Steel DHW, Dinah C, White K, Avery PJ.

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Acta Ophthalmologica 2017, 95(2), 153-157.

Date deposited:

11/09/2017

Embargo release date:

28 August 2017

The relationship between a dissociated optic nerve fibre layer appearance after macular hole surgery and Muller cell debris on peeled ILM.

David H W Steel 1,2

Christiana Dinah 1

Kathryn White 3

Peter J Avery 4

1 Sunderland Eye Infirmary, Queen Alexandra Road, Sunderland, UK

2 Institute of Genetic Medicine, Newcastle University, Newcastle Upon Tyne, UK

3 EM Research Services, Newcastle University, Newcastle Upon Tyne, UK

4 School of Mathematics & Statistics, Newcastle University, UK

Correspondence to:

Mr D Steel

Sunderland Eye Infirmary

Queen Alexandra Road

Sunderland

UK

David.steel@ncl.ac.uk

Abstract

Purpose: A dissociated optic nerve fibre layer (DONFL) is a characteristic change noted in inner retinal morphology after internal limiting membrane (ILM) peeling. It is thought to be due to trauma to Muller cells as the ILM is peeled from their attached end plates. In this study, we aimed to determine the extent and size of Muller cell debris on the retinal side of excised ILM and assess whether this correlated with the extent of DONFL observed post operatively.

Method: Prospective single centre study of a consecutive series of patients undergoing macular hole surgery. Transmission electron microscopy of the ILM was used to assess Muller cell debris and postoperative spectral domain optical coherence tomography (SDOCT) to assess the extent of DONFL. A variety of other pre and postoperative features were also included.

Results: Thirty nine patients were analysed. There were retinal dimples characteristic of DONFL detected on SD-OCT in all 39 eyes. The portion of the retinal side of the ILM specimen covered by cellular debris ranged from 12-49%, with a median of 28%. Using linear regression the percentage of retinal debris, the size of the debris and the postoperative visual acuity were significantly positively associated with the DONFL score. The total R squared for the model was 63.9%.

Conclusion: The extent of DONFL observed postoperatively can be partly explained by the amount of retinal side cellular debris on the retinal side of the peeled ILM. Surgical strategies which minimise this material could reduce the extent of DONFL.

Key words: Internal limiting membrane, Dissociated optic nerve fibre layer, Muller cell, ILM peeling, Macular hole.

Introduction

Peeling of the internal limiting membrane (ILM) is now routinely performed as part of macular hole surgery based on the fact that it improves the rate of hole closure (Spiteri Cornish et al. 2014). However a number of potentially adverse side effects after ILM peeling, including a dissociated optic nerve fibre layer (DONFL) appearance have been described (Tadayoni et al. 2001, Miura et al. 2003, Ito et al. 2005). DONFL is a distinct appearance of the retina observed postoperatively, and appears as multiple arcuate striae on the inner retinal surface restricted to the area of peeled ILM. (Mitamura & Ohtsuka 2005) It is more readily visible with shorter wavelength illumination, and nearly always present on spectral domain optical coherence tomography (SD-OCT) as apparent depressions in the nerve fibre layer (Miura et al. 2005, Kishimoto et al. 2011, Alkabes et al. 2011, Spaide 2012). Whilst the precise mechanism by which DONFL occurs remains unknown, various investigators have suggested possible explanations. The prevailing theory is that DONFL is related to Muller cell foot plate trauma, as the inner processes of these cells form a plexus on the retinal side of the ILM between the nerve fibre layer and the ILM (Tadayoni et al. 2001, Spaide 2012). Trauma to these processes as the ILM is peeled away could result in changes in the regular bundling of the nerve fibre layer, as well as a volume reduction in the ganglion cell layer where the Muller cell bodies reside, resulting in DONFL (Baba et al. 2012). DONFL is not entirely innocuous and has been related to a reduction in central retinal sensitivity and paracentral scotomas. Terasaki et al also reported a limited and delayed recovery of the b-wave amplitude of the focal macula electroretinogram after ILM peeling during macular hole surgery (Nakamura et al. 2009, Terasaki et al. 2001).

Histological examination of the retinal side of the peeled ILM has shown fragments of Muller cells processes. If the theory of DONFL being related to Muller cell trauma is correct then it would be expected that the extent of DONFL should be related to amount of retinal side debris. This would be of significance as a variety of dyes used to peel ILM have been associated with differing degrees of retinal side cellular material (Steel et al. 2015A, Schumann et al 2009).

In this study, we aimed to determine the extent and size of Muller cell debris on the retinal side of excised ILM and assessed whether this correlated with the extent of DONFL observed post operatively.

Method

Consecutive patients undergoing surgery by one surgeon for idiopathic macular holes over an 18 month period were included in the study. Patients with traumatic macular holes, high myopia [>6 dioptres] and previous retinal surgery were excluded from the study. Ethical approval was obtained and informed consent to participate was gained from participants. Patients with less than 3 months follow up, second eyes or inadequate specimens were excluded.

All patients underwent trans-conjunctival 25g vitrectomy with combined phacoemulsification and IOL implantation if phakic. Posterior hyaloid face separation was achieved with aspiration. In cases of stage 4 holes, with a Weiss ring present, the presence of residual vitreous was checked for with diluted triamcinolone staining. Brilliant Blue G [ILM Blue, Dorc international, The Netherlands] was used to stain the macula for less than 10 seconds in all cases. A macular contact lens was used to view the peeling procedure. The ILM was peeled using a pinch technique and 25g end gripping forceps [Grieshaber revolution DSP ILM forceps, Alcon Grieshaber AG, Switzerland]. A peel radius of approximately 1 disc diameter was aimed for in all cases. The number of spontaneous petechial haemorrhages observed as the ILM separated from the retina was recorded and an end of peel photograph of the ILM peeled area taken using the video recording system attached to the microscope to allow the position and distance from the fovea of the haemorrhages to be measured. After removing the ILM from the eye, it was immediately placed in glutaraldehyde and processed for electron microscopy. Either 25 % SF6 or 20% C2F6 gas was used as a tamponade and the patients instructed to position face down for 3 days. Patients were reviewed at 2 weeks and 3 months post operatively. Preoperative and postoperative visual acuity (VA) at 3 months was measured using a standard Snellen acuity chart and converted to logMAR scores for the purposes of statistical analysis. Patients underwent spectral domain optical coherence tomography [SD OCT] imaging on the Heidelberg Spectralis immediately preoperatively and at 3 months post-operatively. A dense horizontal line scan pattern was used preoperatively centred on the macular hole and used to assess the preoperative minimum linear diameter (MLD) of the holes using the Spectralis measuring tools. For the post-operative OCT a 20 by 30 degree horizontal line scan pattern was used centred on the fovea with a line spacing of 240 microns. All scans used a 25 ART setting enabling multisampling and noise reduction over 25 images.

Quantifying DONFL

The number of focal depressions characteristic of DONFL in the retinal surface were counted in each of the central 13 slices of the 3 month post-operative OCT and summed to produce a total DONFL score [Figure 1] as previously described.(Steel et al. 2015B) One observer, masked to their previous count, counted the depressions on two occasions. There was high concordance between the two counts with a correlation coefficient of 0.94. The measurements showed no systematic relationship with the extent of DONFL and the repeatability coefficient was 2.3 which we considered clinically acceptable.

Transmission electron microscopy

Samples were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer. The ILM was enrobed in low-melting point agarose [4%] to form a small block to make the ILM easier to handle. After secondary fixation in 2% osmium tetroxide, the samples were dehydrated in graded acetone, embedded in epoxy resin and polymerised at 60^{o}C. Ultrathin sections of 70nm were taken at 2 levels through the block, stained with uranyl acetate and lead citrate and viewed on a Philips CM100 TEM. Detailed examination of the tissue was then performed to determine the occurrence and size of any cellular debris on the retinal surface of the ILM. Debris was divided up into the following categories. 1) 'Small' - cellular fragments less than 2microns in size with no recognisable cell organelles 2) 'Whole cells' - large fragments greater than 2 microns with recognisable cellular features e.g. nucleus, mitochondria (Figure 2) 3) 'Layers' - a number of cells forming a monolayer with recognisable cellular structures. For estimation of the amount of cellular debris, images were taken at x7900 from 14 randomly sampled areas of the ILM. An unbiased method was used to quantify the amount of debris on the retinal side of the ILM (figure 3) as previously described. (Steel et al. 2014) The same technique was used for estimation of the extent of vitreous side material present on the ILM.

Statistical analysis

Descriptive and statistical analyses were performed using Minitab 17 version 1.0. Patients' demographic characteristics, pre- and postoperative variables are presented in terms of median, upper and lower quartile and range or percentage as appropriate. The DONFL score had an asymmetric distribution with a long tail upwards and hence a square root transformation was used which approximately normalised the distribution. Association between DONFL score and continuous data was assessed using correlations, and between categorical data using two sample t-tests or analysis of variance. Stepwise linear modelling was then used on the square root of the DONFL score to pick out the most important variables. Statistical significance was considered with a p-value of 0.05 or less.

Results

During the study period there were 44 consecutive patients treated. Five were excluded for incomplete follow up (1), specimens being inadequate (2) and second eye status (1) leaving 39 for analysis. A summary of the patients' data is shown in table 1. The mean age of was 70 years old and 29 (74%) were female. Of these, 15 (38%) were pseudophakic and 24(62%) had combined cataract surgery.

Petechial haemorrhages related to ILM separation from the underlying retina were observed in 20 (53%) patients. The number ranged from 0-7 with a median of 0, and 73% were located in the nasal quadrant of the macula.92% were situated greater than 1000 microns from the foveal centre and 80% greater than 1200 microns. There was no significant association between the presence and extent of retinal haemorrhages and the extent of retinal debris on the ILM ($r = 0.27$, $p=0.10$)

There were retinal dimples characteristic of DONFL detected on SD-OCT in all 39 eyes. The mean total DONFL score was 10 (range: 2-26).

The portion of the retinal side of the ILM specimen covered by retinal debris ranged from 12-49%, with a median of 28%. The portion of the vitreous side of the ILM covered by residual material ranged from 0-98%, with a median of 10%.

Using univariate analysis 5 variables were statistically significantly associated with the DONFL score; the size of the macular hole, the number of petechial haemorrhages observed during peeling, the postoperative visual acuity, the percentage of retinal cellular debris on the retinal side of the ILM and the size of the fragments.(Table 1)

Using stepwise linear modelling only the percentage of retinal debris (partial R squared 24.6%, $p<0.001$), the size of the debris (small<whole<layers partial R squared 27.7%, $p<0.001$) and the postoperative visual acuity (partial R squared 11.6%, p=0.002) were significantly associated (all positively) with the DONFL score. The total R squared for the model was 63.9%.

Pre and postoperative visual acuity and macular hole size were significantly related to each other (preoperative Va:postoperative Va , r =0.51; preoperative Va:MLD, r=0.70; Postoperative Va:MLD r=0.56, p<0.001 for all)

Discussion

We found a significant relationship between the percentage and the size of retinal side cellular debris present on the ILM after peeling and the extent of DONFL after surgery. This supports the current theory that it is Muller cell trauma that results in the occurrence of DONFL after peeling. Amongst the many functions of Muller cells in the retina they are thought to provide structural support to the nerve fibre layer. Trauma to their endplates, as occurs during ILM peeling as the ILM is forcibly peeled from the endplates (Wolf 2004), may result in changes to the bundling/arrangement of the NFL, producing DONFL (Tadayoni et al. 2001, Spaide 2012).

There are several important potential confounding factors to consider. Different dyes have been shown to be associated with differing degrees of retinal side material on the ILM (Steel et al. 2015 A). We therefore used the same dye with the same duration of application time in all cases. One surgeon also performed all the peels and used a consistent technique of peeling which may also have some bearing on the amount of material observed after during peeling based on the technique and optimal angle of ILM separation (Steel et al. 2014, Dogramaci et al. 2013). We measured the extent of vitreous side material on the ILM as the presence of ERM has been shown to be associated with a deeper plane of ILM separation but we found no effect of this on the extent of DONFL (Kenawy et al. 2010). We have previously also not found any correlation between the extent of retinal and vitreous side material in macular holes cases perhaps relating to the lesser degrees of ERM (Steel et al 2015 A).

Previous studies have shown that a DONFL appearance is most prevalent in the superior quadrant followed by inferiorly, nasally and then temporally but more commonly the dimples extend through the nerve fibre layer temporally (Nukada et al.2013). This possibly relates to the relative thickness and bundling arrangement in the nerve fibre layer making dimples more obvious in these areas and more likely to extend through it when thinner temporally. An alternative explanation might relate to the ILM being more adherent to the retina in certain areas. One of the weaknesses of our methodology however is that we did not orientate our specimens topographically so cannot comment on whether the amount of retinal side material varied across the ILM surface in any systematic way.

On univariate analysis there was a significant positive relationship between the occurrence of spontaneous haemorrhages as the ILM separated from the retina and the extent of DONFL suggestive that retinal haemorrhages could act as a surrogate marker of a deeper plane of separation. There was also a higher occurrence of retinal haemorrhages on the nasal side fitting with the more frequent occurrence of DONFL in this area. The relationship with DONFL however disappeared on multivariate analysis and there was also no relationship between the number haemorrhages and the extent of retinal debris on the peeled ILM. It is possible that analysis of retinal side debris orientated topographically could show higher values nasally and be correlated with retinal haemorrhages. It is also worth observing that no red blood cells were seen on any ILM specimens.

We found a significant association between worsening postoperative VA and the extent of DONFL. There have been no clear associations between DONFL and reduced postoperative visual outcome described before, although there have been reports of reduced retinal sensitivity in the peeled area and paracentral scotomata (Tadayoni et al. 2012, Ripandelli et al. 2015, Lim et al.2011)

Pilli et al found an association between inner retinal volume in the macular and postoperative visual outcome although the presence of DONFL was not specifically described (Pilli et al.2012). Pre and postoperative visual acuity and MLD were all highly correlated and it is uncertain of the validity of the finding.

Linear regression had a total R squared of 64% using our best model,suggesting that other factors remain to be discovered which affect the extent of DONFL. These might include other facets of surgical trauma, light exposure and dye toxicity. Further study is needed to investigate these and other possible factors.

We quantified the extent of the DONFL defects on SD OCT as previously described. We assessed its intra-observer repeatability which was acceptable. In future studies however the use of en face OCT techniques may allow more precise quantification of the extent of DONFL (Kishimoto et al. 2011, Alkabes et al. 2011, Spaide 2012)

In conclusion the extent of DONFL observed postoperatively after macular hole surgery can be partly explained by the amount of retinal side cellular debris on the retinal side of the peeled ILM. Many factors are known to affect this including the presence of epiretinal membranes but also the peeling technique used and the use of dyes. Further research is needed into these as well as other factors which affect the occurrence of inner retinal trauma after ILM peeling.

Acknowledgements: Presented in part at the Euretina annual meeting 2014, London.
DS has attended advisory boards and received research funding for other studies from Alcon.

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Legends for figures

Figure 1. Method for measuring number of dimples characteristic of DONFL. **A** shows red free image with DONFL visible. **B** shows corresponding infrared image with SD OCT scan line. Dimples seen on B correspond to 2 dimples on red free image (arrows).

Figure 2. Transmission electron microscopy image (X13,500) showing small (asterix) and 'whole' cell (arrows) categories of retinal side cellular debris.

Figure 3. Method for estimating extent of internal limiting membrane (ILM) surfaces covered by cellular material. A grid of 2 micron lines was superimposed on each image and the number of intercepts between the grid line and retinal side of the ILM were counted (**A**). Another grid of 1 micron line length was then superimposed on each image and the number of intercepts between the grid lines and any retinal debris were counted (**B**). The percentage of retinal side surface covered by cellular debris was taken as the number of intercepts on cellular debris/ [number of intercepts on surface x 2] * 100.