

HISTOPATHOLOGY OF SELECT CORNEAS TREATED WITH AGROCHEMICAL FORMULATIONS IN THE BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

This summary presents the histopathology results of select formulations tested in the bovine cornea opacity and permeability (BCOP) assay at the Institute for In Vitro Sciences, Inc. (IIVS), and reflects the findings originally presented in IIVS Study reports 18AC49-AC54.350049 and 18AN58-AN65, AN67-AN68.350049.

The following select agrochemical formulations were tested: A and D tested on 10 May 2018, W tested on 20 April 2022, and X tested on 25 April 2022. Histopathology was performed to determine whether any test article-induced changes in the treated corneas are evident, relative to the negative control-treated corneas. Although histopathology was conducted on negative and positive control-treated corneas in each of the trials, only a single exemplary set (20 April 2022) is presented in this summary for brevity. Nonetheless, it is acknowledged that some variations in corneal sectioning and staining profiles may be observed between histology processing batches.

The table below presents the in vivo observations and the BCOP endpoint scores for the four select formulations. The formulations are presented in order of increasing levels of corneal irritation. As is often observed with testing of surfactant formulations in the BCOP assay, corneal opacities tend to be understated and generally reflect opacities due to stromal swelling. Loss of barrier function as a result of epithelial erosion tends to be reflected in increasing fluorescein permeability scores, and can be key to discriminating among surfactant activity.

Formulation Code	Animals Tested (Driving Classification)	In Vivo Study Result Summary	BCOP Endpoints		
			Opacity Score	Permeability Score (OD ₄₉₀)	IVIS
A	3 (3)	Minimal effects; no positive scores up to 7 days	1.3	-0.01	1.2
W	3 (1)	CO in single animal on D1; cleared by D7	4	0.134	6.0
X	3 (1)	CO in single animal on D1; cleared by D14	5	0.352	10.3
D	1 (1)	CO (score 1) on D21	10.3	0.624	19.7

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of agrochemical formulations to isolated bovine corneas. In brief, bovine corneas, obtained as a byproduct from freshly slaughtered animals, were mounted in special holders and treated with the undiluted agrochemical formulations for a 10-minute exposure. After the 10 minute exposures, the test articles were rinsed from the corneas, and incubated for a post-exposure incubation period of 2 hours,

after which changes in corneal opacity were determined. To evaluate loss of barrier function, a fluorescein solution was applied onto the anterior side of the corneas and the corneas were incubated for 90 minutes. After the medium was removed from the posterior chamber for the fluorescein permeability determination, each cornea was carefully separated from its corneal holder and fixed in 10% neutral buffered formalin.

An *In Vitro* Irritation Score (IVIS) was determined for the agrochemical formulations based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas. The methods and procedures used in this assay were consistent with OECD Test Guideline 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage¹ and the Office of Pesticide Programs, U.S. Environmental Protection Agency “Use of an Alternate Testing Framework for Classification of Eye Irritation Potential of EPA Pesticide Products”². After the BCOP assay, the fixed corneas were transferred to StageBio (Frederick, MD) for embedding, sectioning, and H&E staining. An evaluation of the histological changes was performed by John W. Harbell, Ph.D. (JHarbell Consulting LLC) and Hans Raabe, M.S. (Institute for *In Vitro* Sciences, Inc.) to assess the depth and degree of injury.

SUMMARY OF HISTOPATHOLOGY FINDINGS

Formulation A, neat, 10-minute exposure, 120-minute post-exposure

The corneas treated with Formulation A showed a histological pattern similar to that of the negative control-treated corneas except for a small increase in cytoplasmic vacuolization in the surface squamous cell layer. Based on the depth of injury prediction model of Jester and Maurer (Figure B), injury limited to the squamous epithelial layer is consistent with non- to minimal ocular injury.

Formulation W, neat, 10-minute exposure, 120-minute post-exposure

The epithelium of corneas treated with Formulation W showed detachment of the squamous epithelium as a continuous sheet, suggesting coagulation of the layer. The upper wing cells showed a rounded morphology and vacuolated cytoplasm, but otherwise the epithelium appeared similar to the negative control-treated corneas. The stroma was similar in thickness to the negative control-treated corneas. The stroma directly beneath the anterior limiting lamina appeared similar to the negative control-treated corneas. The stroma at mid-depth appeared similar to the negative control-treated corneas. The lower stroma appeared similar to the negative control-treated corneas. The Descemet's Membrane was

¹ OECD (2020), *Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing.

² Office of Pesticide Programs, U.S. Environmental Protection Agency (2 March 2015) Use of an Alternate Testing Framework for Classification of Eye Irritation Potential of EPA Pesticide Products.
http://www2.epa.gov/sites/production/files/2015-05/documents/eye_policy2015update.pdf

prominent. The endothelium was generally intact similar to the negative control-treated corneas. Based on the depth of injury prediction model of Jester and Maurer (Figure B), injury limited to the upper epithelium without keratocyte damage is consistent with minimal to mild ocular injury.

Formulation X, neat, 10-minute exposure, 120-minute post-exposure

The epithelium of corneas treated with Formulation X showed loss of the squamous and upper wing cells, with occasional loss into the lower wing and basal cell layers. The cells appeared to be lost as individual cells, rather than as a tissue sheet. The remaining wing and basal cells immediately below the areas of loss appeared rounded and hypereosinophilic. In some fields of view where the basal layer was intact, the cells appeared similar to the negative controls while in others the basal cells showed abnormal cellular morphology, hypereosinophilic cytoplasmic staining and pyknotic nuclei. The stroma was thicker than the negative control-treated corneas. The stroma directly beneath the anterior limiting lamina showed mild to moderate collagen matrix expansion especially under those areas with deeper epithelial erosion, while the keratocytes appeared generally similar to those in the negative control-treated corneas. The stroma at mid-depth appeared similar to the negative control-treated corneas. The lower stroma appeared similar to the negative control-treated corneas. The Descemet's Membrane was prominent. The endothelium was generally intact similar to the negative control-treated corneas. Based on the depth of injury prediction model of Jester and Maurer (Figure B), injury limited to the epithelium without notable keratocyte damage is consistent with mild to moderate ocular injury.

Formulation D, neat, 10-minute exposure, 120-minute post-exposure

The corneas treated with Formulation D showed extensive to complete disruption of the corneal epithelium which is inconsistent with the more limited fluorescein passage values. Stromal swelling varied between the treated corneas and was observed to be slightly to appreciably thicker than the negative control treated corneas. The stroma directly beneath the anterior limiting lamina showed marked stromal swelling and loss of viable keratocytes (nuclear condensation and cytoplasmic eosinophilia). The stroma at mid-depth showed moderate collagen matrix swelling and some keratocyte changes. The frequent loss of endothelium with concomitant lower stromal collagen matrix swelling would be consistent with a full thickness severe injury.

INTRODUCTION

The histopathology of test article-treated corneas obtained from the BCOP assay provides a third endpoint in addition to the opacity and fluorescein permeability endpoints. Histopathology is performed to determine whether any test article-induced changes in the treated corneas are evident, relative to the negative control-treated corneas, and in the presence of such changes, the depth and degree of these changes are reported. Accordingly, histopathology can complement the BCOP assay primary endpoints of opacity and fluorescein permeability (loss of barrier function) by elucidating the cytopathic and architectural changes associated with these primary measures, providing evidence of corneal injury

not revealed by the primary BCOP endpoints alone, or confirming the lack of test article-related damage to the cornea.

Depth and Degree of Corneal Injury

Since test materials are applied topically on the outer corneal epithelium, the top-down evaluation follows the potential for lesions to occur dependent upon the penetration of the test chemical into the cornea, and the potential for toxic effects to be induced. Therefore, the evaluation of changes in the treated corneas is performed starting superficially with the squamous epithelium at the site of test article exposure, and progressing deep into the cornea to the endothelium. See Figure A for a micrograph of the untreated Bovine corneal epithelium. Jester et al.^{3,4,5}, Maurer et al.^{6,7,8} and others have shown for a range of chemical classes that depth of injury in the early hours after exposure can be predictive of the eventual degree and duration of the ocular lesions. Their studies were performed in rabbits using the low volume eye test protocol (ten microliters instilled directly onto the center of the cornea)⁹ and the lesions were measured using confocal microscopy. This approach allowed the same lesion to be measured from initial injury through the course of its degree and duration in the same animal. These measurements were supplemented with standard macroscopic observations, standard histology and live/dead staining. They studied several chemical classes and degrees of severity. Their conclusion across the chemical classes was that the initial depth of injury to the cornea at three hours was predictive of the eventual degree and duration (days to clear) for most chemical classes.¹⁰ The exception to the three hour time point is those chemicals that show delayed onset of ocular irritation *in vivo* (peroxides, mustards and other DNA reactive chemicals).¹¹ In these cases, the depth of injury at 24 hours after treatment is used. This approach can be used with the BCOP extended post-exposure protocol.¹² The

³ Jester, JV, Li, HF, Petroll, WM, Parker, RD, Cavanaugh, HD, Carr, GJ, Smith, B, and Maurer, JK. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Investigative Ophthalmology & Visual Science** 39(6):922-936.

⁴ Jester, J. V., Petroll, W. M., Bean, J., Parker, R. D., Carr, G. J., Cavanaugh, H. D., and Maurer, J. K. (1998). Area and depth of surfactant-induced corneal injury predicts extent of subsequent ocular responses. **Investigative Ophthalmology & Vision Sciences** 39, 2610–2625.

⁵ Jester, J. V., Li, L., Molai, A., and Maurer, J. K. (2001). Extent of corneal injury as a mechanistic basis for alternative eye irritation tests. **Toxicology in Vitro** 15:115–130.

⁶ Maurer, J. K., Li, H. F., Petroll, W. M., Parker, R. D., Cavanaugh, H. D., and Jester, J. V. (1997). Confocal microscopic characterization of initial corneal changes of surfactant-induced eye irritation in the rabbit. **Toxicology and Applied Pharmacology** 143:291–300

⁷ Maurer, J. K., and Parker, R. D. (2000). Microscopic changes occurring over time with acetic acid and sodium hydroxide in the rabbit low-volume eye test. **Toxicological Pathology** 28:679–687

⁸ Maurer, J. K., Molai, A., Parker, R. D., Li, L., Carr, G. J., Petroll, W. M., Cavanaugh, H. D., and Jester, J. V. (2001). Pathology of ocular irritation with acetone, cyclohexanol, parafluoroaniline, and formaldehyde in the rabbit low-volume eye test. **Toxicological Pathology** 29:187–199

⁹ Griffith, J. F., Nixon, G. A., Bruce, R. D., Reer, P. J., and Bannan, E. A. (1980). Dose–response studies with chemical irritants in the albino rabbit eye as a basis for selecting optimum testing conditions for predicting hazard to the human eye. **Toxicology and Applied Pharmacology** 55:501–513.

¹⁰ Maurer, JK, Parker, RD, and Jester, JV. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

¹¹ Maurer, J. K., Molai, A., Parker, R. D., Li, L., Carr, G. J., Petroll, W. M., Cavanaugh, H.D., and Jester, J.V. (2001). Pathology of ocular irritation with bleaching agents in the rabbit low-volume eye test. **Toxicological Pathology** 29:308–319.

¹² Curren, R., Evans, M., Raabe, H., Dobson, T., and Harbell, J. (1999) Optimization of the bovine corneal opacity and permeability assay: histopathology aids understanding of the EC/HO false negative materials. **ATLA** 27:344.

studies of Maurer et al and Jester et al are central to the use of the BCOP in general and to histopathology of the treated corneas in particular.^{13,14}

Damage to the cells of each of the three layers of the cornea has very different consequences. The general scheme is presented in Figure B with an overlay on a rabbit cornea. It should be remembered that the scheme is based on the initial lesions associated with the eventual degree and duration of the final ocular irritation category. While these studies were performed with the low-volume eye test rather than the standard Draize dosing scheme, the full range of severities can be achieved with both assays and the predictive principles are the same. The key to predicting severity is the depth of cytotoxic damage because that was what was measured in the *in vivo* studies. Figure A shows the bovine corneal epithelium in detail. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Damage limited to the epithelium can be further divided into minimal loss/damage of the surface squamous epithelium which will repair very quickly (non-irritant) and loss/damage of the wing cell layer which is presented as predictive of slight irritation, again with full repair expected. "For mild irritants, injury extended through the corneal epithelium and included the anterior most, superficial stromal keratocytes, whereas the extent of injury for moderate and severe irritants extended past the anterior most stromal keratocytes and involved deeper layers at times including the corneal endothelium."¹⁵ The difference between moderate and severe is reflected in the depth of keratocyte cytotoxicity in the stroma. Injury to the stromal keratocytes has more serious consequences as damage to these cells initiates the inflammatory process in the stroma. The depth of keratocyte damage determines the balance between regeneration and scarring in this layer. Damage limited to the upper third to half of the stroma is associated with moderate irritation while toxicity extending deeper into the lower stroma (in some cases including the endothelium) would be predictive of severe irritation.

Redden et al. (2009)¹⁶ presented on an evaluation of the BCOP assay with corneal histopathology for predicting the eye irritation potential of a series of anti-microbial products with cleaning claims which had been previously classified *in vivo*. The BCOP histopathology findings showed that anti-microbial cleaning products predicted *in vivo* to have an EPA category IV classification (approximately consistent with a GHS No Label classification) generally induced corneal changes in the *in vitro* assay no deeper than midway through the corneal epithelium. Furthermore, those products predicted *in vivo* to have an EPA category III classification (approximately consistent with a GHS 2B classification) generally induced corneal changes *in vitro* extending no deeper than the upper third of the stroma, where stromal changes typically reflected stromal matrix swelling without appreciable keratocyte pathology. This type of stromal matrix swelling reflects damage to the barrier properties of corneal epithelium which allows the water to pass into the hygroscopic stroma. Those products predicted *in vivo* to have an EPA category II

¹³ Maurer, JK, Parker, RD, and Jester, JV. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

¹⁴ Jester, J. V. (2006) Extent of corneal injury as a biomarker for hazard assessment and development of alternative models for the Draize eye test. **Cutaneous and Ocular Toxicology** 25:41-54.

¹⁵ Ibid Jester (2006)

¹⁶ John Redden, MS; Mark J. Perry, MPH; Timothy Leighton, Jonathan Chen, Ph.D.; Tim McMahon, Ph.D., 5/11/2009. Voluntary Pilot Program to Evaluate Use of a Non-Animal Testing Approach to EPA Labeling For Eye Irritation For Certain Antimicrobial Products With Cleaning Claims.

classification (approximately consistent with a GHS 2A classification) generally induced corneal changes *in vitro* extending no deeper than two thirds of the stroma, where the stromal changes deepest into the cornea were stromal matrix swelling while notable keratocyte pathologies were limited to the anterior third to half of the stroma. Finally, those products predicted *in vivo* to have an EPA category I classification (approximately consistent with a GHS 1 classification) generally induced corneal changes *in vitro* extending into the lower third of the stroma. These changes are damage to the keratocytes as well as any increased stromal swelling. Damage to the endothelium, as reflected by the loss of that cell layer or loss of endothelial function (the prevention of water uptake by the deep stroma), is considered to be predictive of category I damage.

In some cases, quite clear changes are observed in the treated corneas. For example, gross changes or erosion of the corneal architecture, whether limited in depth of injury or as a full corneal thickness injury would be readily apparent. Other lesions may be a bit more subtle, and may simply be limited to changes in nuclear staining, perhaps as a prelude to an apoptotic event. In such cases, these changes may be expected to result in downstream loss of corneal function, which may not be evident by the opacity and permeability endpoints in the standard short-term BCOP assay. While in general, the depth of observed changes in the bovine cornea can be reported, the degree and impact of many of the observed changes may not be fully assessed or known. For example, the observation of abnormal nuclear staining in stromal keratocytes is not readily described in terms of a scale or degree of abnormality. Rather, an attempt is made to present the relative frequency of such observed changes relative to the negative control corneas. Another example would be the swelling of the corneal stroma. Stromal edema may be described in terms of the depth of the observed stromal expansion, but should be characterized by the relative degree of stromal expansion in the upper, mid and lower stroma. Furthermore, stromal edema, resulting from limited loss of epithelial barrier function, in and of itself may not have the same consequences to corneal recovery as stromal edema with loss of keratocytes, or stromal edema occurring in concert with stromal protein precipitation. Therefore, the interpretation of the histopathology should allow for an integration of all of the observed changes, prior to the definitive assessment.

Evaluations of the Corneal Epithelium, Stroma and Endothelium

Damage to the epithelial layer is generally the first change observed in the cornea since the test materials are applied topically to this “unprotected” epithelium. Each “layer” of the epithelium is scored for cell loss or damage. Figure A shows the structure of the epithelium from a control cornea. Changes to the surface epithelium (squamous and upper wing cell epithelium) in the BCOP assay are usually not predictive of lasting corneal changes *in vivo*. The loss of the squamous and upper wing cell layers in the excised bovine cornea by surfactant-based test articles appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo*¹⁷. This type of damage is typically reversible in the rabbit. Many types of surfactants (e.g., sodium lauryl sulfate) are expected to lyse these cells so that they are progressively lost from the epithelial surface. The positive control, ethanol, provides an example of a

¹⁷ Gettings, SD, Lordo, RA, Hintze, KL, Bagley, DM, Casterton, PL, Chudkowski, M, Curren, RD, Demetrulias, JL, DiPasquale, LC, Earl, LK, Feder, PI, Galli, CL, Glaza, SM, Gordon, VC, Janus, J, Kurtz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CFTA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food and Chemical Toxicology** 34(1):79-117.

different type of damage. In this case, the surface squamous epithelial cells are coagulated and fixed in place. The coagulated tissue stains heavily with eosin (see Figure 7). In addition, the wing and basal cells are vacuolated and the adhesion between the basal cells and the basal lamina is disrupted. Such changes can lead to sloughing of the epithelium. Lesions in the deep wing cell and basal cell layers, either by cell lysis or coagulation, are associated with more damage *in vivo*.

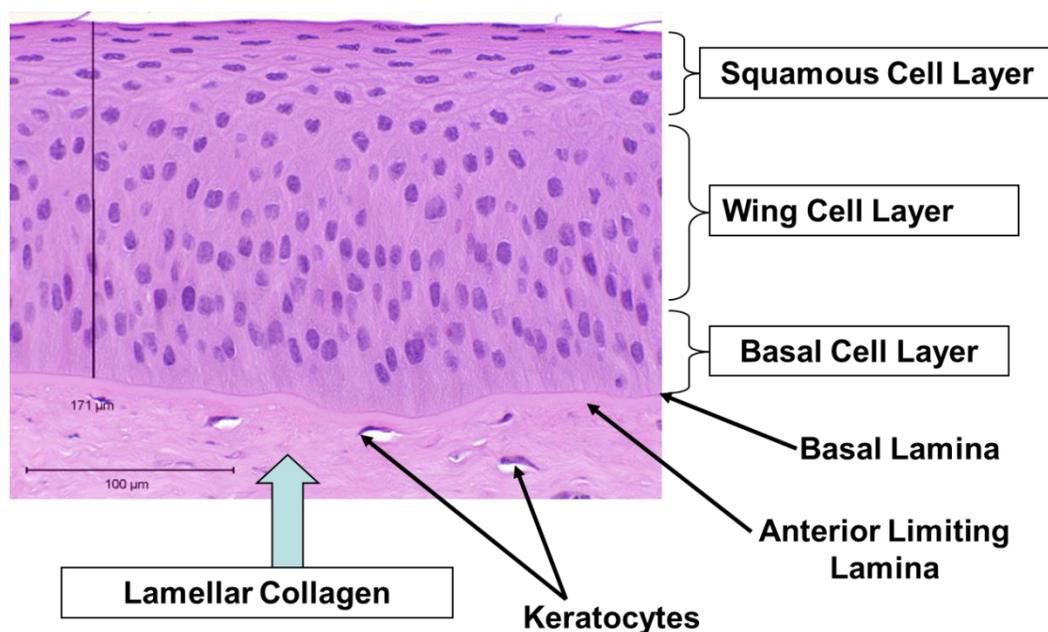


Figure A. An example of a control cornea showing the three layers of the epithelium, the anterior limiting lamina, and the upper stroma (magnification 290 x)

Special effort has been made to detect changes in the stromal elements of the corneas. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling or expansion within the stromal collagen matrix and/or loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected as areas of expansion or separation in the organized collagen matrix. The appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of this stromal expansion may be seen in Figure 8 where the positive control exposure has induced some stromal swelling. The depth and degree of stromal expansion can be indicative of the degree of injury to the cornea and/or penetration of the test article into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix expansion (swelling). Viable epithelium or endothelium will not only slow entry of the water but actively transport it out of the stroma. As the viability of epithelium declines, both the barrier and transport capacities are reduced. Thus, the amount of water accumulating in the upper stroma reflects the damage to the epithelium. When the full epithelial layer is lost over a section of stroma, stromal swelling can increase the stromal thickness by 50% or more over the control corneal stroma. Such changes are often observed when corneas treated with severe irritants are incubated for extended periods after exposure. Loss of the endothelium can also lead to appreciable deep stromal swelling. The loss may result from test article penetration or mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test article exposure tends

to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix expansion will be located in the deep stroma (just above Descemet's Membrane). In contrast, test article-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix expansion will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

In vivo, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through "scar" collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test article-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic hypereosinophilia.

Reactive chemistries such as peroxides or sulfur mustard¹⁸ show a delayed onset of irritation *in vivo* and in the BCOP. To allow more complete expression of damage to the cornea, a 20 to 24 hour post exposure period is used. Concurrent negative control corneas are employed. The corneal epithelium is rich in antioxidants and catalase enzyme. Therefore, the damage to the corneal epithelium may be more limited compared to the stromal keratocytes. A pattern of pyknotic keratocyte nuclei and cytoplasmic hypereosinophilic staining is characteristic of this type of exposure. There is also a generalized decrease in the density of keratocytes suggesting apoptotic cell loss.¹⁹

Since depth of injury (both extracellular and cellular) to the stroma is important in the analysis, a means to describe the depth of injury is necessary as part of this analysis. Determining this depth is not always a straightforward process. Because of the topical application of the test article to the epithelium, one would expect that exposure to the stroma would progress from the area just under the anterior limiting lamina down through the stroma to Descemet's Membrane. There is no external inflammatory process *in vitro*, so one might also expect the progression of damage to follow the progression of exposure. That means that damage to the stroma should first appear close to the anterior limiting lamina. As the damage to the stroma increases, deeper layers might be involved. This implies that one would want to express damage as progressing from the anterior (anterior limiting lamina) to the posterior (Descemet's Membrane) and express it as a fraction of the total stromal depth involved. However, collagen matrix expansion can (and often does) increase total stromal thickness. Thus, measurements of the depth of a

¹⁸ McNutt, P., Hamilton, T., Nelson, M., Adkins, A., Swartz, A., Lawrence, R., Milhorn, D. (2012) Pathogenesis of acute and delayed corneal lesions after ocular exposure to sulfur mustard vapor. *Cornea* 31:280-290.

¹⁹ Harbell (unpublished)

stromal lesion can be complicated by the change in overall stromal thickness. To account for stromal swelling, this depth may be estimated from the percentage of the stromal cross section that remained undamaged (starting at the posterior border). For this report, depth of stromal damage is reported simply in terms of relative depth (e.g., upper, middle and deep stroma).

The depth of initial injury as a predictor of the degree and duration of the resulting corneal lesion is summarized in Figure B. The histological changes, when they occur, will be reported to address the depth of injury through each tissue of the cornea. As discussed above, damage associated with severe injury may and often does extend past the mid-stromal depth to include the endothelial cell layer. However, not all severe irritants will produce damage to the endothelium. Thus the arrow extends only to the deep stroma. It should be understood that damage in this context refers to cytotoxicity rather than just increased collagen matrix swelling.

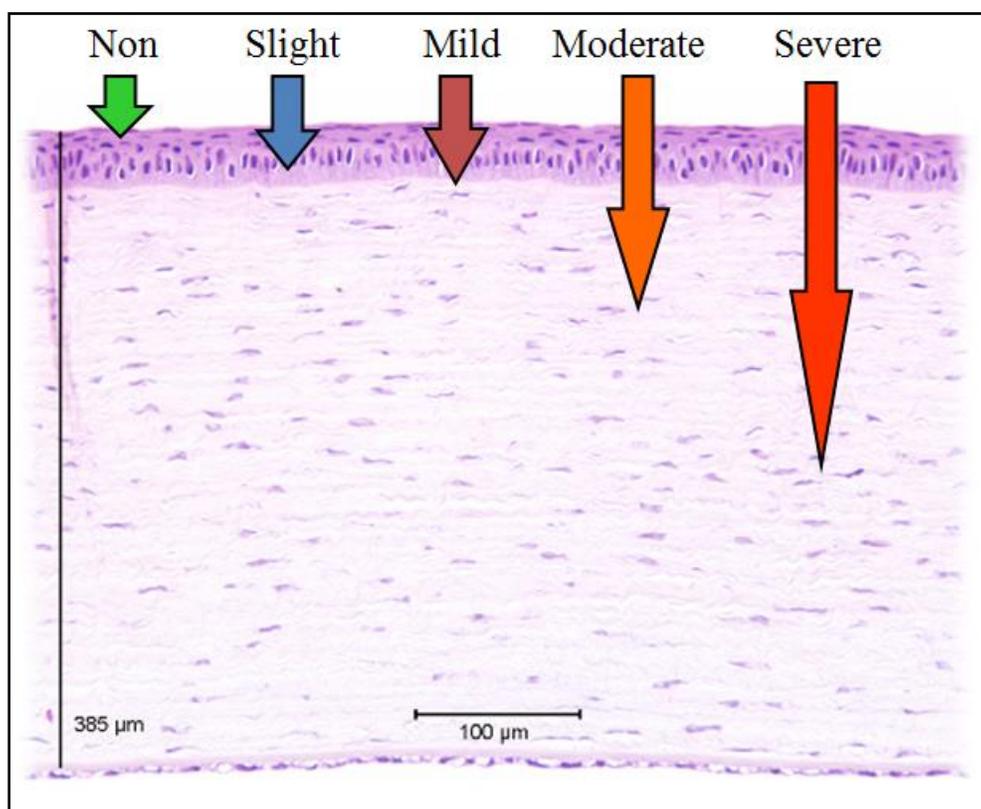


Figure B. Summary of the initial depth-of-injury prediction model from Jester and Maurer

MATERIALS AND METHODS

Tissue Collection and Preparation

Cornea samples were fixed in formalin as described in the protocol and processed according to the methods report by StageBio (Frederick, MD).

Histologic Evaluation

Negative control corneas were processed with the test article-treated corneas as a common histology batch process. The histology of the negative control corneas may thus be used to evaluate the quality/acceptability of the slides within the processing batch. Prior to conducting the evaluation of test article-induced histopathology, the quality of the Hematoxylin and Eosin (H&E) stained corneal sections must be evaluated, so that the nature and degree of the artifacts of both the BCOP assay and the histology processing can be assessed. To this end, the negative control slides are used to detect artifacts at the batch level. They are also used to assess “normal” staining (degree of hematoxylin or eosin in each layer/cell type), tissue architecture and general thickness.

Slides were thoroughly examined by microscopic evaluation. Each cross section of each cornea within a treatment group was observed first under low magnification for an overall assessment of the quality of the tissue sections for conducting the histopathology. Corneal sections were evaluated across the entire section from one crush zone to the other (the crush zone is the outer corneal perimeter where the cornea was mounted against an O-ring within the corneal chamber, and is readily apparent in the histology sections). It is not uncommon for areas within a tissue section to have aberrations or artifacts of processing which preclude those areas from being used in the histopathology. Furthermore, occasional processing artifacts may render an entire tissue section, or both sections from a cornea, unacceptable for use in the histopathology. In general, the routine observation of these artifacts will not be reported.

Photomicrography for Representative Illustration of Corneal Changes

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Routinely five photomicrographs are prepared for each test article-treated group. A low power image of the cornea is used to measure corneal thickness and show the overall state of the cornea. Four high magnification micrographs (epithelium, stroma directly beneath the anterior limiting lamina, mid-depth of the stroma and the deep stroma/endothelium) are prepared. Images were captured using a Spot Digital Camera and Spot 4.0.8 software (Diagnostic Instruments, Inc., Sterling Heights, MI). The color balance of the images was corrected by the software to better represent the colors that would be seen through the microscope. Photomicrographs taken of the epithelium often overexpose the stroma. This leads to the impression that the stroma shows greater areas of stromal expansion. Stromal changes are better represented in micrographs where the stroma is the central figure of the image.

With the digital camera and associated software, it is possible to measure distances within the captured

image. This feature can be used to measure corneal stromal thickness directly. Such measurements require a true cross-section of the cornea (i.e., a perpendicular section relative to the corneal surface plane). In some cases, a true cross-section is not available. The Descemet's Membrane thickness is used to estimate how close the section is to a true cross section. The appearance of an unusually thick membrane suggests that the section was cut at a tangent to the true cross section (or from a more mature animal). If the measured corneal section did not appear to be a true cross section, the observation was noted. Even in a true cross section, the isolated cornea is not of uniform thickness but rather shows peaks and valleys. Therefore, an effort has been made to select "representative" cross-sections that are neither extreme "peaks" nor "valleys" for measurement of corneal thickness. The values obtained should be considered "representative" of the treatment group rather than strict quantitative measures. Many more measurements would be required to provide a quantitative comparison. The stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

Negative control (deionized water), 10-minute exposure, 120-minute post-exposure incubation (20 April 2022) (Figures 1-5)

Epithelium: The negative control-treated epithelium was composed of three layers (Figure 2). The basal cell layer was a well-formed, columnar-cell region directly attached to the basal lamina immediately anterior to the anterior limiting lamina. The basal cells were always tightly attached to each other. Several layers of wing cells covered the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei.

Stroma: The cross section of a negative control-treated cornea, showing the general thickness of the whole cornea and stroma is shown in Figure 1. The stromal elements showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining to narrow, elongated and condensed with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. Rare cells, with eosinophilic cytoplasmic staining, were observed. Collagen bundles were generally parallel and well ordered. The upper stroma immediately under the anterior limiting lamina is presented in Figure 3, and the mid stroma is presented in Figure 4.

Endothelium: The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well maintained as shown in Figure 5.

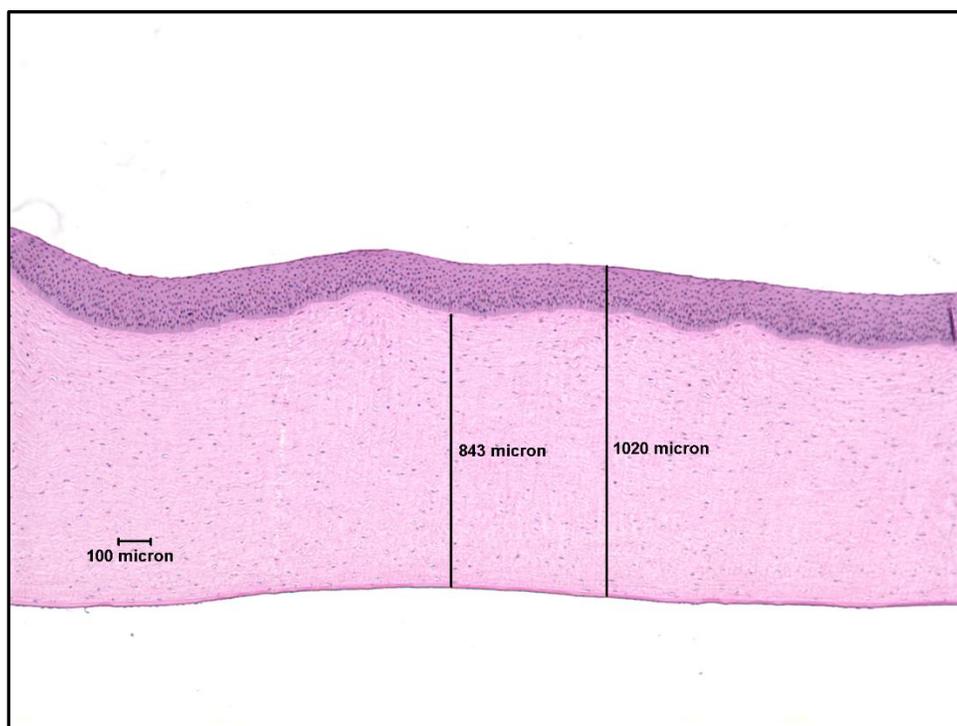


Figure 1 Negative Control – Full thickness



Figure 2 Negative Control – Epithelium

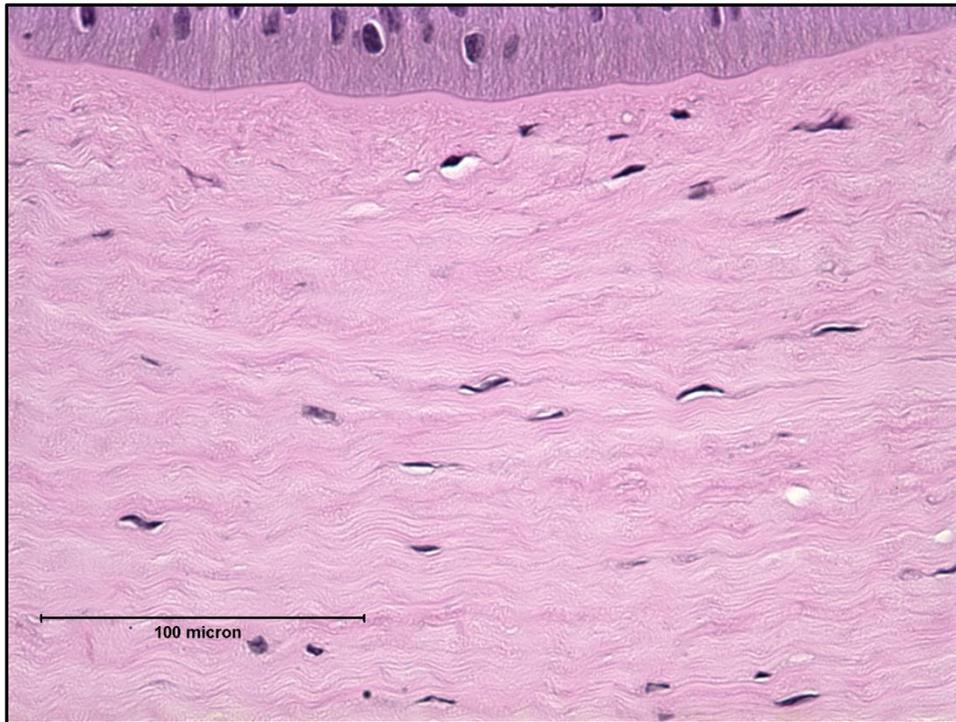


Figure 3 Negative Control – The stroma directly beneath the anterior limiting lamina

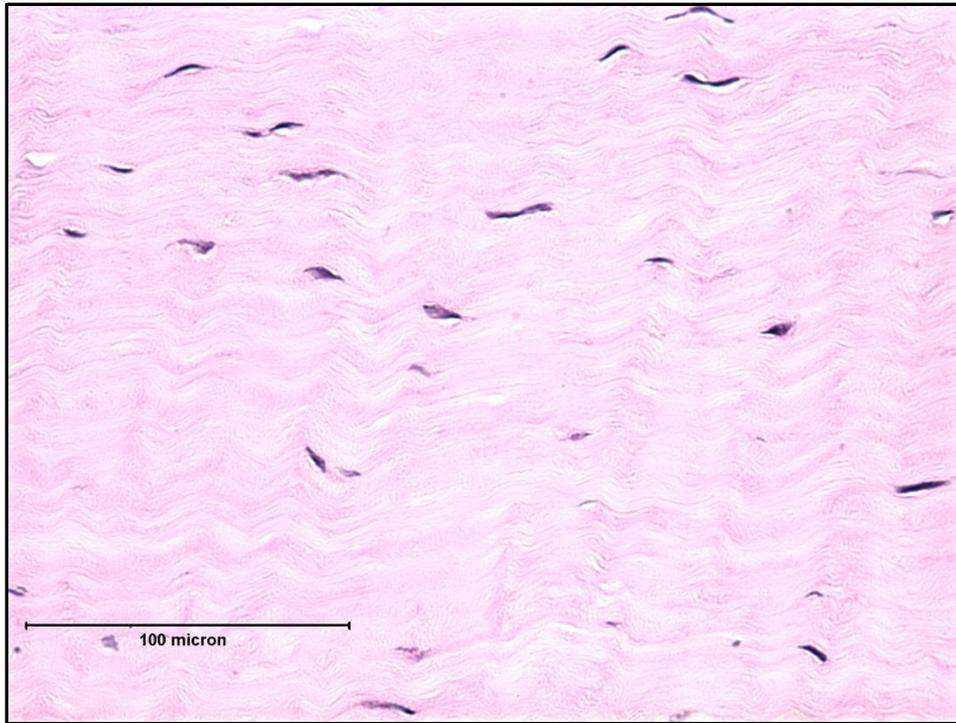


Figure 4 Negative Control – The stroma at mid-depth

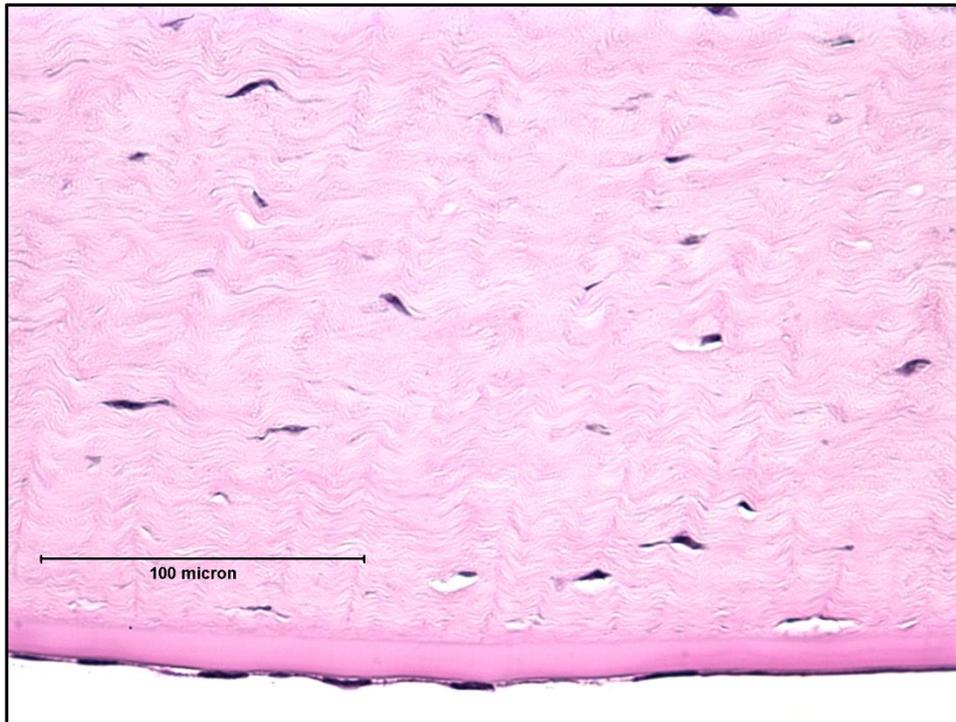


Figure 5 Negative Control – The lower stroma, Descemet's Membrane and endothelium

Positive control (100% Ethanol), 10-minute exposure, 120-minute post-exposure incubation (20 April 2022) (Figures 6-10)

Epithelium: The positive control corneas showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei. The loss of adhesion between the basal cells (cell to cell) and the basal lamina was marked. The epithelium was probably not viable at the time of fixation (Figure 7).

Stroma: Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 6). In the stroma directly below the anterior limiting lamina, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation (Figure 8). Below this zone, moderate/marked collagen matrix expansion extended past 50% depth. In the uppermost stroma, there was a decrease in the density of viable keratocytes as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization with a progression towards keratocytes showing condensed nuclei deeper into the stroma. In the stroma below mid depth, moderate stromal swelling and keratocyte nuclear enlargement/vacuolization were evident as shown in Figure 9.

Endothelium: The Descemet's Membrane was prominent. The endothelium was generally intact (similar to the negative control-treated corneas) (Figure 10).

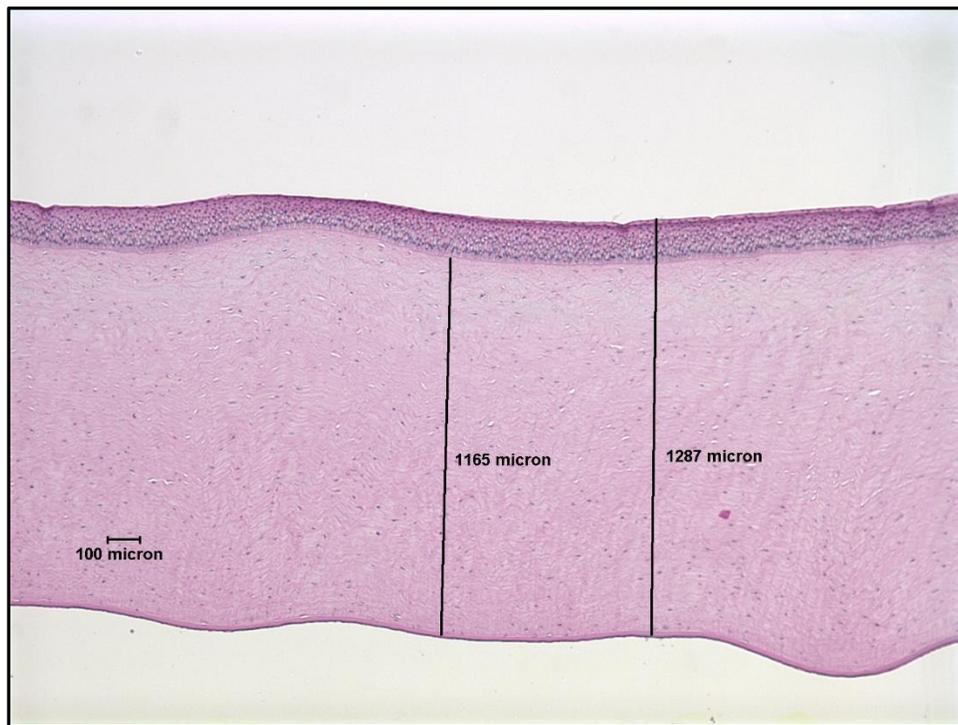


Figure 6 Positive Control – Full thickness



Figure 7 Positive Control – Epithelium

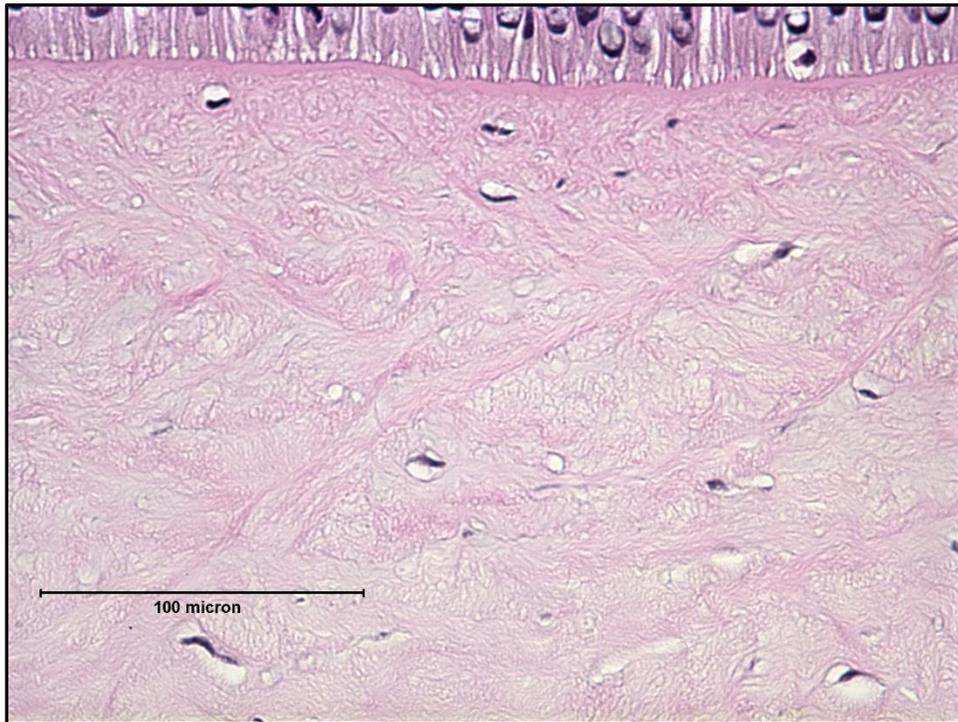


Figure 8 Positive Control – The stroma directly beneath the anterior limiting lamina

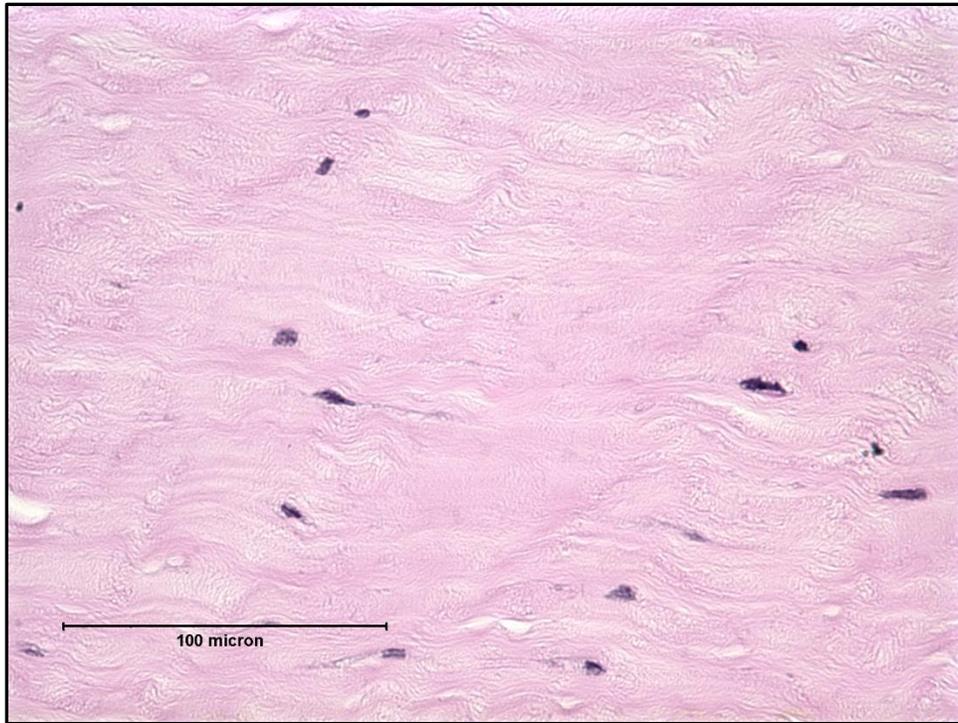


Figure 9 Positive Control – The stroma at mid-depth

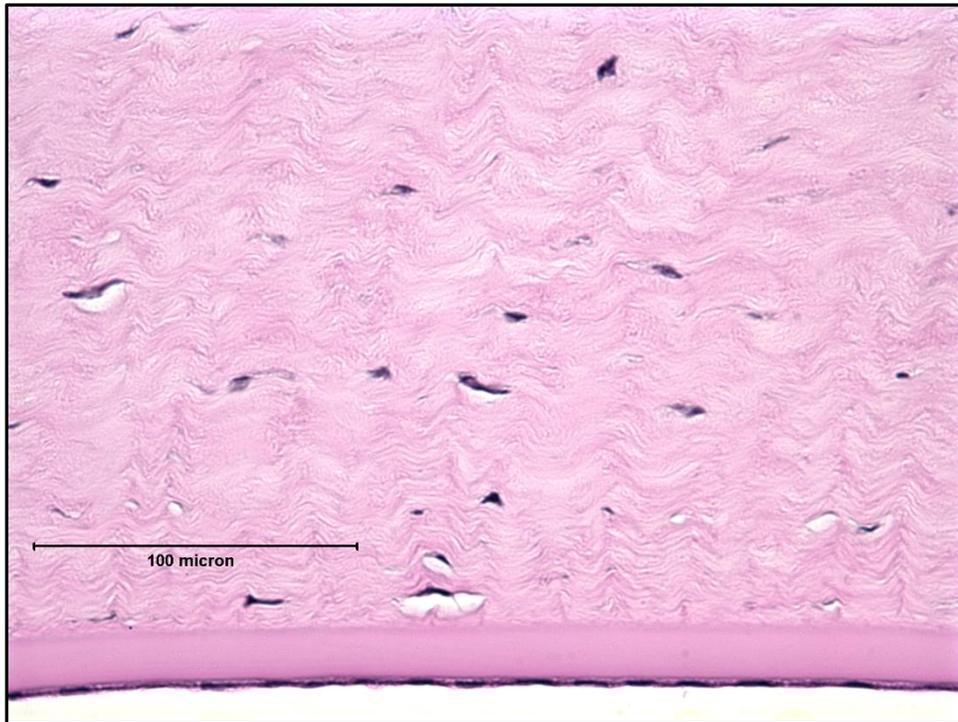


Figure 10 Positive Control – The lower stroma, Descemet's Membrane and endothelium

Formulation A, 10-minute exposure, 120-minute post-exposure incubation (10 May 2018)
(Figures 11-15)

Epithelium: The epithelium of corneas treated with Formulation A was similar to that of the negative control-treated corneas except for a slight increase in the presence of small vacuoles in the surface squamous epithelium. (Figure 12)

Stroma: The stroma was similar in thickness to that of the negative control treated corneas (Figure 11). The stroma directly beneath the anterior limiting lamina was similar to that of the negative control treated corneas (Figure 13). The stroma at mid-depth was similar to that of the negative control treated corneas (Figure 14). The lower stroma was similar to that of the negative control treated corneas.

Endothelium: The Descemet's Membrane was prominent. The endothelium was similar to that of the negative control treated corneas as shown in Figure 15.

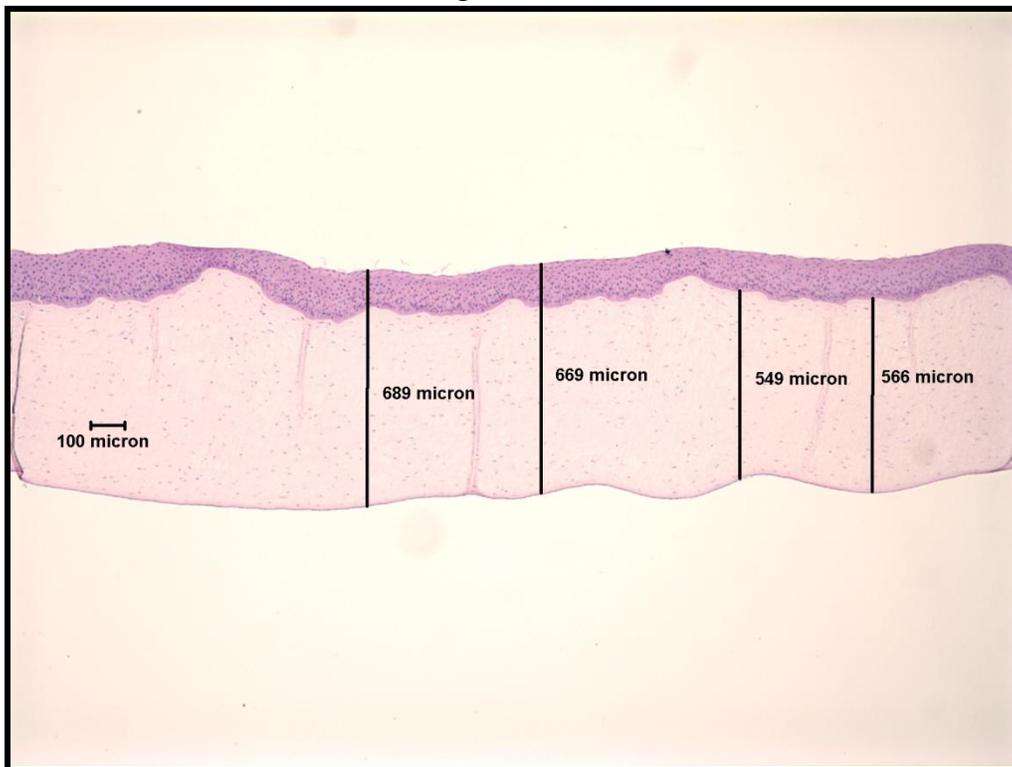


Figure 11 Formulation A - Full thickness



Figure 12 Formulation A – Epithelium

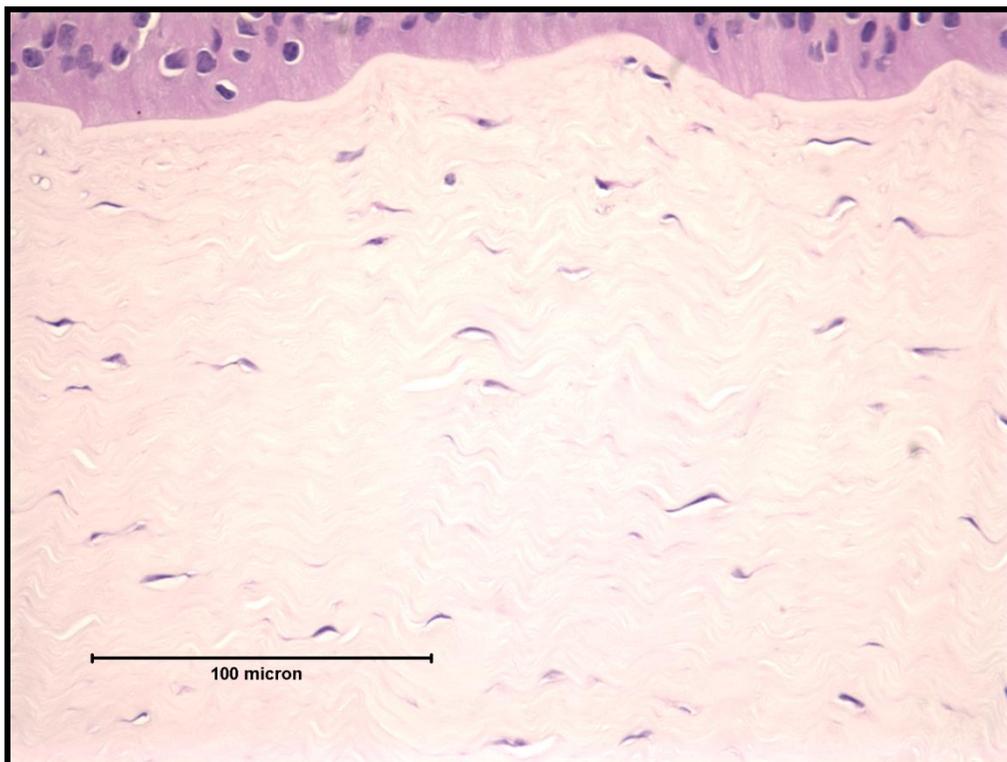


Figure 13 Formulation A - The stroma directly beneath the anterior limiting lamina

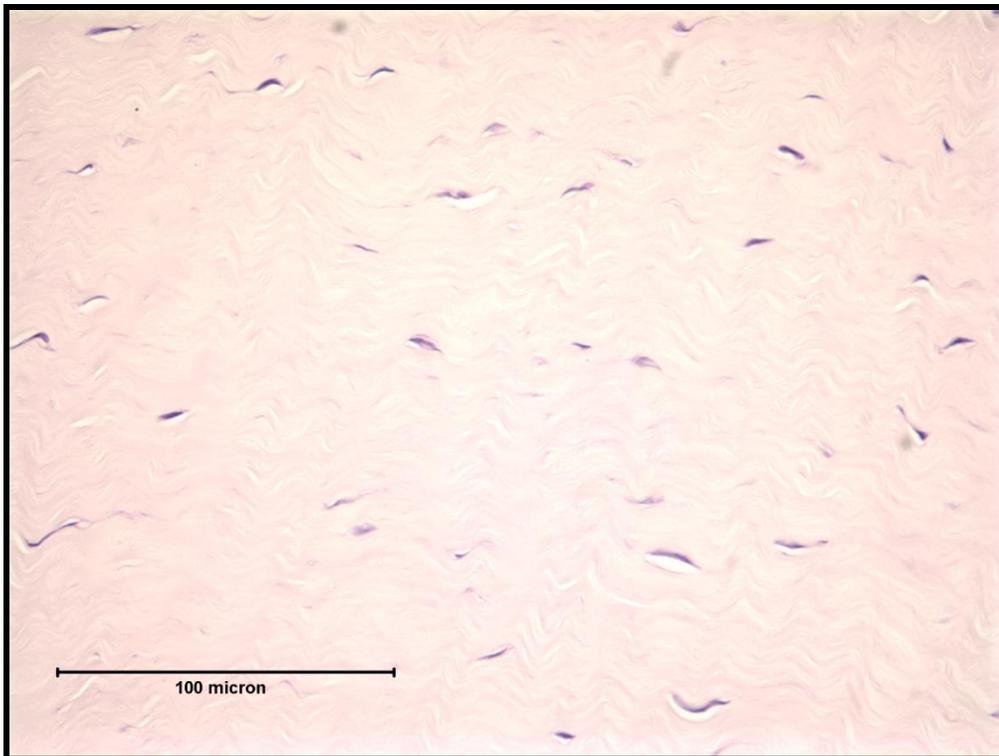


Figure 14 Formulation A - The stroma at mid-depth

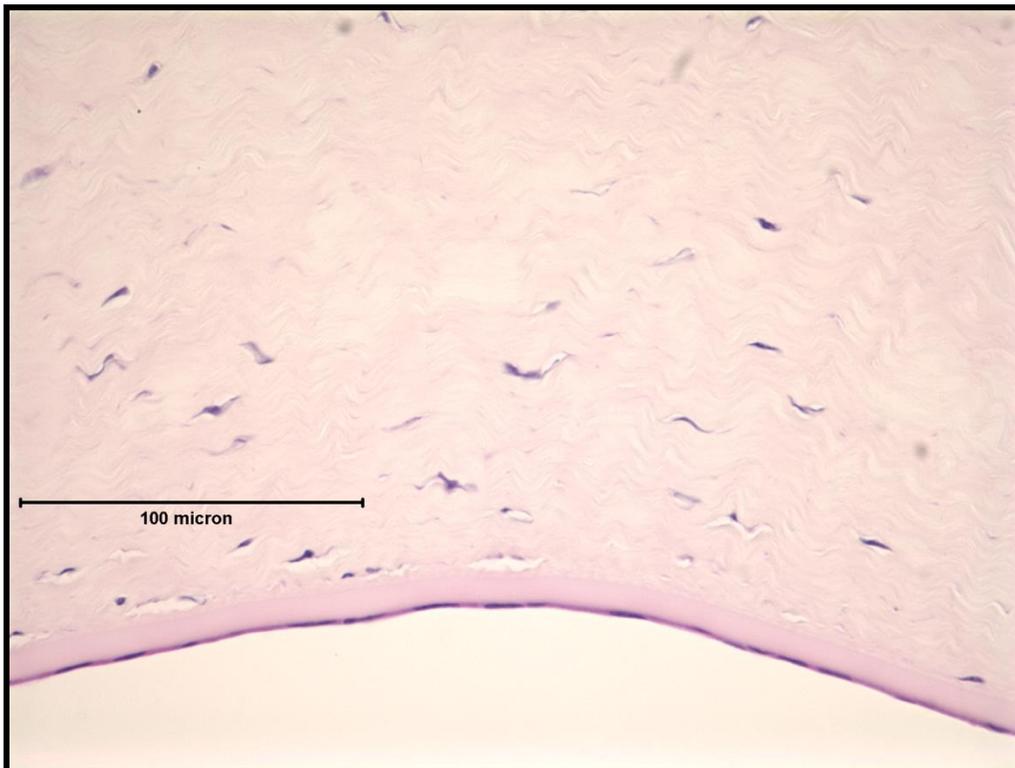


Figure 15 Formulation A - The lower stroma, Descemet's Membrane and endothelium

Formulation W, Neat, 10-minute exposure, 120-minute post-exposure incubation (20 April 2022)
(Figures 16-20)

Epithelium: The epithelium of corneas treated with Formulation W showed detachment of the squamous epithelium as a continuous sheet, suggesting coagulation of the layer. The upper wing cells showed a rounded morphology and vacuolated cytoplasm, but otherwise the epithelium appeared similar to the negative control-treated corneas (Figure 17).

Stroma: The stroma was similar in thickness to the negative control-treated corneas (Figure 16). The stroma directly beneath the anterior limiting lamina appeared similar to the negative control-treated corneas (Figure 18). The stroma at mid-depth appeared similar to the negative control-treated corneas (Figure 19). The lower stroma appeared similar to the negative control-treated corneas.

Endothelium: The Descemet's Membrane was prominent. The endothelium was generally intact (similar to the negative control-treated corneas) (Figure 20).

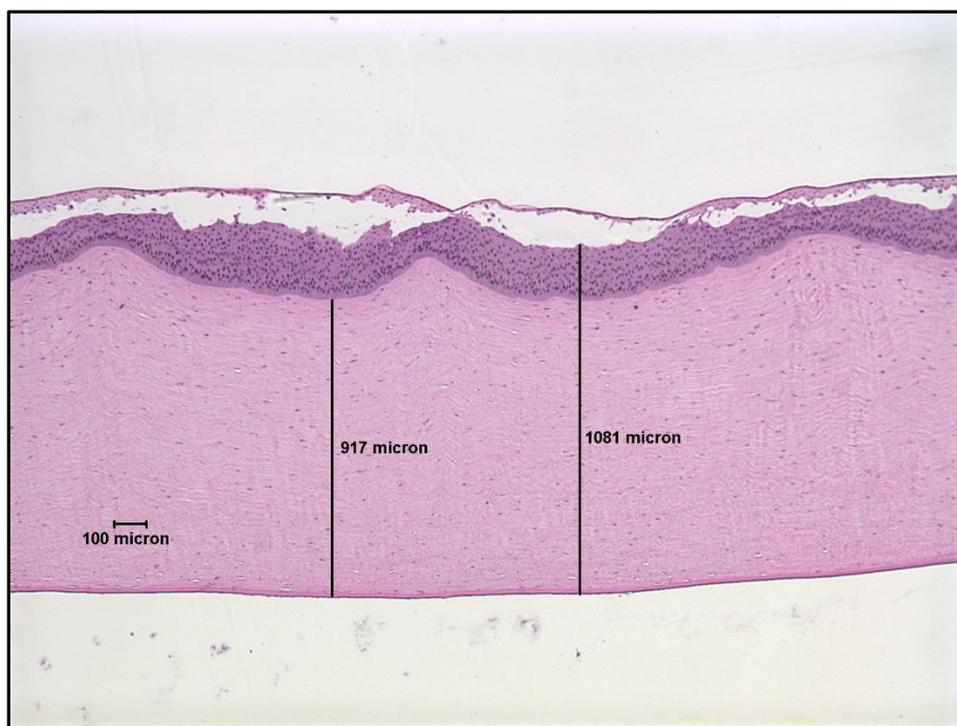


Figure 16 Formulation W - Full thickness



Figure 17 Formulation W – Epithelium

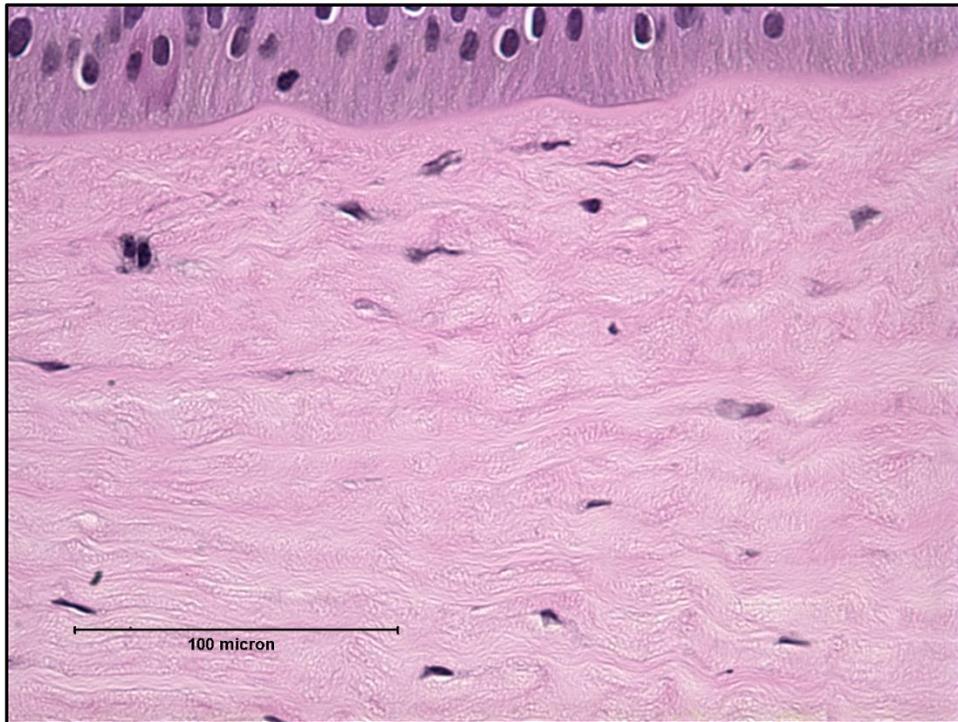


Figure 18 Formulation W - The stroma directly beneath the anterior limiting lamina

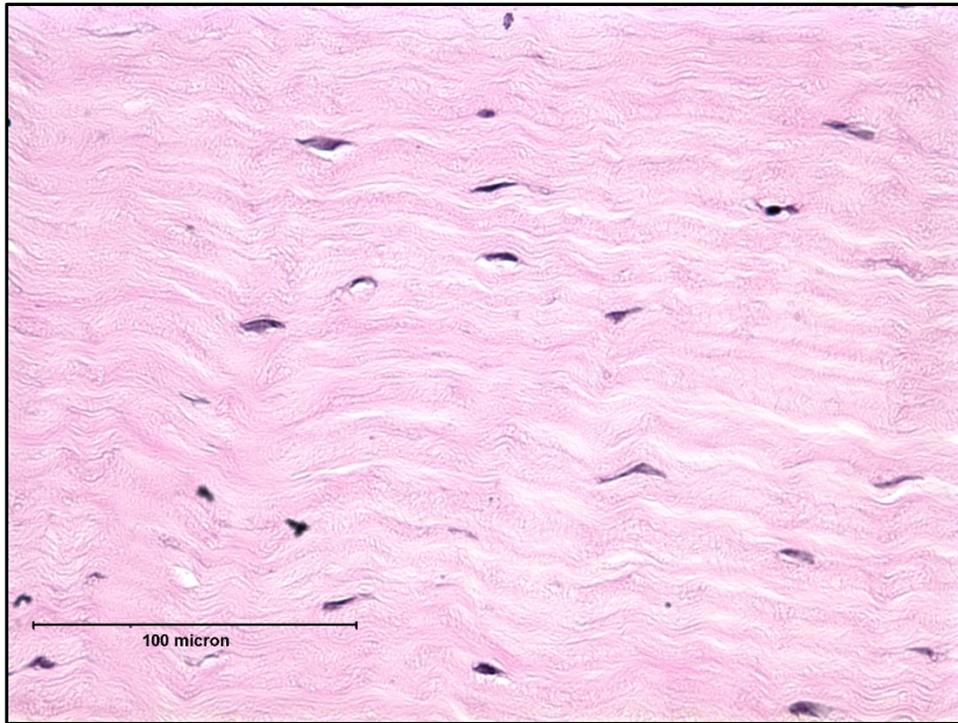


Figure 19 Formulation W - The stroma at mid-depth



Figure 20 Formulation W - The lower stroma, Descemet's Membrane and endothelium

Formulation X, Neat, 10-minute exposure, 120-minute post-exposure incubation (25 April 2022)
(Figures 21-25)

Epithelium: The epithelium of corneas treated with Formulation X showed loss of the squamous and upper wing cells, with occasional loss into the lower wing and basal cell layers. The cells appeared to be lost as individual cells, rather than as a tissue sheet. The remaining wing and basal cells immediately below the areas of loss appeared rounded and hypereosinophilic. In some fields of view where the basal layer was intact, the cells appeared similar to the negative controls while in others the basal cells showed abnormal cellular morphology, hypereosinophilic cytoplasmic staining and pyknotic nuclei (Figure 22).

Stroma: The stroma was thicker than the negative control-treated corneas (Figure 21). The stroma directly beneath the anterior limiting lamina showed mild to moderate collagen matrix expansion especially under those areas with deeper epithelial erosion, while the keratocytes appeared generally similar to those in the negative control-treated corneas (Figure 23). The stroma at mid-depth appeared similar to the negative control-treated corneas (Figure 24). The lower stroma appeared similar to the negative control-treated corneas.

Endothelium: The Descemet's Membrane was prominent. The endothelium was generally intact (similar to the negative control-treated corneas) (Figure 25).

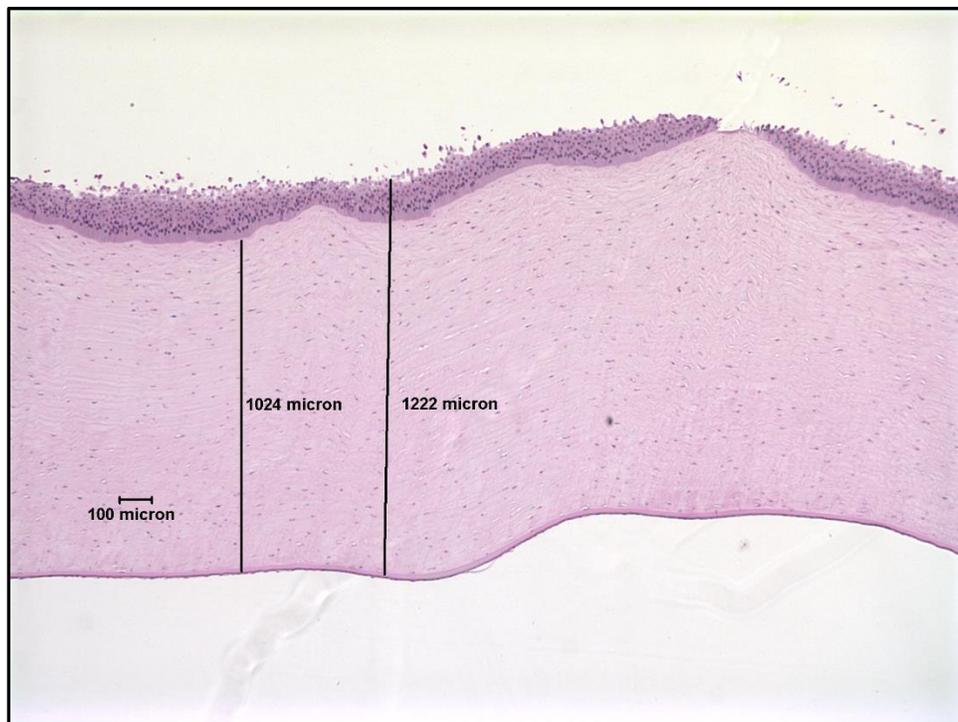


Figure 21 Formulation X - Full thickness



Figure 22 Formulation X – Epithelium

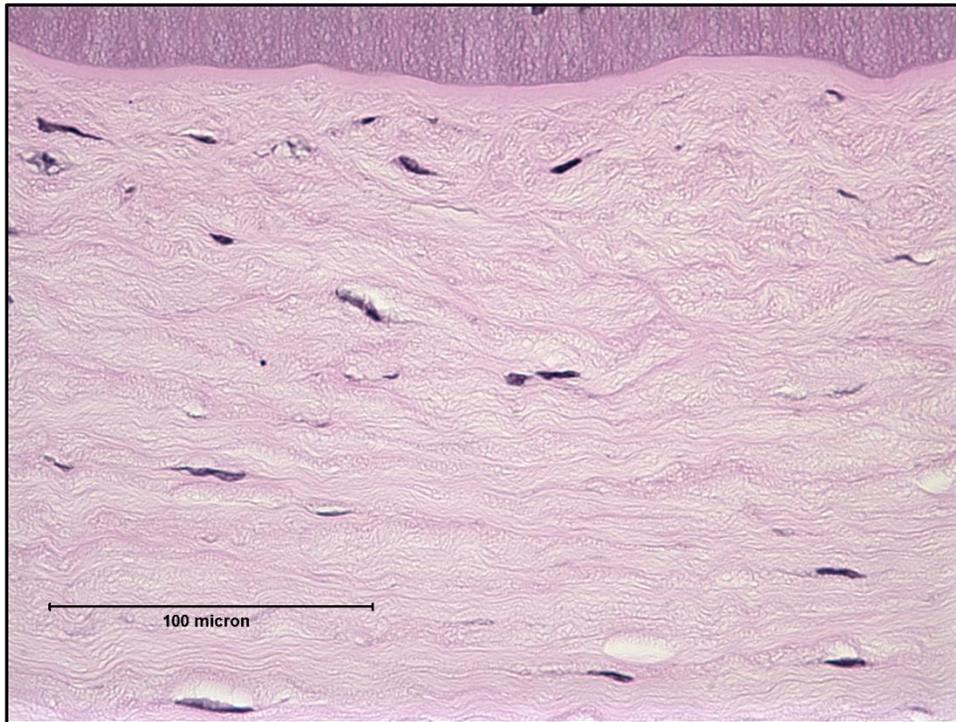


Figure 23 Formulation X - The stroma directly beneath the anterior limiting lamina

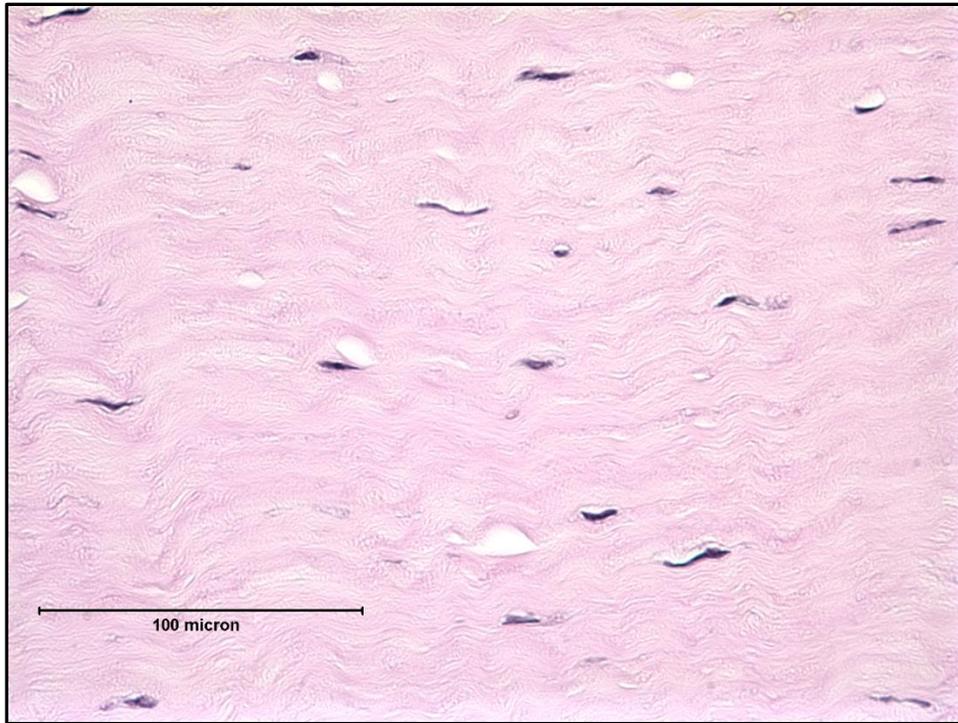


Figure 24 Formulation X - The stroma at mid-depth

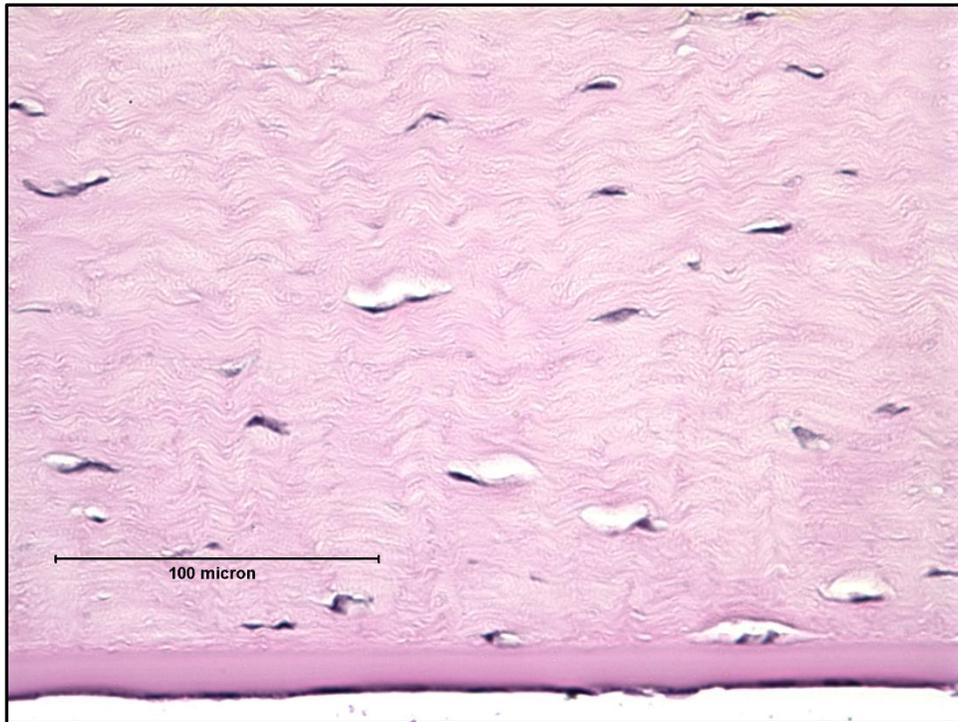


Figure 25 Formulation X - The lower stroma, Descemet's Membrane and endothelium

Formulation D, Neat, 10-minute exposure, 120-minute post-exposure incubation (10 May 2018)
(Figures 26-32)

Epithelium: The epithelium of corneas treated with Formulation D showed extensive to complete disruption of the corneal epithelium which is inconsistent with the more limited fluorescein passage values. This is particularly true of cornea 14 which showed a net optical density value of 0.401 while cornea 17 showed the highest value (0.911). The epithelium remaining on cornea 14 is shown (Figure 28).

Stroma: The stroma was slightly (cornea 14) to appreciably (cornea 17) thicker than the negative control treated corneas (Figures 26 and 27). The stroma directly beneath the anterior limiting lamina showed marked stromal swelling and loss of viable keratocytes (nuclear condensation and cytoplasmic eosinophilia (Figures 29 and 30). The stroma at mid-depth showed moderate collagen matrix swelling and some keratocyte changes (Figure 31). The deeper stroma showed slight collagen matrix swelling suggesting that the loss of endothelial barrier integrity occurred before fixation.

Endothelium: The Descemet's Membrane was prominent. The endothelium was damaged in many fields. This pattern of deep stromal swelling and endothelial cell damage is consistent with a full thickness injury to the treated corneas. The damaged endothelium is shown in Figure 32.

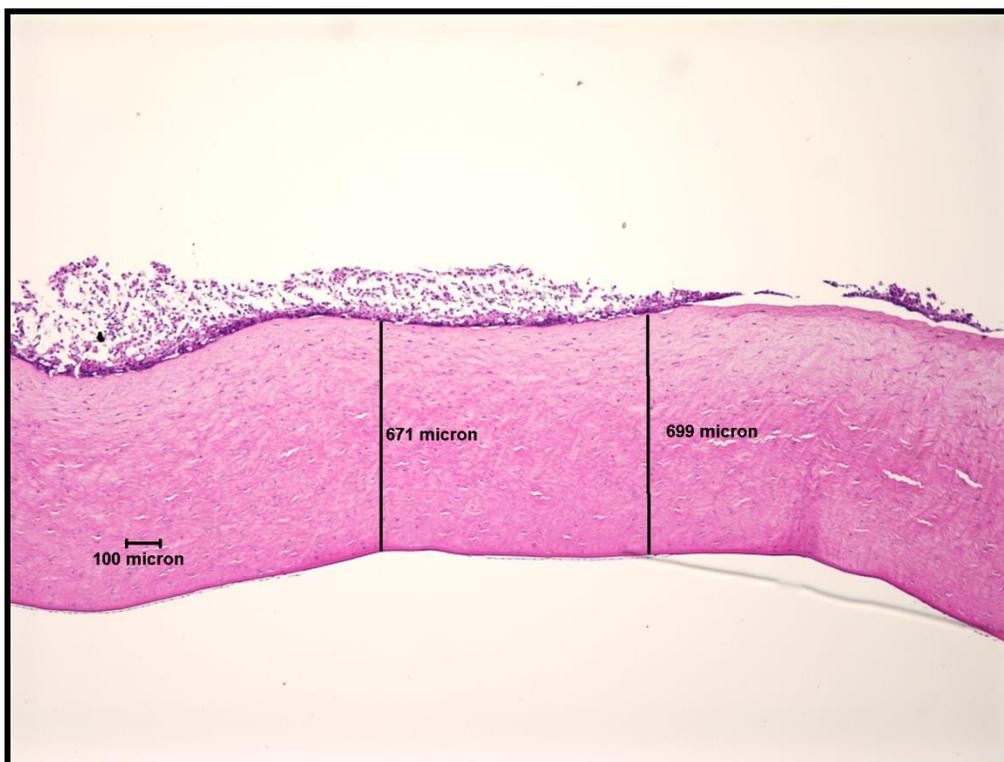


Figure 26 Formulation D - Full thickness (Cornea 14)



Figure 27 Formulation D - Full thickness (Cornea 17)

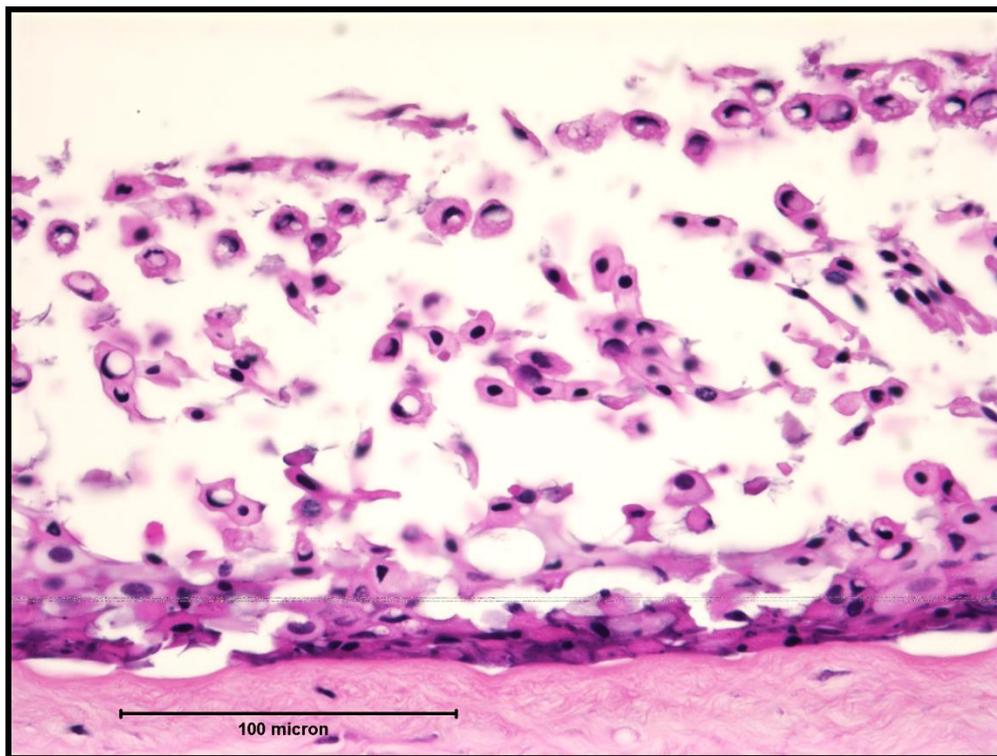


Figure 28 Formulation D - Epithelium (cornea 14)

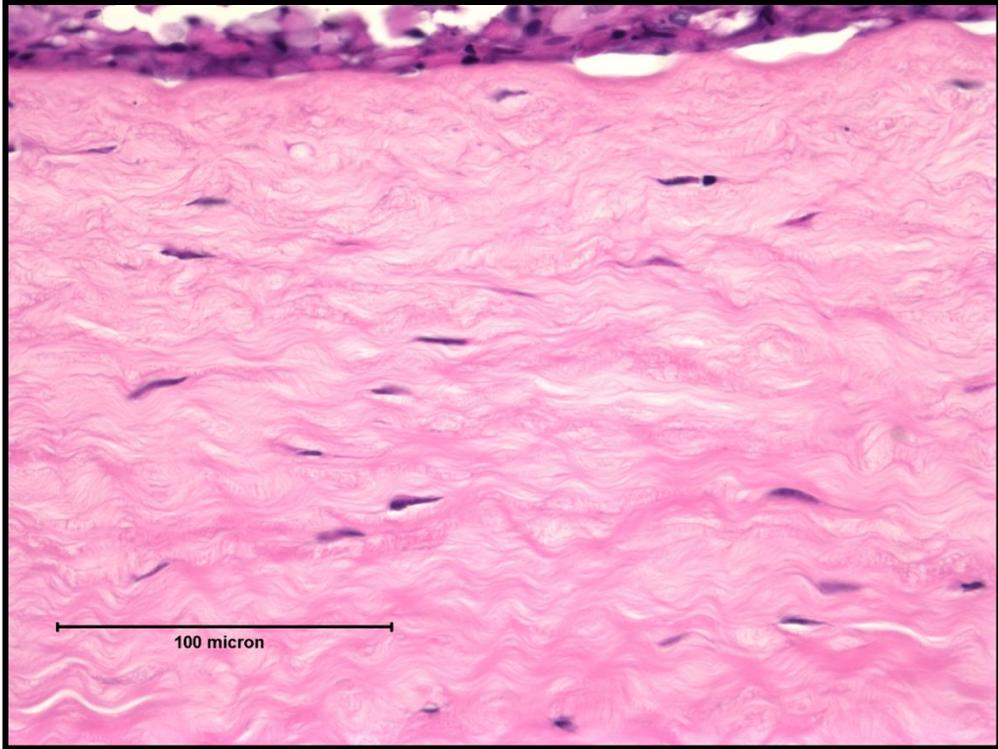


Figure 29 Formulation D - The stroma directly beneath the anterior limiting lamina (cornea 14)



Figure 30 Formulation D - The stroma directly beneath the anterior limiting lamina (cornea 17)

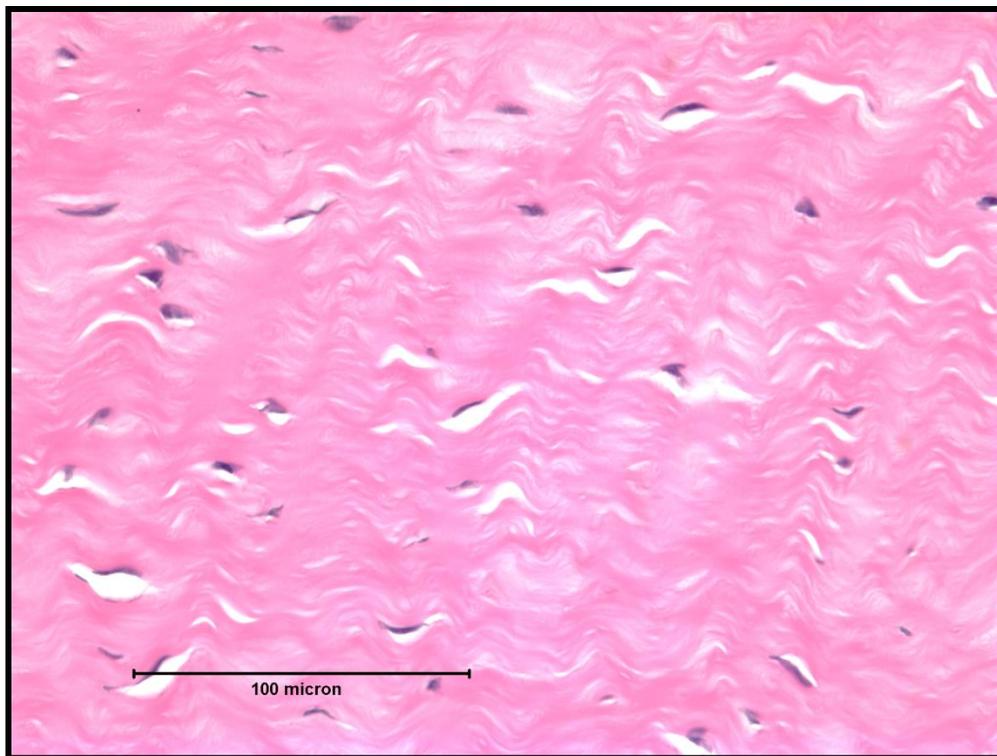


Figure 31 Formulation D - The stroma at mid-depth (cornea 14)



Figure 32 Formulation D - The lower stroma, Descemet's Membrane and endothelium (cornea 17)