye as a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and neurosensory abnormalities play etiologic roles. The inflammatory process leading to infiltration of immune cells with resulting damage to the corneal nerves is supplied primarily by the ophthalmic terminal branch of the trigeminal nerve. These branches transform to the subepithelial nerve plexus between the anterior stroma and Bowman’s layer. The nerves arising from the plexus penetrate the Bowman’s layer and generate corneal subbasal nerve (CSN) fibers extending through basal epithelial nerve cells. CSN fibers have substantial function on corneal homeostasis and have a primary role in the maintenance of ocular surface sensation and maintaining epithelial integrity via regulation of epithelial cell proliferation and wound healing. Several diseases including diabetes mellitus, keratocoroma, herpetic keratitis, limbal stem cell deficiency, aging, long-term use of contact lens, and corneal surgery might result in CSN damage. Persistent environmental stress induces ocular surface inflammation due to secretion of several chemical mediators from keratocytes, keratoconjunctival epithelial cells, leading to infiltration of immune cells with resulting damage to the corneal nerves.

Basal tear secretion is regulated by the cold thermoreceptors of the corneal trigeminal ganglion neuron. In addition, the mechanonociceptor and polymodal nociceptor neurons in the cornea are associated with pain sensation in dry eye disease.

In vivo confocal microscopy (IVCM) is a noninvasive technique that enables cellular assessment of the cornea; it is commonly used for the differential diagnosis and follow-up of various corneal diseases. This technique also provides high-resolution images of the CSN. Quantitative measurement of CSN density using IVCM is a standardized and very common parameter in the assessment of corneal nerve alterations in humans. In addition, the tortuosity; reflectivity of nerve fibers; and the number of dendritic cells (DCs) can also be evaluated by IVCM and provide valuable information in dry eye disease.

Several studies used IVCM to evaluate healthy and pathologic CSN in humans. Long-term diabetes has been also
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reported to be associated with morphologic changes of corneal nerves such as increased tortuosity. Although many mice models to investigate the pathogenesis of dry eye disease exist, there is a paucity of information in relation to CSN morphologic alterations in dry eye mouse models.

The aim of this study was to investigate the morphologic changes and distribution of the CSN plexus in wild-type (WT) mice after environmental dry eye stress (EDES) using IVCM.

**Materials and Methods**

**Animals and Implementation of EDES**

We examined 22 eyes of 8-week-old WT male mice (Balb/c; n = 11) in this study. WT mice were purchased from the CLEA Japan (Osaka, Japan). Five 8-week-old male mice were used as controls and six mice were exposed to EDES for 5 days (from 9:00 AM to 2:00 PM). The EDES mouse model was designed to mimic adverse conditions in office work by modifying the setting from a previous rat swing board model from our laboratory. In this modified new EDES model, the mice were placed in individual narrow compartments at a distance of 5 cm from the air fan.

**Surface Vital Staining Assessment**

After fluorescein dye instillation, the cornea was divided into three zones (upper, middle, and lower) and each zone had a staining ranking score between 0 and 3 points. The cornea was washed with 5 μL of phosphate-buffered saline and the same procedure was performed for lissamine green dye. Corneal vital stainings were recorded by a digital camera-equipped microscope using the same settings (exposure time) for all mice.

**Tear Film Stability Assessment**

The tear film break-up time (TBUT) was measured to evaluate tear film stability using an SL-15 (Kowa, Tokyo) portable slit-lamp microscope. First, 1 μL of 2% fluorescein solution was instilled. Excess fluorescein was wiped from the lateral canthus. Then, one researcher applied an air puff with a 1 mL syringe to induce a blink and the other researcher observed the TBUT with the handheld slit lamp after the blink response. After a natural blink, the TBUT was evaluated three times and the mean of these measurements was calculated.

**In Vivo Laser Scanning Microscopic Examination and Image Selection**

Before IVCM investigations, the animals were anesthetized with an intraperitoneal injection of 6 mg/mL of ketamine and 4 mg/mL of xylazine. While examining one eye after anesthesia, a carbomer 2% gel (comfort gel, Dr. Mann Pharma; Fabrik GmbH, Berlin, Germany) was applied to the other cornea to avoid exposure and dryness. We used a moveable mouse holder that we created from cardboard boxes to immobilize the mice during measurements. The Heidelberg Retina Tomograph II (HRT) with the Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) was used for corneal surface layer examination. HRT uses a 670-nm red wavelength diode laser source, and provides 400 × 400 μm real-time images of the cornea with a lateral resolution of 1 μm/pixel. The resolution of each recorded frame was 384 × 384 pixels, 8 bits of data, and a 128-bit binary floating point format. The total period of IVCM assessment was approximately 5 minutes per eye and at least 7 sequences, each containing 80 images (each image representing an area of 160.00 μm²), were obtained from each eye. Two experienced researchers selected four nonoverlapping IVCM images per cornea for CSN fiber assessment according to focus quality, high-resolution absence of motion, and pressure-induced artifact, which allowed distinction of nerves from the background. Two researchers were totally blinded to which group the images belonged (Fig. 2).

**Image Analysis**

Four parameters were investigated: the density of nerve fibers (NFD); tortuosity and reflectivity of CSN; and DC density. Image analysis was performed by two researchers (CS, TK). NFD was analyzed by tracing the CSN on raw TIFF images using semiautomated NeuronJ software (National Institutes of Health, Bethesda, MD, USA).

The NFD was determined as the total length of CSN fibers within a frame. Four different representative images for each cornea were evaluated in pixels separately. The mean NFD for each cornea was calculated by averaging these total values.

The grading of nerve tortuosity was performed at the subbasal layer. In their study, Oliveira-Soto et al. classified human corneal nerve tortuosity into four grades. Based on this study, we defined a new four-grade scale for mice CSN fibers. Nerves longer than 50% of the width frame underwent tortuosity examination (Fig. 3).

The grading of nerve reflectivity was again based on the previous report by Oliveira-Soto et al. Similarly, only the

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**Figure 1.** Representative photograph of the environmental dry eye stress mice model. WT mice were exposed to constant low humidity air flow aimed at the face. Mice were placed in individual narrow compartments at a distance of 5 cm from the air fan.
nerves longer than 50% of the width frame underwent reflectivity examination (Fig. 4).

For DC density assessment, four representative images from the CSN plexus area and basal epithelial layer were examined to measure the density of epithelial DCs.

Statistical Analysis
The Wilcoxon signed-rank test was performed to compare tear quantity, vital staining scores, and CSN parameters before and after EDES. The Spearman correlation coefficient was calculated to evaluate the association of CSN parameters between two examiners. A $P$ value less than 5% was considered to be statistically significant.

RESULTS
Aqueous Tear Secretion Quantity, Tear Function, and Vital Staining Alterations
The mean weight-adjusted aqueous tear secretion quantity significantly decreased after exposure to EDES ($P = 0.0019$; Fig. 5A). The mean TBUT at 3 days after EDES exposure (1.25 ± 0.35 seconds) was significantly shorter than before EDES exposure (2.7 ± 0.42 seconds; $P = 0.002$) (Fig. 5B). We also examined the change in corneal fluorescein and lissamine green staining scores before and 3 days after exposure to EDES. The mean fluorescein staining scores increased from 0.7 ± 0.82 points before EDES to 2.16 ± 1.11 points after exposure to EDES. The corneal lissamine green staining scores increased from 1.2 ± 1.81 before EDES to 4.83 ± 1.33 after exposure to EDES. EDES exposure significantly increased the corneal fluorescein ($P = 0.005$) and lissamine green staining scores ($P = 0.0003$; Fig. 6).

Subbasal Nerve Fiber Alterations After EDES
A total of 88 images including 687 nerves were examined for NFD by two examiners. The mean NFD was 2813 ± 762 pixels/frame, which decreased to 1898 ± 286 pixels/frame after EDES. The mean NFD value assessment between the two examiners showed a strong correlation ($R^2 = 0.83058$, $P < 0.0001$; Fig. 7A). We demonstrated that the NFD showed a marked decrease after 3 days of EDES in the corneas of WT mice ($P = 0.0071$; Fig. 8A).

We evaluated the tortuosity and reflectivity of 269 nerves from 88 images according to our new grading scale. The mean...
subbasal nerve tortuosity grade was 0.81 ± 0.33, which tended to increase to 0.96 ± 0.40 after exposure to EDES ($P = 0.307$; Fig. 8B). The subbasal corneal nerve reflectivity grade was 0.83 ± 0.37, which changed to 0.78 ± 0.43 after exposure to EDES ($P = 0.758$; Fig. 8C). In comparison to pre-EDES, the tortuosity and reflectivity mean grade values were not significantly different after exposure to EDES. The tortuosity and reflectivity average grade assessments between the two examiners showed a strong correlation ($R^2 = 0.8046$, $P < 0.0001$, and $R^2 = 0.89776$, $P < 0.0001$, respectively; Figs. 7B, 7C).

The density of DCs was 12.62 ± 5.9 cells/mm$^2$ before exposure to EDES, which increased to 15.93 ± 5.30 cells/mm$^2$ after exposure. Compared with pre-EDES, the average density of corneal DCs was significantly different after exposure to EDES ($P = 0.026$; Fig. 8D). The assessment of density of DCs between the two examiners showed a strong correlation ($R^2 = 87339$, $P < 0.0001$; Fig. 7D).

**DISCUSSION**

Desiccating stress in ocular mucosal epithelial tissue associated with decreased amount of tear and elevated tear osmolarity has been reported to induce local inflammation and peripheral nerve damage. $^{20}$ Local inflammation and nerve damage causes short- and long-term genetic and molecular alterations in primary sensory nerves. $^{29}$ Corneal superficial nerves are intensely and superficially present on the corneal surface of the sensory nerve terminals among the epithelial cells. Thus, corneal superficial nerves are easily affected by environmental factors (such as air pollution, low humidity); trauma (cataract and refractive surgery); and ocular surface diseases (pterygium, conjunctivochalasis, keratoconus). $^{50–52}$ In previous animal studies, changes in ocular surface temperature and decrease in tear secretion have been reported to be associated with alterations in the intensity and structure of CSN and epithelial nerve terminals. $^{53,54}$ Similarly, CSN changes were reported in the number of CSN fibers, tortuosity, budding pattern, and reflectivity in patients...
with tear deficiency associated with a wide range of ethnologic factors. The Tear Film & Ocular Surface Society DEWS II report states that when the tear film becomes unstable, mechanical trauma related to eye blinking affects the corneal surface epithelial cells, resulting in damage to terminal corneal nerve branches. As a result, a degeneration/regeneration cycle is initiated that causes structural changes in the cornea and conjunctiva nerve fibers.

**Figure 4.** Representative grade metric (0–4) images for reflectivity. We developed a new quantitative grade scale for CSN reflectivity, according to a previous study of Oliveira et al. and with respect to the background. (A) Grade 0. The CSN fibers appear with very low brightness; distinguishing from the background was difficult. (B) Grade 1. The CSN fibers are minimally brighter than the background. (C) Grade 2. The CSN fibers show moderate radiance. (D) Grade 3. The CSN fibers appear pretty well reflective; with definite brightness of the nerve. (E) Grade 4. The CSN fibers are easily distinguishable from the background; appearing strongly bright compared with the grade 3 nerve fibers. Scale bar: 50 μm.

**Figure 5.** Aqueous tear production and tear film stability assessment. (A) The mean aqueous tear production quantity decreased significantly in the Balb/c mice after 3 days of EDES application (P = 0.0019). (B) The mean tear break-up time values decreased significantly with EDES application in the Balb/c mice (P = 0.002).
Recent research has focused on the neurobiologic effects of dry eye disease. However, it is challenging for researchers to observe ocular surface nerve fiber morphology in vivo. IVCM provides real-time and high-resolution images and recently has been widely used for the in vivo real-time examination of corneal microstructures. IVCM also provides extensive information regarding corneal neuropathic alterations in dry eye disease, and morphologic changes of CSN plexus in other ocular surface diseases (Aggarwal S, et al. IOVS 2014;55:ARVO E-Abstract 1468).40

There are several studies employing the IVCM in the evaluation of the qualitative and quantitative alterations of corneal nerves in dry eye disease.41,42 These studies are generally focused on corneal CSN density. Although a reduction was detected in corneal nerve density in the majority of these studies,36,43–46 one study reported increased nerve density in patients with Sjögren syndrome.47 Tuominen et al.38 and Hoşal et al.48 compared CSN density between dry eye disease and control subjects and they did not find any differences. These discrepancies in the findings are considered to be associated with the severity and different stages of dry eye disease, differences in neural regeneration/degeneration patterns, differences in the extent of inflammation, and

![Comparison of fluorescein staining scores before and after environmental dry eye stress](image1.png)

![Comparison of lissamine green staining scores before and after environmental dry eye stress](image2.png)

**Figure 6.** Representative anterior segment photographs and time-wise alteration of corneal fluorescein and lissamine green staining before and after environmental dry eye stress. (A) Anterior segment photographs indicating alterations in fluorescein vital staining after environmental dry eye stress in WT mice. The deterioration in fluorescein staining after environmental dry eye stress application can be seen clearly. (B) Anterior segment photographs indicating alterations in lissamine green vital staining after environmental dry eye stress in WT mice. The deterioration in lissamine green staining after environmental dry eye stress application can be seen clearly. (C) Alterations of fluorescein staining scores with environmental dry eye stress in the WT mice. Note the significant increase in the fluorescein scores in the WT mice after 3 days of environmental dry eye stress application. *P < 0.05. (D) Alteration of lissamine green scores with environmental dry eye stress in the WT mice. Note the significant increase in the lissamine green scores in the WT mice after 3 days of environmental dry eye stress application. *P < 0.05.
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**Figure 7.** Quantitative approach differences between two examiners. Graphs for (A) nerve density, (B) tortuosity grade, (C) reflectivity grade, and (D) DCs density show the strong correlation between measurements of the two examiners.

**Figure 8.** Quantitative analysis of IVCM images for nerve density, tortuosity, reflectivity, and DCs. (A) Note the significant decrease in CSN density of WT mice after 3 days of environmental dry eye stress application. *P < 0.05. (B, C) The CSN tortuosity and reflectivity showed no significant difference after 3 days of environmental dry eye stress application. (D) Note the significant increase in DC density of WT mice after 3 days of environmental dry eye stress application. *P < 0.05.
variations at the level of paralgesia and allodynia due to repetitive effects on the corneal nerves. Although several aforementioned human studies reported new insights using IVCM in dry eye disease, no dry eye studies have been performed in mice, probably due to the technical difficulties and availability of IVCM. Reichard et al. conducted a study using IVCM on normal WT mice and characterized age-related changes in CSN morphology.

In our study, we found that IVCM was a useful device that provided detailed and high-resolution images in the visualization of corneal nerves in mice. We detected a reduction in CSN density and an increase in dendritic cell density in WT mice after exposure to environmental stress over a 3-day period. Morphologic parameters that are commonly evaluated with CSN density are tortuosity, reflectivity, inflammatory cell density, and beading pattern. In these previous human studies, alterations were detected in these parameters and it was considered to be a result of neural regeneration associated with CSN damage. IVCM studies performed in patients with dry eye disease revealed increased density of other immune cells together with DCs. Increase in DC density was found to be associated with clinical symptoms in these patients. In the previous reports, dendritic antigen-presenting cells in the epidermis (Langerhans cells) were noted to have an affinity to the cutaneous nerves during regeneration in the skin via neuropeptides and neurotransmitters. Hosoi et al. showed that amount and pattern of activated Langerhans cells in the skin might vary according to the innervation status under normal and pathologic circumstances. Additionally, Choi et al. and He et al. hypothesized that the secreted nerve products, including neuropeptides and neurotransmitters as vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating peptide, and calcitonin gene-related peptide released from the main corneal nerve fibers can modulate the function of Langerhans cells. In the current study, we did not detect a significant change in tortuosity and reflectivity after exposure to EDES. These results may be due to the inadequate duration for nerve regeneration since EDES was applied for a short period of time. Since previous reports showed increased nerve tortuosity in chronic diseases such as diabetic neuropathy, keratoconus, dry eye disease, and Meibomian gland dysfunction, it is possible to detect alterations in these parameters with long-term exposure to EDES in mice. Moreover, Tepelus et al. compared CSN tortuosity and reflectivity between a control group and dry eye patients with and without Sjögren syndrome and they reported an increase in tortuosity and a reduction in nerve reflectivity in the dry eye patients. In other words, tortuosity and reflectivity changes may also be alterations associated with the chronicity of the underlying disorder.

In this study, we used the Balb/c mice strain because CSN density is higher in Balb/c mice compared with same-aged C57BL/6 mice. The limitations of this research were that only male mice were used to avoid hormonal changes of menstrual cycle, and the short period of EDES applied to assess the acute alterations on the cornea. Further studies should also focus on female mice and different mice strains, and involve long-term EDES.

Additionally, our EDES mouse model can be used to evaluate the effects of drugs used in the treatment of dry eye disease in future studies.

**Conclusions**

In conclusion, even short-term exposure to EDES caused changes in CSN morphology in WT mice. The changes in CSN fibers such as increased reflectivity or tortuosity may be representative of more chronic alterations in the mice after exposure to EDES.

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**References**


