Experimental Eye Research 171 (2018) 92–100

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

# Recovery of the sub-basal nerve plexus and superficial nerve terminals after corneal epithelial injury in mice

Laura E. Downie, Cecilia Naranjo Golborne, Merry Chen, Ngoc Ho, Cam Hoac, Dasun Liyanapathirana, Carol Luo, Ruo Bing Wu, Holly R. Chinnery*

*Department of Optometry and Vision Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia*

ARTICLE INFO

*Keywords:*
Corneal nerve
Image analysis
Epithelium
Re-innervation
Sub-basal nerve plexus
Nerve tracing
Thresholding

ABSTRACT

Our aim was to compare regeneration of the sub-basal nerve plexus (SBNP) and superficial nerve terminals (SNT) following corneal epithelial injury. We also sought to compare agreement when quantifying nerve parameters using different image analysis techniques. Anesthetized, female C57BL/6 mice received central 1-mm corneal epithelial abrasions. Four-weeks post-injury, eyes were enucleated and processed for PGP9.5 to visualize the corneal nerves using wholemount immunofluorescence staining and confocal microscopy. The percentage area of the SBNP and SNT were quantified using: ImageJ automated thresholds, ImageJ manual thresholds and manual tracings in NeuronJ. Nerve sum length was quantified using NeuronJ and Imaris. Agreement between methods was considered with Bland-Altman analyses. Four-weeks post-injury, the sum length of nerve fibers in the SBNP, but not the SNT, was reduced compared with naïve eyes. In the periphery, but not central cornea, of both naïve and injured eyes, nerve fiber lengths in the SBNP and SNT were strongly correlated. For quantifying SBNP nerve axon area, all image analysis methods were highly correlated. In the SNT, there was poor correlation between manual methods and auto-thresholding, with a trend towards underestimating nerve fiber area using auto-thresholding when higher proportions of nerve fibers were present. In conclusion, four weeks after superficial corneal injury, there is differential recovery of epithelial nerve axons; SBNP sum length is reduced, however the sum length of SNTs is similar to naïve eyes. Care should be taken when selecting image analysis methods to compare nerve parameters in different depths of the corneal epithelium due to differences in background autofluorescence.

## 1. Introduction

The densely innervated mammalian cornea has been elegantly described in humans (Muller et al., 2003) and mice (He and Bazan, 2016). Large nerve trunks enter the cornea at the periphery, branching just beyond the limbus and continuing to branch extensively towards the central cornea. Beneath the epithelium, nerve axons form a sub-epithelial plexus and turn at right angles to pierce Bowman's layer. Once within the epithelium, nerve axons run parallel to the cornea at the level of the basal epithelium, referred to as the sub-basal nerve plexus (SBNP). Nerve fibers, branching from the sub-basal plexus, extend processes perpendicularly towards the tear film, terminating throughout the epithelial layers. The superficial nerve terminals (SNT) terminate just before the outermost epithelial cells. Stimulation of the corneal nerves can result in pain and a number of other reflexive defence mechanisms, such as blinking and tear production. Furthermore, corneal nerves provide trophic support to maintain the homeostasis of the corneal epithelium and the functional integrity of the ocular surface (Marfurt et al., 2010).

Over the past 15 years, the increased use of *in vivo* confocal microscopy (IVCM) to examine the living human cornea has led to a surge in studies reporting the effect of a range of systemic and ocular diseases on the number and distribution of corneal nerves (Cruzat et al., 2017; Chinnery et al., 2017). Corneal nerve imaging can be performed non-invasively, to detect early neuropathic changes in people with conditions such as diabetes (Malik et al., 2003) and small fiber neuropathy (Bucher et al., 2015). IVCM has revealed alterations to the density and distribution of SBNP axons in people with dry eye disease (Labbe et al., 2013), surgically induced neurotrophic keratitis (Lambiase et al., 2013), herpes simplex keratitis (HSK) (Hamrah et al., 2010) and interstitial keratitis following acute bacterial and acanthamoeba infection (Cruzat et al., 2011). Despite IVCM providing valuable clinical information about corneal health, one of its major limitations is the inability to resolve smaller diameter nerve fibers and the SNTs (Marfurt

* Corresponding author.
*E-mail address:* holly.chinnery@unimelb.edu.au (H.R. Chinnery).

https://doi.org/10.1016/j.exer.2018.03.012
Received 10 July 2017; Received in revised form 10 March 2018; Accepted 12 March 2018
Available online 14 March 2018
0014-4835/ 

et al., 2010; Tervo et al., 2016). As a result, most clinical studies have focused upon characterizing changes within the SBNP only, with few *ex vivo* accounts considering the relationship between the density of the SBNP and the SNT (He et al., 2010; Cai et al., 2014).

The correlation between corneal nerve regeneration and restoration of corneal sensation is of clinical importance, as some surgical procedures and corneal pathologies result in damage to the sensory nerves. There have been contrasting findings reported with respect to refractive surgery, such as photorefractive keratoplasty (PRK) and Laser In-Situ Keratomileusis (LASIK), with some studies demonstrating a direct correlation between SBNP density (measured using IVCM) and corneal sensation (Linna et al., 2000), and others finding little or no association (Bragheeth and Dua, 2005; Stachs et al., 2010). As such, whether there is a direct correlation between the SBNP and the overlying nerve supply to the superficial epithelium, and whether this is associated with sensory function, remains questionable.

The use of rodent models to study corneal nerve architecture offers the advantage of being able to examine and quantify the nerve supply to the SBNP and the STN using *ex vivo* immunofluorescence microscopy or histology. Following rotating burr debridement of the central corneal epithelium in mice, which causes denervation of the SBNP, corneal regeneration is incomplete after four weeks (Pajoohesh-Ganji et al., 2015). After stromal transection injury in mice, the density of nerve axons in the SBNP returns to baseline by six weeks (Namavari et al., 2011). In both of these models of sterile corneal injury, it is unclear how the rate of recovery of the SNT relates to the regeneration of the SBNP. We compared the sum length of nerves in the SBNP and corresponding overlying SNT, in the peripheral and central cornea, in both naïve mice and in mice following a central corneal abrasion. We undertook correlation analyses to determine whether changes in the sum length of the SBNP was associated with a concomitant change in the SNT.

A notable difference between *ex vivo* histological analysis of corneal nerves compared with *in vivo* analysis using IVCM is the number of nerves visible for quantitation, with the latter technology vastly underestimating the number of nerve fibers due limitations in resolution (He et al., 2010). In mice, IVCM analysis of corneal nerve fibers also largely underestimates the density of the SBNP (Reichard et al., 2015). With the improved visibility of nerve architecture using *ex vivo* techniques, such as immunofluorescence staining with confocal microscopy (to allow visualization of both the SBNP and overlying SNT), a large number of nerve axons need to be quantified. This has led to the adoption of a range of methods for corneal nerve quantitation, including manual tracing (Reichard et al., 2015; Leckelt et al., 2016) using an open source software such as NeuronJ (Meijering et al., 2004), automated thresholding of the corneal nerve staining area using ImageJ (Gao et al., 2016; Dong et al., 2017) or three dimensional (3D) volume analysis using more sophisticated image-processing tools, such as Imaris software (Cai et al., 2014; Yorek et al., 2015). In view of the considerable difference in time required to perform image analyses using these different methods (e.g., several seconds for an automated threshold in ImageJ compared with ~45 min when manually tracing nerves visible within a field (Guimaraes et al., 2016)), the aim of this study was to compare several image analysis techniques and measure the level of agreement when quantifying a range of corneal nerve parameters, including nerve fiber staining area and total nerve fiber length, in both the SBNP and SNT.

## 2. Material and methods

### 2.1. Animals

Female C57BL/6 mice were purchased from the Animal Resources Centre (Canning Vale, WA, Australia) and housed under specific pathogen-free conditions at the Florey Institute of Neuroscience and Mental Health. All animal procedures were approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### 2.2. Corneal injury

Mice were deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The central corneal epithelium (1 mm diameter) was debrided using a corneal rust ring remover (Algerbrush II; Alger Equipment Co., Lago Vista, TX, USA) to create a circular injury, as previously described (Downie et al., 2016). A 1 μL drop of sterile saline was applied to the corneal surface of each eye to prevent drying.

### 2.3. Spectral-domain optical coherence topography (SD-OCT)

Four weeks after sterile corneal injury, the anterior segment was examined using SD-OCT to ensure there were no clinical signs of chronic corneal inflammation (i.e., inflammatory cells, corneal edema or epithelial erosions). Anesthetized mice were placed on the animal imaging mount and rodent alignment stage (AIM-RAS) attached to an SD-OCT imaging device (Bioptigen Envisu R2200 VHR; Bioptigen, Inc., Durham, NC, USA). Volumetric 3 mm × 3 mm rectangular scans of the anterior segment (1000 A-scans/100 B-scans) were captured using an OCT device with an 18-mm telecentric lens (Envisu R-2200; Bioptigen, NC, USA). Central corneal thickness was determined by measuring the distance from the tear film to endothelium using ImageJ software, as previously described (Downie et al., 2014).

### 2.4. Wholemount immunofluorescence staining

Dissected corneas were fixed in cold (4 °C) Zamboni's fixative (2% formaldehyde and 15% saturated picric acid) for 2 h. Tissue flat mounts were washed in phosphate-buffered saline (PBS) then incubated in 20 mM EDTA for 60 min at 37 °C, followed by a blocking buffer (PBS + 3% bovine serum albumin +0.3% Triton X-100) for 60 min at room temperature. To stain nerves, tissue flatmounts were incubated overnight at 4 °C in blocking buffer containing the primary antibody rabbit anti-human PGP9.5 (1:1000; #CL95101 Cedarlane Laboratories, Ltd., Burlington, NC, USA). The next day, corneas were washed three times with PBS and then incubated in goat anti-rabbit Alexa Fluor 647 (1:500; #A21244 ThermoFisher Scientific, Carlsbad, CA, USA) for 2 h at room temperature. Stained corneas were transferred onto a coverslip in aqueous mounting medium and mounted onto glass slides with the epithelium facing up.

### 2.5. Confocal imaging and image analysis

Corneas were imaged using confocal microscopy with a 40x objective lens (Confocal Laser Scanning Platform SP8; Leica Microsystems, Buffalo Grove, IL, USA). Two full thickness epithelial z-series (Fig. 1A & D: 1 μm step sizes; 512 × 512 pixel resolution; 290 μm × 290 μm) were collected using from the central (i.e., within the central 1 mm of the cornea) and peripheral cornea (i.e., between 1.5 mm and 2mm from the central cornea). The depth of z-series from the superficial epithelium to the basal epithelium ranged between 14 and 17 μm. Separate z-stacks were created for the SBNP (Fig. 1B) and SNT (Fig. 1C), by generating z-projections of the superficial and basal epithelial layers.

Each image was analyzed by three different masked observers (MC, YH, CH, DL, CL and RBW) using ImageJ software (Schindelin et al., 2012) (for auto-thresholds and manual thresholds), the NeuronJ plugin (for manual nerve tracings) (Meijering et al., 2004) and Imaris software (Biplane, Zurich, Switzerland); all data were averaged to obtain a final representative value. The sum length of the corneal nerves was quantified using the manual trace function (NeuronJ, Fig. 1G & K) and the filament tracing function in Imaris (Fig. 1H, L & M). The percentage area occupied by corneal nerves was quantified separately for the SBNP