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Mapping the Nerve Architecture of Diabetic Human Corneas

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Abstract

Purpose

Objective—To investigate the entire human corneal nerve architecture of donors with different durations of insulin-dependent diabetes mellitus (IDDM).

Design—Experimental study.

Participants and Controls—Sixteen fresh human eyes from 8 diabetic donors (aged 43–66 years old, with IDDM for 2–17 years), and 12 eyes from 6 normal donors (aged from 44–67 years old) were obtained from National Disease Research Interchange (NDRI).

Methods—After fixation, corneas were stained with mouse monoclonal anti- β tubulin III antibody and images were acquired to build a whole view of the corneal nerve architecture. The same corneas were used for both whole mount and cross-section examination.

Main outcome measures—Corneal epithelial nerve density was calculated based on the whole mount view of the central area. The number of stromal nerves was calculated by counting the nerve trunks at the corneo-scleral limbus of the entire cornea. Differences between diabetic and normal corneas in epithelial nerve densities and main stromal nerve numbers were compared by paired-samples *t* test.

Results—The diabetic eyes presented numerous neuropathies in areas where the epithelial nerve bundles emerged. A striking pathologic change was the presence of abundant nerve fiber loops in the stroma. These loops seemed to form by resistance presented by the basement membrane, which may prevent penetration of stromal nerve branches into epithelia. There was no difference in the numbers of main stromal nerve trunks between corneas from diabetic and normal donors, but there was a significant decrease in central epithelial nerve density in the diabetic corneas. We did not find an age effect on this decrease. Instead, it was significantly affected by 5 or more years of IDDM.

Conclusions—This is the first study showing an entire view of the nerve architecture in human diabetic corneas. The decreased epithelial nerve density may result from the abnormalities of stromal nerve architecture and is affected by 5 or more years of IDDM. Although compensation

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for some nerve regeneration takes place, the alterations in the stromal nerves can explain the poor healing and persistent epithelial defects seen in diabetic patients.

Keywords

corneal sensitivity; diabetic keratopathy; stromal nerves; epithelial nerves; Insulin-dependent diabetes mellitus; immunofluorescence of corneal nerves

Introduction

Human corneal nerves penetrate the cornea in a radial form through the stroma, where they bifurcate and advance to the epithelium, appearing as long bundles, fine branches and nerve terminals¹. The cornea is densely innervated and the structure of the corneal nerves is important to maintaining a healthy ocular surface. These nerves modulate the blink response and release neuropeptides, neurotrophins and growth factors that have regenerative properties^{2–7}. Several studies have shown that diabetes alters the structure of corneal nerves^{8–13}. Damage to corneal nerve fibers is responsible for most of the symptoms experienced by diabetic patients with cornea keratopathy; these symptoms include decreased corneal sensitivity, recurrent corneal erosions, delayed corneal epithelial healing, persistent epithelial defects, and neurotrophic corneal ulcers^{14–18}. Changes in corneal nerve morphology have been studied in diabetic rats by light and electron microscopy⁸. More recently, *in vivo* confocal microscopy (IVCM) has been used as a non-invasive technique to study corneal nerves affected by different pathologies, including diabetic neuropathy¹⁹. By analysis of corneal nerve fiber density and morphology, it has been reported that there is a reduction of epithelial nerve fiber bundles per image and an increase in nerve tortuosity of diabetic patients compared with control subjects^{9–13}. However, a more detailed structure of corneal nerves is limited due to technical reasons. For example, the finest nerve fibers (diameter less than 0.5 μ m) cannot be detected and the stromal nerve density cannot be measured by IVCM because the stromal nerves follow an oblique course in the cornea^{9,12,13}.

We have developed a modified method of immunofluorescence staining and imaging that provides a three dimensional map of the entire architecture of the human corneal nerves in both the epithelium and the stroma¹. In the present study, we used this method to examine the nerve structure in human donor corneas with different lengths of IDDM and compared epithelial nerve density and number of stromal nerves with normal corneas.

Materials and Methods

Human Eye Specimens

This study was conducted according to the tenets of the Declaration of Helsinki. Sixteen fresh human eyes from 8 diabetic donors (aged 43–66 years old, with insulin-dependent diabetes mellitus (IDDM) for 2–17 years), and 12 eyes from 6 normal donors (aged from 44–67 years old) were obtained from the National Disease Research Interchange (NDRI). The eyes were kept in a wet chamber and shipped to our laboratory on ice. The donors had no history of eye disease, contact lens wear, ocular surgery, or other systemic diseases that might have affected the corneas. The eyes were examined by slit lamp biomicroscopy and surgical microscopy to exclude possible mechanical damage of corneal central epithelia during eyeball excision.

Tissue preparation, Immunofluorescence Staining and Imaging

The procedure for tissue immunostaining and acquisition of images has been previously described¹. After the position of the cornea was defined and marked, the tissue was excised along the sclero-corneal rim and fixed in 4% paraformaldehyde for 24 hours at 4°C. Corneas

were carefully washed with 0.1M phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (PBS-BSA) three times for 5 minutes each, placed in a 24-well plate (one cornea/well) and incubated with 10% normal goat serum plus 0.3% Triton X-100 solution in PBS for 1 hour at room temperature to block non-specific binding. This was followed by incubation with a neuronal specific mouse monoclonal anti- β III-tubulin antibody (Tuj1, MMS-435P, 1:3000, Convince Antibody Services Inc, Berkeley, CA) in 0.1M PBS containing 1.5% normal goat serum plus 0.1% Triton X-100 for 72 hours at 4°C. After thorough washing with PBS-BSA (4 × 15 minutes), the corneas were incubated with the secondary antibody Alexa Fluor® 488 goat anti-mouse IgG (1:1500, Molecular Probes, Eugene, Oregon) for 24 hours at 4°C. Previous studies have shown no staining when omitting the first antibody^{20, 21}. Corneas were then mounted in a bottle cap with a central hole of 12.5 mm interior diameter, which was inversely positioned in a well of a 24-well plate where the bottom had been marked with an “X” in the center of the well. The apex of the cornea was positioned at the intersection of the “X” and covered with 0.1M PBS. Consecutive images, from the center to the periphery as well as from the anterior to the posterior cornea, were acquired in a time-lapse mode with a fluorescence microscope (Nikon Eclipse TE200) equipped with a Photometrics digital camera (CoolSNAP™ HQ) using MetaVue imaging software. The images, recorded on the same plane at adjoining points, were merged using Photoshop imaging software (Adobe, Mountain View, CA) and then pasted onto a Microsoft Office PowerPoint template to build the whole view of the corneal epithelial nerve architecture (as shown in Fig 1 A, E and Fig 2).

To obtain a whole image of the stromal nerves, consecutive images were captured in a time lapse mode with a stereoscopic zoom microscope (Nikon SMZ 1500) set at the low magnification of 4 × (objective lens). The images were merged to build a complete view of corneal stromal nerves (as shown in Figs 3A and 6; Fig 4 is available at <http://aaojournal.org>).

For transected images of corneal nerves, 20 μ m cryostat sections were prepared from the samples after finishing the whole mount examination using the same methods as described previously^{1, 21}.

Data Analysis

Corneal epithelial density is higher in the center cornea and is not affected by gender¹. To investigate the distribution of epithelial nerves in diabetic corneas, we compared the nerve density between the diabetic and normal corneas in a 3 mm radius area starting at the apex (the central zone). A total of 16 images per cornea were taken at a magnification of 20x from each of the four quadrants within the central zone. To acquire better contrast, the images were changed to grayscale mode and placed against a white background using Photoshop imaging software. The nerve fibers of each image were carefully drawn with four-pixel lines following the course of each fiber. Percentage of nerve area was quantified for each image using the image analysis program. The number of stromal nerves was counted at the corneo-scleral junction based on whole mount images. Differences between diabetic and normal corneas in corneal epithelial nerve densities and main stromal nerve numbers were compared by paired-samples *t* test and differences between age groups in central nerve densities were compared by analysis of variance (ANOVA); $p < 0.05$ was considered a statistically significant difference. Quantification of nerve density and number was done by a person unaware from which donor the images were taken.

Results

Epithelial nerve density and structure in diabetic corneas

The main characteristics of the donor corneas are summarized in Table 1 (available at <http://aaojournal.org>). The average post-mortem time and preservation-in-wet-chamber time, which represents the time interval between death and fixation, were not significantly different between normal and diabetic donors (4.2 ± 1.5 vs. 3.2 ± 1.2 , $P = 0.19$, and 34.8 ± 6.7 vs. 33.8 ± 7.6 , $p = 0.8$, t -test, respectively).

To examine the effect of diabetes on changes in epithelial nerve densities at different ages, we compared the central epithelial nerve densities in corneas of donors from the following age groups: 43–45 years old (4 eyes/6 eyes; normal/diabetes); 54–58 years old (4 eyes/4 eyes); and 63–67 years old (4 eyes/6 eyes) (Fig 5A). A significant decrease ($p < 0.01$, t -test) was observed in all three age groups of the diabetic donors compared to those of the normal donors. In the normal eyes, there was a gradual decrease in nerve density with aging, but the differences between the groups were not significant ($P > 0.05$). We have previously reported significant decrease of central epithelial nerve density in normal eyes of older donors (75–80 years old)¹. Similar results were also detected in the IDDM donors. To test the effect of diabetes duration in epithelial nerve density, we compared the central epithelial nerve densities as a function of time of IDDM. There was a very significant ($p < 0.01$) decrease in central epithelial nerve density between 2 and 5 years of IDDM (Fig 5B). This decrease was maintained up to 17 years of IDDM.

Figure 1A shows the whole mount view of the epithelial nerve architecture of the two eyes of a 64-year-old normal male donor. As previously described¹, long epithelial nerve bundles originate from the branches of the stromal nerves and run from the periphery to the center, converging into an area within the inferior nasal quadrant (merging area). Highlighted images (2B, C and D) show the details of the epithelial nerve architecture in the center and merging areas. As a comparison, Figure 1E is an image of the whole mount view of the epithelial nerve architecture from the right eye of a 45-year-old male with IDDM for two years. Highlighted images (Fig 1G) show the detailed structure of the epithelial nerve network in the merging area. There is already a moderate decrease in the numbers of long epithelial nerve bundles, and the nerves that course from the periphery to the center show increasing tortuosity with irregular nerve beading when compare to normal corneas. Some parts of the bundles show increased thickness (Fig 1F, arrows). In addition, neuropathic lesions are detected in the sub-epithelial area (Fig 1F, circled area).

In corneas from mid- to long-term diabetics, we observed many areas without epithelial innervation. Around these areas, we often found newly-regenerated epithelial nerves. The image in Figure 2 shows the whole mount view of the epithelial nerve architecture from the left eye of a 58-year-old female with IDDM for 13 years. Highlighted images (Fig 2A–C) show the decrease in epithelial nerve density compared to a normal cornea, with many areas devoid of innervation. In the merged area, a few short bundles of newly regenerated nerves are observed (Fig 2C). In Figure 2B, a new regenerated nerve is shown coming from the stroma. In order to re-innervate the epithelium, two types of nerve regeneration occur. One is derived from the underlying stromal nerve branches. As shown in Figure 6 A and B (available at <http://aaojournal.org>), which displays the diabetic cornea from the same donor as in Figure 2, the tips of the branches from the stroma penetrate the basement membrane into the epithelia, where they form a new epithelial nerve plexus. This innervation occurs in patches throughout the entire cornea. The second type of epithelial nerve regeneration is done by the neighboring epithelial nerve bundles (Fig 6C, available at <http://aaojournal.org>), which sprout nerve buds (i) that grow to connect with each other and form a new epithelial

nerve network (ii, iii). Nerve terminals appear in these areas (iv) but are less abundant than those in normal corneas¹.

Stromal nerve architecture in diabetic corneas

There was no significant difference in the number of main stromal nerve trunks that enter the cornea (34 ± 4.3 vs. 32.7 ± 3.5 , $P=0.38$, t -test) between normal and diabetic corneas. As previously reported¹, the nerves penetrate the stroma in a radial pattern with a structure similar to a tree with thick trunks dividing into branches. The nerves ran toward the center of the stroma and divided into several branches along the course. Some of the branches connected to each other and formed a stromal nerve network. Figure 3A shows a whole mount view of the stromal nerves from the right eye of a 43-year-old male donor with IDDM for 5 years. A remarkable change with respect to healthy corneas is the abundance of nerve fiber loops from the periphery to the center. These loops derive from the tips of stromal nerve branches and appear in the anterior stroma (Fig 3B). The topographic images from the same area in Figure 3C show the architecture of nerve loops at different stromal depths. There are also numerous neuropathies formed in the anterior stroma (Fig 3A, arrows). They are localized in the superficial stroma near the basement membrane (Fig 3 D, E).

A whole mount view of the stromal nerve structure from the two eyes of a 44-year-old female with IDDM for 7 years is shown in Figure 4 (available at <http://aaojournal.org>). As mentioned previously, no difference was found in the number of main stromal nerve trunks entering into the cornea, but there was an obvious decrease in the number of subdivisions and a shortage of connections between the stromal nerve branches in the diabetic corneas in comparison with the normal corneas¹. A massive number of nerve fiber loops are present in the anterior stroma of the right cornea, which is highlighted in the montage of images (Fig 4, inlet A, available at <http://aaojournal.org>). In the left cornea, where no obvious loops were seen, there was a corneal ulceration present in the inferior quadrant close to the center. Highlighted images show the tortuous stromal nerves present at the bottom of the ulcerating area (Fig 4C, available at <http://aaojournal.org>), and few epithelial nerves at the edge of the ulcerating area (Fig 4B, available at <http://aaojournal.org>).

One observed feature of the diabetic stromal nerve architecture was the extensive presence of neuropathic lesions, which were also found in the early- and mid-term diabetic corneas (Figs 3 and 4; Fig 4 is available at <http://aaojournal.org>). The greater number of lesions affect the distal part of the stromal nerve branches at the place where they penetrate the basement membrane and transit to epithelial nerve bundles (Table 1, available at <http://aaojournal.org>). They appeared as irregular lesions located at the anterior stroma beneath the basement membrane (Fig 3 D, E). Occasionally we observed that a whole bundle was involved in the nerve degeneration. The whole-mount view in Figure 7 shows the pathologic morphology recorded from a 56-year-old male donor with IDDM for 7 years; the image displays a nerve bundle from the trunk to the branches in the anterior stroma. Segmental demyelination (Fig 7 C, D) was seen in the main stromal nerve trunks, and Schwann cell detachment (Fig 7 A, B) was detected in the distal part of the branches.

Discussion

Studies by electron microscopy and IVCM have shown morphologic alterations in corneal nerves in diabetic patients^{8-13, 22}, but the detailed pathology of the entire corneal nerve architecture remains unclear. Using a modified method of immunofluorescence staining and imaging, we recently provided, for the first time, a three dimensional map of the architecture of the human corneal nerves in both the epithelium and the stroma and demonstrated that aging decreases the density of central epithelial nerves¹. In the present study, we used this

method to investigate the architecture of corneal nerves in donors with IDDM. To exclude the aging factor as much as possible and evaluate the effect of diabetic condition on corneal innervation, we prepared age-matched diabetic and non-diabetic corneas.

Morphologic changes of epithelial innervation were noted in the diabetic corneas at an early stage of the disease. In comparison with the normal control, the epithelial nerve bundles were obviously thicker and the courses of the nerves from the periphery to the center present more tortuosity in the diabetic cornea. These findings are in agreement with prior studies by IVCN in which there was a significant difference in nerve thickness between the diabetic nerves and normal control¹². Thickening of nerve fiber bundles is the result of advanced glycation end products (AGEs) accumulation because diabetic corneas are exposed to increased glucose concentrations²³⁻²⁵. AGE deposition induces a thickened basement membrane and may also play a role in the corneal nerve bundle thickening observed in the early stages of the disease²⁴.

A significant decrease in central corneal epithelial nerve density was found in all corneas with diabetes. There was also a progressive decrease in central epithelial nerve density along with the duration of diabetes from 2 to 17 years. The most significant reduction of nerve fibers was noted between 2 and 5 years of IDDM. One thing to take into consideration is that the analyzed samples recorded the times that the donors were treated with insulin, but the variation in duration of the disease without treatment is not known. This could be why we did not find statistically significant differences between 5 and 17 years of IDDM. Another explanation could be that stromal nerves that penetrate the epithelium are affected earlier in the disease and reach a plateau after a critical period of loss between 2 and 5 years. We found numerous fiber loops in the stroma of a cornea with 5 years of IDDM. This points to the importance of early and controlled treatment in diabetic patients.

Corneal innervation provides important protective and trophic functions for the cornea, and a reduction in nerve density results in delayed wound healing²⁶⁻²⁷. We found that in diabetic corneas the areas with ulceration have less epithelial innervations (see Fig 4B, available at <http://aojournal.org>). The reduction in epithelial nerve fiber density for diabetic corneas might explain slower closure of epithelial defects and the formation of ulcers in the tissue.

Although several IVCN studies have shown a decrease in epithelial nerve density in diabetic corneas, the mechanisms underlying this phenomenon remain unknown. In the present study, quantitative analysis of corneal nerve distribution based on the whole mount images shows that there was no difference in the number of nerve trunks entering the stroma between corneas from diabetic and normal donors, but there was a significant decrease in central epithelial nerve density in the diabetic corneas. We attributed this reduction to the abnormalities of the stromal nerve architecture. As we have reported recently¹, central epithelial innervation is supplied by branches of the stromal nerves. These stromal nerves enter the cornea like trunks of a tree that divide into several branches and run towards the anterior stroma, subsequently dividing into smaller branches. The tips of these branches, which are predominantly present within the peripheral area, penetrate the basement membrane into the epithelium and give place to long bundles, which constitute the epithelial nerve network. One of the most striking features in the stromal nerve architecture of diabetic corneas was the presence of abundant nerve fiber loops in the anterior stroma. There are two possible mechanisms that result in the formation of these nerve loops. One is the changes in the composition of components of the extracellular matrix (ECM) in the diabetic corneal stroma. Corneal nerves course the stroma through collagens, proteoglycans and fibronectin²⁸⁻³⁰. These ECM components are known to be up-regulated in diabetes and play an important role in axonal navigation and outgrowth^{31, 32}. Therefore, imbalance in their expression within the environment of navigating axons may disturb the course of nerve

outgrowth during nerve regeneration. A second mechanism may consist in the resistance offered by the basement membrane, which may prevent the stromal nerve branches from penetrating into the epithelia. Early studies by electron microscopy and histochemistry have shown alterations in thickness and molecular components of basement membrane in diabetic corneas³³⁻³⁶. It is conceivable, therefore, that increased thickness and altered molecular components in the basement membrane may contribute to increase resistance of nerve fiber penetration, which leads to the formation of nerve fiber loops and a decrease in epithelial bundle density.

The extensive presence of neuropathies in the diabetic stroma is another feature observed in the current study. Previously, we have shown that age-related nerve lesions occur in the peripheral area of normal corneas¹. Here, we show that the neuropathic lesions are found extensively in the diabetic cornea. Those neuropathies look like the age-related nerve lesions in appearance, but are not limited to the peripheral area and have little or no relation to the age of the donor. Most of the diabetic neuropathies occurred at the anterior stroma near the basement membrane in the area where the stromal nerve branches will penetrate the epithelium. Therefore, diabetic neuropathies damaged the epithelial nerve bundles at their points of emergence and subsequently led to decreased epithelial innervations. We also observed a few cases where a whole stromal nerve was degenerated.

One interesting finding is the appearance of regenerated nerves, which were found in all the diabetic corneas regardless of the severity or duration of diabetes. According to the regenerating nerve source and site, it can be identified as epithelial nerve regeneration or stromal nerve regeneration. Epithelial nerve regeneration happened when an epithelial bundle was destroyed, which left an area without innervation. In an effort to compensate for the lack of innervation, the surrounding epithelial bundles sprouted fine fibers, which spread in the empty space and contacted with each other to construct a new epithelial nerve network. In diabetic corneas, a less dense array than in healthy corneas of nerve terminals budded from the network to re-innervate the epithelial cells. This explains the decrease in corneal sensitivity observed in diabetic patients^{9, 17}. In fact, diabetic patients show lower sensitivity than normal subjects to mechanical, chemical, heat and cold stimuli, demonstrating that the disease affects all class of sensory nerves³⁷. Stromal nerve regeneration occurred at the distal part of stromal nerve branches, where the newly-regenerating nerve fibers sprouted and grew upward to form a nerve plexus in the superficial stroma or within the epithelia. New epithelial nerve bundles originated from the nerve plexus to supply the epithelia.

In the current study, there are no available data about the diet and the treatments that the donors followed, but the coexistence of nerve regeneration with neuropathy in all the diabetic corneas suggests the balance between nerve damage and repair may play a critical role in the development of corneal neuropathy. Clinically, reducing nerve damage by optimal glycemic control, by topical application of an aldose reductase inhibitor, or by promoting nerve regeneration should be useful in treating diabetic keratopathy. Growth factors, such as nerve growth factor (NGF), have been shown to regulate corneal nerve repair³⁸. In rabbits, NGF or pigment epithelium-derived factor (PEDF) in combination with docosahexaenoic acid (DHA), a ω -3 fatty acid, stimulate nerve regeneration, restore sensitivity and increase epithelial wound healing after experimental refractive surgery^{20,21,39,40}. We recently discovered that neuroprotectin D1 (NPD1), a docosanoid derived from DHA⁴¹, is a very potent nerve regenerative factor in rabbits after nerves are damaged by a stromal dissection (Cortina MS, He J, Russ T, Bazan NG, Bazan HE. Topical Treatment with Neuroprotectin D1 Increases Corneal Nerve Regeneration after Experimental Surgery. Paper presented at: ARVO 2011 Annual Meeting; May 2, 2011).

Although there are no studies on diabetic corneas, this could represent an important future treatment to improve regeneration of nerves damaged by this disease.

In summary, by using a recently-developed, modified method of immunofluorescence, we were able for the first time to reveal a detailed map of the entire architecture of diabetic human corneal nerves in both the epithelium and the stroma. The abundant presence of neuropathic lesions and nerve fiber loops in the anterior stroma reduced corneal epithelial innervations either by damaging corneal epithelial nerve bundles at their emergence or by preventing nerve from penetration into epithelia, thereby contributing to the pathomechanisms of diabetic keratopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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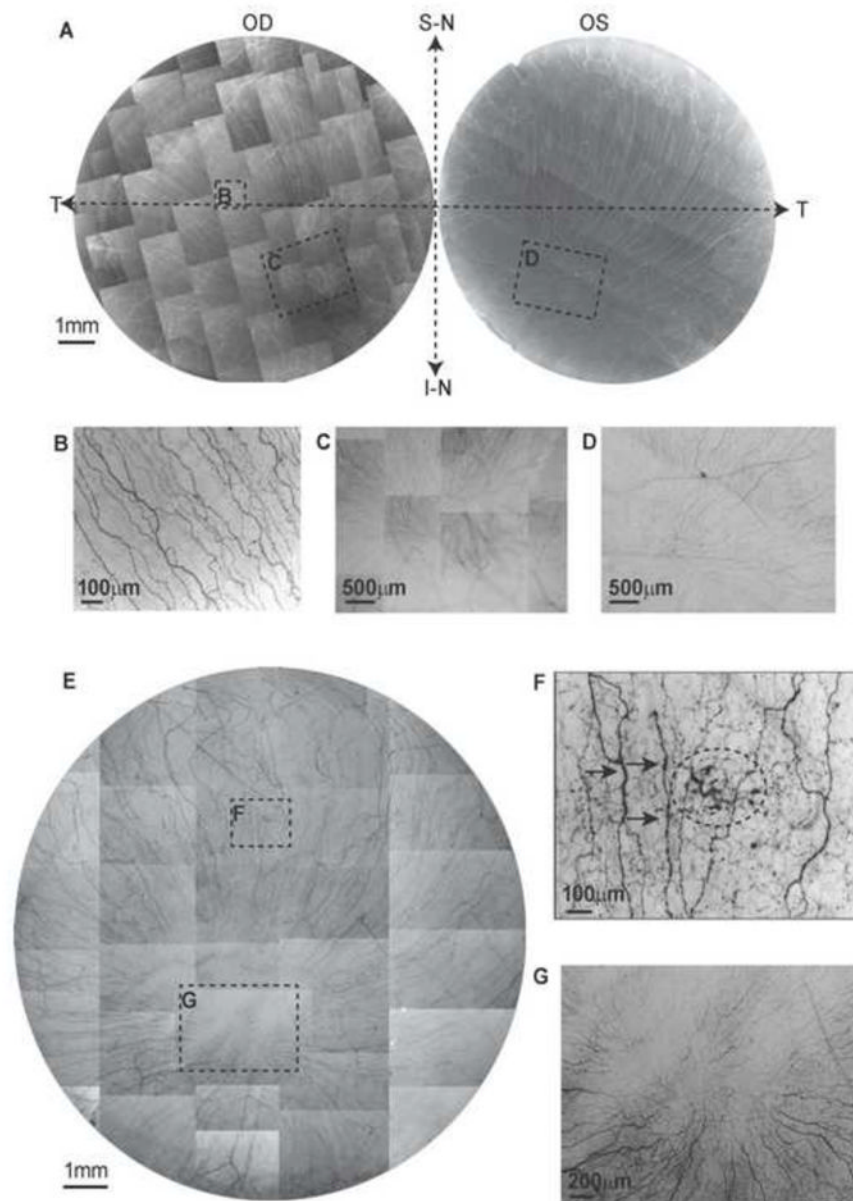


Figure 1. Whole mount view of corneal epithelial nerve distribution in normal and diabetic corneas at early stage of the disease. **A)** Images were acquired in a time-lapse mode with a Nikon Eclipse TE200 and with a 5X lens in compliance with the natural shape of the cornea from the two eyes of a 64-year-old normal male donor. OD: oculus dexter (right eye); OS: oculus sinister (left eye); S-N: superior nasal quadrant; I-N: inferior nasal quadrant; T: temporal quadrant. The epithelial nerve bundles ran in a radial pattern from the periphery to merge in an area within the inferior-nasal quadrant beyond the corneal apex. Highlighted images showed the detailed architecture of the epithelial nerve distribution in the central (**B**), and merging areas (**C**, **D**). **E)** Images were taken from the right eye of a 45-year-old male with insulin-dependent diabetes mellitus (IDDM) for two years. Highlighted images show the detailed nerve distribution in the converging area (**G**). **F)** Image F, outlined with the small

frame, shows the increased thickness in parts of the nerves (arrows) and a sub-epithelial neuropathy (circle).

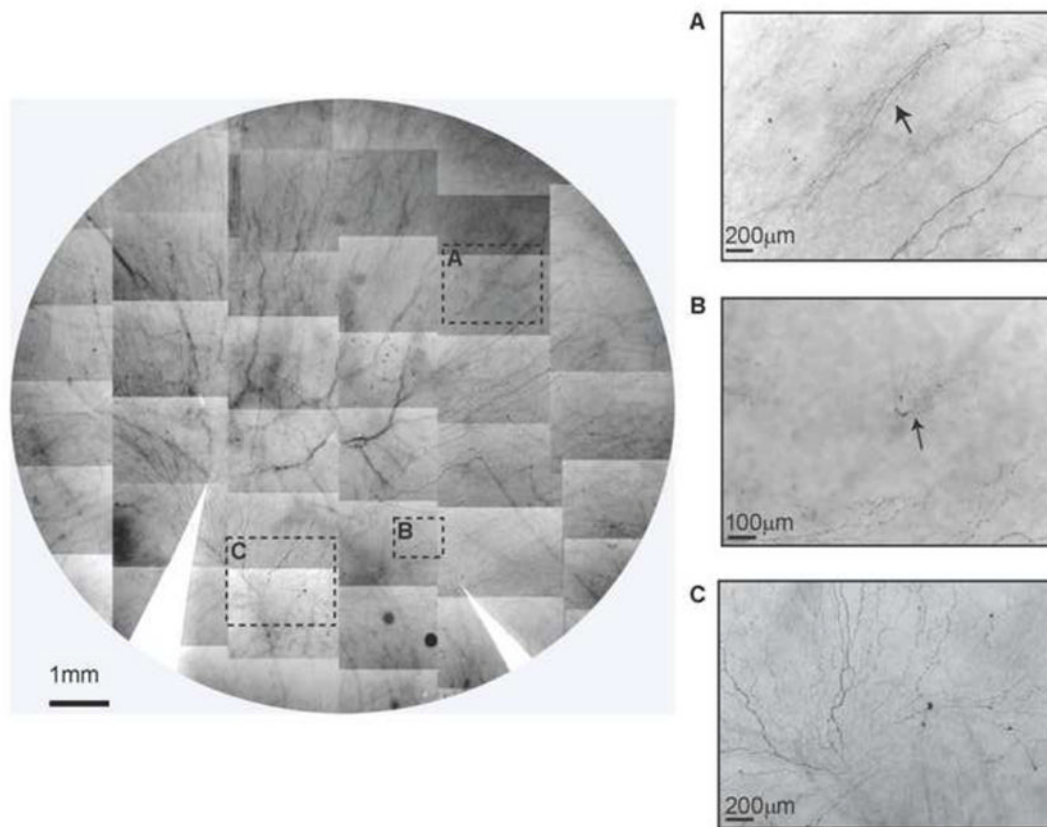


Figure 2.

Whole mount view of corneal epithelial nerve distribution in a diabetic cornea after prolonged insulin-dependent diabetes mellitus (IDDM). Images were taken from the left eye of a 58-year-old female with IDDM for 13 years. The montage of images outlined a frame that shows the nerve distribution in detail. The images (A, B) show newly-regenerating epithelial nerve leashes (arrows) with abundant empty spaces without innervations. Image C shows the detailed nerve distribution in the merging area. The long bundles running from the superior quadrants converge in a counter-clock pattern at the inferior quadrant close to the limbal area. Several short nerve bundles, representing newly regenerating nerves, are visible in the lower part.

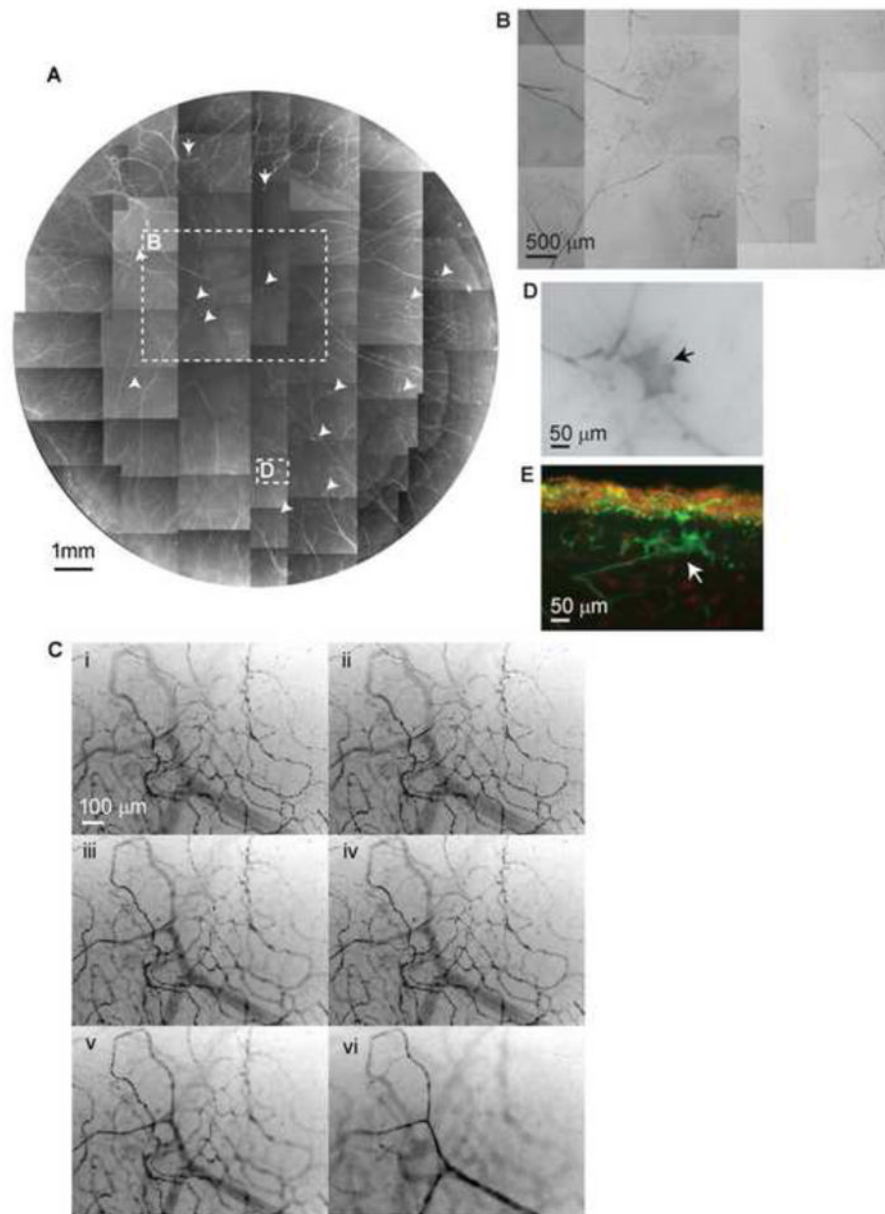


Figure 3. **A)** Whole mount view of corneal stromal nerve distribution in a diabetic cornea taken from a 43-year-old male with insulin-dependent diabetes mellitus (IDDM) for 5 years. Numerous nerve fiber loops and neuropathic lesions (arrows) are present in the whole cornea. **B)** Highlighted image shows the nerve fiber loops present in the center. **C)** Topographic images of the same area show the detailed architecture of corneal nerve loops at different depths. Images were recorded in time-lapse mode from the stroma surface (I) to the middle of the stroma (VI). **D)** and **E)** are representative images of diabetic neuropathies in a whole mount and in a transected view.

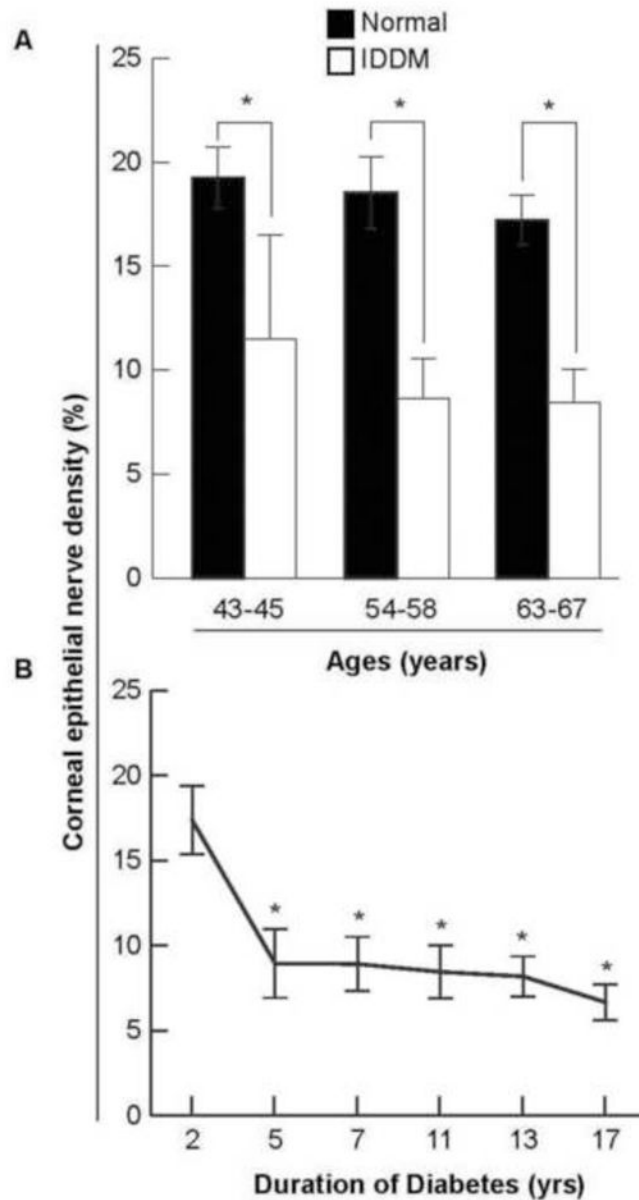


Figure 5.

A) Age-paired comparative analysis of corneal epithelial nerve density between normal and diabetic human eyes. The corneal epithelial nerve density was significantly decreased in the diabetic eyes when compared to the normal. Data are expressed as average percent \pm standard deviation (SD) of 64 images from 4 corneas in the normal groups and 64–96 images from 4–6 corneas in the diabetic groups (* $p < 0.01$). **B)** The effect of insulin-dependent diabetes mellitus (IDDM) duration on corneal epithelial nerve density. A significant decrease (* $p < 0.01$) was observed in the corneas of patients diagnosed with IDDM for 5 years or more. Data expressed as average percent \pm SD, with each time point having 32 images analyzed from two eyes except for 5 years and 7 years time points, both of which have 64 images analyzed from 4 eyes.

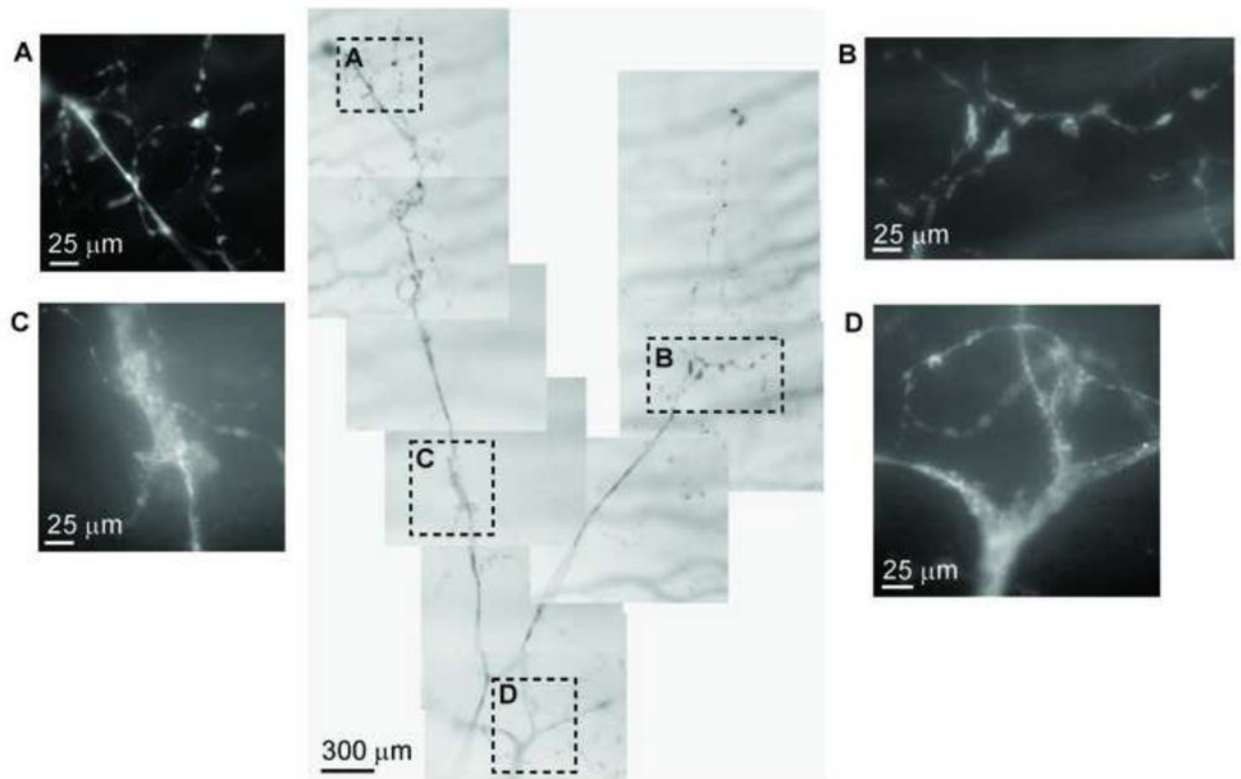


Figure 7. Corneal stromal nerve degeneration. The images reveal the montage of a main stromal nerve trunk together with its branches of a 56-year-old male donor with insulin-dependent diabetes mellitus (IDDM) for 7 years. Highlighted images show the details of nerve degeneration at different parts of the nerve.