Ex vivo confocal microscopy of human corneal nerves

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ABSTRACT

Aims To evaluate the distribution, morphometry and the postmortem changes of the central and peripheral human corneal nerves by ex vivo laser-scanning confocal microscopy (EVCM).

Methods 24 eyes from 14 cadavers were retrieved at different time intervals after death and examined by EVCM. Five regions were examined in each eye: central, superior, inferior, temporal and nasal. In each region, corneal nerve images were categorised according to their anatomical location in the cornea into sub-basal, stromal and limbal nerves. Five nerve parameters were measured: density, orientation, diameter, numbers and branching pattern.

Results Ex vivo confocal scanning of a motionless eye allows high quality imaging and tracking of corneal and limbal nerves. Stromal nerves from the sub-Bowman’s plexus perforate the Bowman’s zone and terminate in bulb-like structures, from each of which a leash of sub-basal nerves arises. Following death, sub-basal nerve parameters showed significant changes. The density decreased from 9.23 ± 4.18 to 4.5 ± 0.07 mm/mm², the diameter from 4.01 ± 0.81 to 2.08 ± 0.20 μm, the numbers from 8.3 to 1.0 and branching pattern from 39.38% to 0% (p<0.05) from day 1 to day 5 postmortem. Stromal and limbal nerves showed no significant changes in their density and diameter.

Conclusions This study establishes a direct link between sub-basal nerves and the sub-Bowman’s nerves via distinct terminal bulbs. Limbal nerves are the thickest, are seen in all quadrants and can be traced to the corneal centre. The sub-basal nerve plexus rapidly degenerates after death but stromal and limbal nerves survive during the first five days after death.

INTRODUCTION

The human cornea is one of the most richly innervated structures in the body and is richly supplied by sensory and autonomic nerves deriving primarily from the ophthalmic division of the trigeminal nerve.1-11 Radially oriented nerve fibre bundles enter the mid-stroma at the limbus and give rise to branches that innervate the anterior and mid-stroma. Under Bowman’s layer, they form a loose plexus from which branches perforate Bowman’s zone and form the sub-basal nerve plexus, fibres from which terminate within the superficial epithelial cells.

Conical nerves have been studied extensively by light and electron microscopy, utilising different histochemical12-14 and immunohistochemical15-19 methods. However, these techniques require the tissue samples to be sectioned, so considerable information about their spatial arrangement is often lost.20 Confocal microscopy has offered an unparalleled opportunity to study corneal nerves in vivo,21-24 but is limited by the small field of view, and normal microscadic eye movements affect the image quality. Most invivo studies have reported descriptions of nerves in the central cornea. To study corneal nerves in more detail and over a larger area, we considered the possibility of ex vivo confocal examination of freshly obtained cadaver eyes. This eliminated artefacts due to involuntary eye movements and also provided information on the degeneration pattern of human corneal nerves over time.

MATERIALS AND METHODS

The research was approved by the local research ethics committee. Twenty four postmortem eyes from 14 patients (7 women, 7 men; mean age 76.5 years, range 60–92 years) were obtained by enucleation at different time intervals after death. The specimens were collected in the period April 2008 to August 2009. All eyes were considered unsuitable for transplantation due to lack of consent, delayed time to enucleation, cancer related deaths or patients with cognitive disorders. The orientation of the eyes was marked prior to enucleation. The research followed the tenets of the Declaration of Helsinki.

For the purpose of evaluation of postmortem corneal nerve changes, the eyes were classified into five groups based on the time of enucleation and examination after death: 4 eyes were examined within 24 h after death (group 1), 7 eyes between 24 and 48 h (group 2), 7 eyes between 2 and 3 days (group 3), 4 eyes between 3 and 4 days (group 4), and 2 eyes between 4 and 5 days (group 5).

Confocal microscopy examination

Immediately after enucleation, the eyes were transferred in sterile Dulbecco’s phosphate buffered saline solution (Sigma-Aldrich, UK) to the examination room. The eyes were examined by laser scanning confocal microscope (Heidelberg Retina Tomograph II Rostock Corneal Module (RCM); Heidelberg Engineering, Heidelberg, Germany).

To facilitate the examination, the whole globe was held in front of the microscope by a clamp holder specifically designed for such purpose. This had three joints (including one ball-and-socket joint) which allowed accurate positioning and orientation of the eye for examination. Pressure-free contact with the cornea was monitored during the examination using a coloured digital camera system that gives a side view of the microscope objective and eye. The average time for examining each eye was about 30 min.

Additional movie clips are published online only. To view these files please visit the journal online (http://bjo.bmj.com).
Image analysis
To study both the central and peripheral corneal nerves, five regions were examined sequentially in each eye: central, superior, inferior, temporal and nasal. The central area of 5×5 mm² was considered as the central region. The side camera of the confocal microscope was used to ensure exact positioning. As the cadaver eyes lacked any natural micro-saccadic movements it was possible to precisely locate the confocal microscope probe. Moreover, identification of the palisade structures provided morphological localisation of the limbus. Any nerves located in the palisade region were classed as limbal nerves. Nerves located central to the limbus but outside the 5×5 mm² central zone of the cornea were classed as peripheral nerves. It was also possible to track the peripheral mid-stromal nerves towards the limbus. These were characterised by thicker hyper-reflective nerve bundles situated within a homogenous hypo-reflective stroma, occasional hyper-reflective clump like structures, dark striae and absence of normal stromal and keratocyte architecture. Some of these limbal stromal in-vivo confocal microscopy (IVCM) features have been reported previously.

In each region, corneal nerve images were categorised according to their anatomical location within the cornea, into sub-basal, stromal and limbal. Each image was then analysed using image processing and analysis software programs (GNU Image Manipulation Program (GIMP) V.2.6.2, Free Software Foundation; and ImageJ V.1.31, Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). The software applications were used to measure corneal nerve length and diameter (in pixels) and orientation (in degrees). A plugin software called NeuronJ was used with ImageJ to facilitate the tracing and quantification of corneal nerves semi-manually (figure 1). Different parameters, adopted from previous studies, were used to analyse corneal nerves:

1. Nerve fibre density (NFD): total length of all nerve fibres and branches within a frame in mm per mm².
2. Thickness: the frame was zoomed in on the monitor to 200% and five measurements of the width for sub-basal nerve fibres and three measurements for stromal and limbal nerves were taken.

Figure 1  Semi-manual tracing of the sub-basal nerve fibres using NeuronJ, an imageJ plugin software. Scale bar=100 μm.

Figure 2  Measurement of the stromal nerve orientation using the GNU Image Manipulation Program (GIMP).

3. Orientation: the mean value of the angle formed by the nerves with respect to the horizontal plane. Orientation of a nerve was horizontal (if the angle measured between 0° and 30°), oblique (between 31° and 60°) or vertical (between 61° and 90°) (figure 2). For the sub-basal plexus, the main nerve fibres rather than their interconnecting twigs were considered for orientation.
4. Number of nerves: defined as the sum of the nerve branches observed within a frame.
5. Branching patterns within the frame: expressed as the percentage of branches per total number of nerve fibres within a single frame.

Statistical analysis
Data were analysed using an analysis tool pack for Microsoft Excel 2007, SPSS V16.0; p<0.05 was taken as the threshold of statistical significance. One way repeated measures analysis of variance (ANOVA) was performed to investigate the differences in the corneal nerve parameters among the five groups. The Student t test for independent samples was used to establish whether any differences between stromal and limbal nerve diameter were significant.

RESULTS
A total of 366 images were selected. The average number of frames per eye was 15. The distribution of images according to their location within the corneal layers and topography was as follows: 94 images (25%) were of sub-basal nerves, 218 images (60%) were of stromal nerves and 54 images (15%) were of limbal nerves. With respect to topographical distribution of images, 71 images (19%) were from the central cornea, 61 images (17%) were from the superior cornea, 78 images (21%) were...
from the inferior cornea, 83 images (23%) were from the nasal cornea and 75 images (20%) were from the temporal cornea.

### Postmortem changes

#### Sub-basal nerves

**Density**

The density of sub-basal nerves in group 1 was measured at 9.25±4.48 mm/mm². Thereafter it showed a significant decline in group 2 (4.44±5.56 mm/mm²) (p<0.05). However, no significant changes were observed among groups 2, 3 and 4. In group 5, the sub-basal nerve density was 0.45±0.07 mm/mm² (p<0.05) (table 1).

**Diameter**

The average sub-basal nerve diameter was measured at 4.01±0.81 μm in group 1, which then significantly decreased to 3.26±0.78 μm in group 2. No statistically significant change was observed in the diameter of sub-basal nerves among groups 2, 3 and 4. Thereafter, the diameter of sub-basal nerves showed a significant decrease to 2.08±0.20 μm (p<0.05).

**Number**

The mean number of nerves in group 1 was 8.3, which then significantly declined to 3.83 in group 2 (p<0.05). No statistically significant change was observed in the number of sub-basal nerves among groups 2, 3 and 4. The average number of nerves in group 5 was 1.00 (p<0.05).

**Branching pattern**

In group 1, branching pattern was 39.38±25.52% (figure 3A,B), which then showed a significant decrease to 17.23±22.52% in group 2 (p<0.05). No statistically significant difference was noted in branching pattern among groups 2, 3 and 4. No nerve branches were observed in group 5 (figure 4A,B) (p<0.05).

Advanced degeneration was characterised by loss of continuity and granularity of the sub-basal nerves (figure 5A,B). An interesting and novel observation was that sub-basal nerves terminated in bulb-like or rounded bright structures just above the level of Bowman’s zone (figure 6A,B). These were present as clusters of 2–6 bulbs. Converging posterior extensions from bulbs of each cluster terminated in a large stromal nerve trunk. Volume scanning of the region related to these clusters revealed that they were in continuation with anterior stromal (sub-Bowman’s) nerves (movie clips 1, 2, 3 and 4). These bulbs survived longer than the nerves that originated from them. At places bulbs could be seen with no fibres emanating from them.

### Stromal and limbal nerves

Stromal and limbal nerves were detected in all regions examined.

**Density and diameter**

No significant changes were observed in the density and the diameter of stromal and limbal nerves over 5 days postmortem. Furthermore, both types of nerves showed no obvious signs of degeneration, for example granularity or loss of continuity. The average stromal nerve density was 1.77±0.79 mm/mm² and the average diameter was 8.58±3.1 μm.

Tracing of corneal nerves from the centre of the cornea towards the periphery showed a gradual increase in the diameter of stromal nerves. The average diameter of stromal nerves in the central cornea was 7.75±2.58 μm while their average diameter in the peripheral cornea was 8.84±3.27 μm. At the extreme periphery, limbal nerves were the largest nerves observed throughout the cornea. They measured up to 60 μm in diameter (average was 27.60±9.7 μm) and were significantly larger than stromal nerves (p<0.05). Figure 7 shows the variation in the thickness of corneal nerves in different layers within the cornea.

Table 1: Results of postmortem quantitative ex vivo confocal microscopy of human corneal nerves

<table>
<thead>
<tr>
<th>Group</th>
<th>Density (mm²/mm²), mean±SD</th>
<th>Diameter (μm), mean±SD</th>
<th>No. of nerves, mean±SD</th>
<th>Branching pattern (%)</th>
<th>Density (mm²/mm²), mean±SD</th>
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<th>No. of nerves, mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>9.23±4.48</td>
<td>4.01±0.81</td>
<td>8.30±4.5</td>
<td>39.38±25.52</td>
<td>1.98±1.02</td>
<td>9.48±3.91</td>
<td>3.43±0.64</td>
</tr>
<tr>
<td>Group 2</td>
<td>4.44±3.56</td>
<td>3.26±0.78</td>
<td>3.83±3.54</td>
<td>17.23±22.32</td>
<td>1.80±0.86</td>
<td>8.23±3.00</td>
<td>2.48±0.77</td>
</tr>
<tr>
<td>Group 3</td>
<td>3.74±3.27</td>
<td>3.03±0.93</td>
<td>3.65±2.69</td>
<td>16.54±21.10</td>
<td>1.84±0.73</td>
<td>9.00±3.14</td>
<td>2.58±0.78</td>
</tr>
<tr>
<td>Group 5</td>
<td>3.47±3.49</td>
<td>3.30±0.61</td>
<td>2.37±2.22</td>
<td>14.14±20.00</td>
<td>1.46±0.58</td>
<td>8.08±2.93</td>
<td>2.05±0.50</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.45±0.07</td>
<td>2.08±0.20</td>
<td>1.00±0.00</td>
<td>0%</td>
<td>1.69±0.75</td>
<td>8.29±2.88</td>
<td>2.43±0.85</td>
</tr>
</tbody>
</table>

*Group 1, within 24 h postmortem; group 2, 24–48 h postmortem; group 3, 2–3 days postmortem; group 4, 3–4 days postmortem; group 5, 4–5 days postmortem.*

Advanced degeneration was characterised by loss of continuity and granularity of the sub-basal nerves (figure 5A,B). An interesting and novel observation was that sub-basal nerves terminated in bulb-like or rounded bright structures just above the level of Bowman’s zone (figure 6A,B). These were present as clusters of 2–6 bulbs. Converging posterior extensions from bulbs of each cluster terminated in a large stromal nerve trunk. Volume scanning of the region related to these clusters revealed that they were in continuation with anterior stromal (sub-Bowman’s) nerves (movie clips 1, 2, 3 and 4). These bulbs survived longer than the nerves that originated from them. At places bulbs could be seen with no fibres emanating from them.

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No significant changes were observed in the density and the diameter of stromal and limbal nerves over 5 days postmortem. Furthermore, both types of nerves showed no obvious signs of degeneration, for example granularity or loss of continuity. The average stromal nerve density was 1.77±0.79 mm/mm² and the average diameter was 8.58±3.1 μm.

Tracing of corneal nerves from the centre of the cornea towards the periphery showed a gradual increase in the diameter of stromal nerves. The average diameter of stromal nerves in the central cornea was 7.75±2.58 μm while their average diameter in the peripheral cornea was 8.84±3.27 μm. At the extreme periphery, limbal nerves were the largest nerves observed throughout the cornea. They measured up to 60 μm in diameter (average was 27.60±9.7 μm) and were significantly larger than stromal nerves (p<0.05). Figure 7 shows the variation in the thickness of corneal nerves in different layers within the cornea.
Orientation of corneal nerves

Table 2 shows the orientation of sub-basal, stromal and limbal nerves in the five different topographical areas within the human cornea. The majority of the sub-basal nerves were oriented obliquely and to a lesser extent vertically, in all corneal regions including nasal and temporal.

Stromal nerves were seen in the central cornea where their orientation was mainly horizontal. Stromal and limbal nerves were arranged predominantly vertically in the superior and inferior corneal regions and predominantly horizontally in the nasal and temporal regions.

DISCUSSION

Postmortem changes of human corneal nerves have been studied elegantly by Muller et al using light microscopy. They reported that 13.5 hours after death, the majority of the sub-basal nerve fibres had degenerated or gone. Our study reports the changes in stromal nerves as well and highlights an important structure at the junction of the stromal nerves with the sub-basal plexus. In our study, the sub-basal nerve plexus was the most affected by postmortem degeneration. The sub-basal nerve branches showed rapid disintegration and the branching pattern significantly declined 24 h after death. Over the first four days after death, the sub-basal nerves showed a noticeable decrease in the density, diameter and number and they were hardly distinguishable by the fifth day. It is worth noting that degenerated nerves below the resolution of confocal microscope (1 μm) could not be studied. Nevertheless, patchy degeneration was noted from the first day postmortem. This might explain the results reported by Muller et al who used very small samples of 1–2 mm² for en face examination using light microscopy. They could have missed sites where the nerves were present and they completely missed the detection of the bulbs.

The greatest difference in the sub-basal nerve density between groups 1 and 2 was due to the rapid degeneration of the nerve branches by the second day postmortem. This was also reflected in the branching pattern. It has been shown that there is a high correlation between the number of nerves and nerve density in all corneal regions. As far as changes in the sub-basal nerves in groups 2, 3 and 4 are concerned, there was an increasing number of areas where total nerve loss was observed with each passing day after death, but in areas where sub-basal nerves were found and measured in these groups, no significant difference was found in these parameters. It is likely that progressive degeneration continued to occur but was not detectable by confocal microscopy. Nevertheless, the overall structural appearance of the main sub-basal nerves was maintained in parts of the cornea during the first five days postmortem.

Several confocal studies have shown that the sub-basal nerve density in normal individuals can vary depending on the type of invivo confocal microscope used. Reported sub-basal nerve densities using laser scanning confocal microscope were as high as 25.9±7.0 mm/mm² and 21.6±5.98 mm/mm². Nerve diameter in normal individuals ranged from 0.52±0.23 to

Figure 4 Sub-basal nerve plexus shows lack of branches which have largely degenerated by the second day postmortem. The main sub-basal nerves are also thinner than those in figure 3. Scale bar=100 μm.

Figure 5 Advanced degeneration of the sub-basal nerves with loss of continuity (white arrowheads) and granularity (white arrows). Scale bar=100 μm.
In our study, the sub-basal nerve density in the first day postmortem was markedly reduced but the nerve diameter was within the normal range in the nerves that survived. Theoretically, scanning the same corneas over time could possibly give more precise figures but we found that sequential examination of the same specimen resulted in accelerated disintegration and sloughing of the corneal epithelium with injury to the sub-basal plexus. Pathologically, the postmortem changes can be explained by failure of axon transport, resulting in degeneration of vulnerable distal regions of long axons. Degeneration appears to advance proximally towards the nerve cell body (dying-back).31

The termination of the sub-basal nerves into characteristic bright bulb-like thickenings and the relation of these structures to underlying stromal nerves in normal corneas is unique to the current study. This strongly correlates with our recent histological demonstration of budding and branching pattern of sub-Bowman’s nerves in the anterior stroma.32 Similar structures were also reported in patients with Fuchs endothelial dystrophy where it was suggested that they related to the disease condition.20

Figure 6 Termination of sub-basal nerves into bright bulb-like structures at the level of basal epithelial cells. Scale bar=100 μm.

Figure 7 Variation in thickness of corneal nerves in different regions within the cornea: (A) sub-basal nerves (4.01±0.81 μm); (B) central stromal nerve (7.75±2.38 μm); (C) peripheral stromal nerve (8.84±3.27 μm); (D) limbal nerve (27.60±9.7 μm). Scale bar=100 μm.
We did not notice any obvious signs of structural changes in, or alterations in density and diameter of the stromal and limbal nerves in the first 5 days after death. In one study by Szaflik, corneal nerves including sub-basal nerves were distinguished in 5 days cold preserved corneas from eye bank using white light confocal microscopy.33

In our study, the average stromal nerve density was 1.77±0.79 mm/mm². This figure is different from the results obtained by other investigators using a slit scanning confocal microscope (0.45±0.11 mm/mm² (Simó Mannoni et al 27) and 3.61±1.04 mm/mm² (Oliveira-Soto and Efron21)). However, our measurement of the average stromal nerve diameter was 8.58±3.1 μm (7.75±2.58 μm centrally; 8.84±3.27 μm peripherally). These (ex vivo postmortem) measurements are comparable to the results obtained in several invivo studies using a slit scanning confocal microscope (Hosal et al, 9.65±1.93 μm; Benítez del Castillo et al, 7.64±2.54 μm; Oliveira-Soto, 6.3±1.8 μm).21 30 34 Other investigators reported a lower stromal nerve diameter.27 35 The variation in the results might be due to different kinds of confocal microscopes used with different light sources and resolution and/or the fact that stromal nerves run obliquely, relative to the en face sections of confocal images. Therefore, obtaining an en face image through the centre of the nerve is not always possible, especially when involuntary eye movements are present.25

There are no previous estimates of limbal nerve parameters in the literature. It has been reported that scanning corneal limbus for the evaluation of deep structures was not possible due to the limitation of current technology. For the evaluation of deep structures was not possible due to the limitation of current technology. It has been reported that scanning corneal limbus for the evaluation of deep structures was not possible due to the limitation of current technology. However, we have managed to scan this region and obtain good quality images of limbal nerves at the extreme periphery of the cornea.

The majority of the main sub-basal nerves were oriented obliquely and to less extent vertically in all corneal regions including nasal and temporal sides, fibres with horizontal orientation being the least. The current study does not support the finding by Muller et al that the sub-basal nerve bundles in central and mid-peripheral cornea run first in the 9–3 o’clock direction, then after bifurcation in the 12–3 o’clock direction and after a second bifurcation again in the 9–3 o’clock direction.

In general, the orientation of the stromal and limbal nerves is perpendicular to the corresponding corneoc-scleral junction. These nerves enter the cornea from all quadrants and move anteriorly towards the centre of the cornea, giving rise to branches that innervate the anterior and mid-stroma.

In conclusion, this study establishes a direct link between sub-basal nerves and the sub-Bowman’s nerves via distinct terminal bulbs. Limbal nerves are the thickest, are seen in all quadrants and can be traced to the corneal centre. The sub-basal nerve plexus rapidly degenerates postmortem but stromal and limbal nerves survive during the first five days after death.

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### REFERENCES


