

FINAL REPORT

Study Title

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Product Identity

X
Y

Author

Janet Luczak, M.G.A.
Study Director

Study Completion Date

March 6, 2007

Testing Facility

Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Laboratory Project Number

06AA45-AA46.350064

Laboratory Project ID

4315

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10 (d) (1) (A), (B), or (C).

Company:

Company Agent:

Date: _____

Signature: _____

Title

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study meets the requirement of 40 CFR § 160 with the following exceptions:

The identity, strength, purity, composition, and stability or other characteristics to define the test or control substances have not been determined by the testing facility. A certificate of analysis was not provided by the sponsor.

The stability of the test or control substances has not been determined by the testing facility under the test conditions and is not included in the final report.

Submitter:

Date: _____

Sponsor:

Date: _____

Study Director:

Janet Luczak, M.G.A.
Study Director

Date: _____

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QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 06AA45-06AA46.350064

Study Director: Janet Luczak, M.G.A.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. EPA GLP Standards (40 CFR 160) and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	07-Feb-06	07-Feb-06	07-Feb-06
Final Opacity Measurement	07-Feb-06	09-Feb-06	13-Feb-06
Draft Report and Data	29&30-Nov-06	30-Nov-06	11-Dec-06
Final Report	05-Mar-07	05-Mar-07	05-Mar-07

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

Date

STUDY PERSONNEL

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Study Director:

Janet Luczak, M.G.A.
Study Director

Date

Laboratory Personnel:

Principle Investigator:
(Histopathology)

Stewart B. Jacobson, DVM, DACVP
Charles River Laboratories, Pathology Associates
(PAI)-Maryland

Laboratory Supervisor:

Gregory Moyer, M.B.A.

Primary Technical Staff:

Nathan Wilt, B.S.
Allison Hilberer, B.S.
Valerie Deoudes, B.S.
Nicole Barnes, B.S.
Jennifer Nash, M.S.
Allyson Morrison, M.S.

STUDY REPORT

Study Title: Bovine Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Sponsor:

Test Facility: Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Test Substance Identification

IIVS Test/Reference Substance Number	Sponsor's Designation	Physical Description	Expiration Date	Receipt Date	Storage Conditions *
06AA45	X	clear colorless non-viscous liquid	NP**	1/24/06	room temperature
06AA46	Y	clear colorless non-viscous liquid	NP**	1/24/06	room temperature

* Protected from exposure to light

**Not Provided

Study Initiation Date: February 7, 2006

Experimental Start Date: February 7, 2006

Experimental End Date: February 23, 2006

Study Completion Date: March 6, 2007

Study Objective: To evaluate the potential ocular irritancy/toxicity of the test substance using the Bovine Corneal Opacity and Permeability Assay (BCOP)

Test Method: Bovine Corneal Opacity and Permeability Assay (BCOP)

Test System: Bovine Corneas

STUDY MATERIALS

Media and Reagents:	Minimum Essential Media (EMEM) without phenol red containing 1% FBS and 2mM L-glutamine (Complete MEM)
	Minimum Essential Media (EMEM) with phenol red containing 1% FBS and 2mM L-glutamine (Complete MEM) (used for rinsing only)
	Hanks' Balanced Salt Solution (HBSS) including Ca^{++} , Mg^{++} , and containing Pen/Strep
	Fluorescein (4 mg/mL)
	10% Formalin (Fixative)
Equipment:	Opacitometer Molecular Devices Vmax Kinetic Microplate Reader Corneal Holders 32°C Incubator Micropipettors(calibrated)

TEST METHOD

Preparation of Test Substance

As instructed by the Sponsor, each test substance was administered to the test system without dilution. The pH of the test substances was determined using pH paper (EMD Chemicals Inc.). Initially, each of the test substances was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each of the test substances was added to 7.5 to 14 pH paper with 0.5 pH unit increments to obtain a more precise pH value. The pH values obtained from the narrower range pH paper are recorded in Table 1.

Preparation of the Test System

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. Trueth & Sons, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at $32 \pm 1^\circ\text{C}$ for a minimum of 1 hour.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacimeter. Three corneas, whose initial opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test substance number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

The liquid test substances, X and Y, were tested neat. An aliquot of 750 μL of the test substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. Each treated cornea was completely covered with the test substance. One group of three corneas was incubated in the presence of the test substances at $32 \pm 1^\circ\text{C}$ for 3 minutes. A second group of three corneas was incubated in the presence of the test substances at $32 \pm 1^\circ\text{C}$ for 10 minutes. The positive and negative control substances were tested on three corneas at $32 \pm 1^\circ\text{C}$ for 10 minutes. After the 3 and 10-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test substance. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at $32 \pm 1^\circ\text{C}$. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to the chamber number. Aliquots of 360 μL from the numbered tubes were placed into their designated wells on a 96-well plate. The

optical density at 490 nm (OD_{490}) was determined using a Molecular Devices Vmax kinetic microplate reader. If the OD_{490} value of a control or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 μ L sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours.

The fixed corneas were transferred to Charles River Laboratories, Pathology Associates (PAI)-Maryland for embedding, sectioning and staining. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin block so that a section of each half could be cut and placed on a single slide. Each slide was then stained with hematoxylin and eosin. Slides were provided to the pathologist for evaluation.

PROTOCOL CHANGES

Protocol Amendments: Protocol Amendment I and II (see Appendix A)

CONTROLS

Positive Control: Ethanol (Pharmaco, 200 proof, USP) (tested without dilution)

Negative Control: Sterile, Deionized Water (Quality Biological)

STUDY ACCEPTANCE CRITERIA

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that was within two standard deviations of the historical mean.

DATA ANALYSIS

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD₄₉₀ for the blank wells was calculated. The mean blank OD₄₉₀ was then subtracted from the raw OD₄₉₀ of each well (corrected OD₄₉₀). Any dilutions that were made to bring the OD₄₉₀ readings into the linear range of the platereader (OD₄₉₀ should be less than 1.500) had each diluted OD₄₉₀ reading multiplied by the dilution factor. The final corrected OD₄₉₀ of each treatment group and the positive control was then calculated by subtracting the average corrected OD₄₉₀ of the negative control corneas from the corrected OD₄₉₀ value of each treated cornea:

Final Corrected OD₄₉₀ = (raw OD₄₉₀ – mean blank OD₄₉₀) – average corrected negative control OD₄₉₀

The mean OD₄₉₀ value of each treatment group was calculated by averaging the final corrected OD₄₉₀ values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD₄₉₀ Value)

STUDY RETENTION

Upon completion of the final report, all raw data, copies of the reports, slides and tissue blocks will be maintained by the Institute for In Vitro Sciences, Inc.

STUDY RESULTS AND DISCUSSION

Opacity and Permeability Results

Table 1 summarizes the opacity, permeability, and *in vitro* score for the test substance or reference substance at each exposure time. Table 2 summarizes the opacity, permeability, and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 39.9 to 64.5), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test/Reference Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score	pH
2/7/06	06AA45	X	Neat	3 minutes	4.3	2.494	41.7	12.0
				10 minutes	6.7	5.016	81.9	
	06AA46	Y	Neat	3 minutes	32.7	2.152	65.0	12.5
				10 minutes	32.3	2.836	74.9	

Table 2
BCOP Results of the Positive Control

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score
2/7/06	Ethanol	10 minutes	29.3	1.120	46.1

Histological Evaluation

The negative control treated corneal epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. 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. Asymmetric clear spaces (“nuclear halos”) were occasionally observed around nuclei in the wing and basal cell layers. These were an artifact of tissue processing and unrelated to treatment. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei. The stromal elements in the negative control treated corneas 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. Rarely, cells with eosinophilic cytoplasmic staining were observed. Collagen bundles were generally parallel and well ordered. The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well maintained.

The positive control treated 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. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas. In the stroma directly below Bowman's Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate/marked collagen matrix expansion extended past 50% depth. In the upper 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. In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia. The endothelial cells were generally intact (similar to negative control treated corneas).

Corneas treated with the test substance, X, for three minutes exhibited diffuse loss of the squamous and wing layers of the epithelium. Fragmentary remnants of the basal layer were necrotic and not viable. The stroma was similar in thickness to the positive controls. There was marked expansion of the upper 50% of the stroma and minimal to mild expansion of the lower stroma (full thickness damage). There were moderate increases in keratocytes with pyknotic nuclei in the upper 50% of the stroma and in keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma. The endothelium was generally intact. Multifocally, there were endothelial cells with enlarged nuclei and vacuolated cytoplasm.

Corneas treated with the test substance, X, for ten minutes showed a complete loss of the epithelium and the superficial stroma and the exposed stroma was uniformly blanched (unstained). The stroma was similar in thickness to the positive controls. There was marked expansion of the upper 50% of the stroma and mild to moderate expansion of the lower stroma (full thickness damage). There was a marked increase in keratocytes with pyknotic nuclei in the upper half of the stroma. There were scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma. The endothelium was generally intact. Diffusely, endothelial cells had enlarged nuclei and vacuolated cytoplasm.

Corneas treated with the test substance, Y, for three minutes showed diffuse loss of the squamous and upper wing layers and uniform blanching of the exposed surface epithelium. The

remaining epithelium was hypereosinophilic (coagulation necrosis) with granular nuclear chromatin and brightly eosinophilic nucleoli. The epithelium was not viable. The stroma was similar in thickness to the positive controls. There was marked expansion of the upper 50% of the stroma and mild expansion of the lower stroma (full thickness damage). There was a moderate increase in keratocytes with pyknotic nuclei in the upper half of the stroma. There were scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma. The endothelium was generally intact. Diffusely, endothelial cells had enlarged nuclei and vacuolated cytoplasm.

Corneas treated with the test substance, Y, for 10 minutes exhibited diffuse loss of the squamous and wing layers and uniform blanching of the exposed surface epithelium. The remaining epithelium was hypereosinophilic (coagulation necrosis) with granular nuclear chromatin and brightly eosinophilic nucleoli. The epithelium was not viable. The stroma was similar in thickness to the positive controls. There was marked expansion of the upper 50% of the stroma and mild to moderate expansion of the lower stroma. Throughout the stroma there was a moderate increase in keratocytes with pyknotic nuclei and scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm. The endothelium was generally intact. Diffusely, endothelial cells had enlarged nuclei and vacuolated cytoplasm.

Histopathological evaluation of the corneas for this study was performed at Charles River Laboratories, Pathology Associates (PAI)-Maryland. The report detailing the findings can be found in Appendix C.

STUDY CONCLUSION

The following classification system was established by Sina et al.¹ based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials.

In Vitro Score:

from 0 to 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

The *in vitro* scores for the test substance, X, were 41.7 (3 minute exposure) and 81.9 (10 minute exposure), primarily resulting from extreme increases in fluorescein permeability. Based on the classification established by Sina *et al* (1995), the test material would be classified as a severe irritant. This classification is supported by the histopathological evaluation. The depth and degree of injury extended through the full thickness of the cornea. Histological evaluation showed complete loss of the epithelium in the 10 minute exposure as well as expansion throughout the stroma. These observations are typically associated with the high fluorescein permeability values and are evidence of loss of corneal barrier function. Additionally, there was a moderate increase in keratocytes with pyknotic nuclei in the upper 50% of the stroma and in keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma. The endothelium exhibited cells with enlarged nuclei and vacuolated cytoplasm.

The *in vitro* scores for the test substance, Y, were 65.0 (3 minute exposure) and 74.9 (10 minute exposure), resulting from moderate increases in both opacity and fluorescein permeability. Based on the classification established by Sina *et al*(1995), the test material would be classified as a severe irritant. This classification is supported by the histopathological evaluation. The depth and degree of injury extended through the full thickness of the cornea. Histological evaluation showed a diffuse loss of the squamous and wing layers and uniform blanching of the exposed surface epithelium. These observations are typically associated with the high fluorescein permeability values and are evidence of loss of corneal barrier function. The remaining epithelium was hypereosinophilic (coagulation necrosis) with granular nuclear chromatin and brightly eosinophilic nucleoli. The epithelium was not viable. Coagulation of the epithelium is typically associated with notable increases in opacity. There was marked expansion of the upper 50% of the stroma and mild to moderate expansion of the lower stroma (full thickness damage). Throughout the stroma there was a moderate increase in keratocytes with pyknotic nuclei and scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm. The endothelium exhibited cells with enlarged nuclei and vacuolated cytoplasm.

¹Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

REPORT SUBMITTED BY:

Study Director

Date

APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
06AA45	14	2	4	2	2.7		
Neat	15	3	10	7	7.7		
3 minutes	17	3	5	2	2.7	4.3	2.9
06AA45	19	4	11	7	7.7		
Neat	21	4	12	8	8.7		
10 minutes	22	3	6	3	3.7	6.7	2.6
06AA46	24	4	42	38	38.7		
Neat	25	5	35	30	30.7		
3 minutes	26	5	33	28	28.7	32.7	5.3
06AA46	28	3	38	35	35.7		
Neat	29	4	33	29	29.7		
10 minutes	33	4	35	31	31.7	32.3	3.1
Neg. Control	1	5	4	-1	NA		
Sterile, DI water	4	5	5	0	NA		
10 minutes	8	5	4	-1	NA	-0.7	
Pos. Control	10	5	30	25	25.7		
Ethanol	11	4	40	36	36.7		
10 minutes	12	4	29	25	25.7	29.3	6.4
	*16	4					
	*20	3					
	*23	7					
	*31	6					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
 NA - Not Applicable

Performed on February 7, 2006

Study No. 06AA45-AA46.350064

PERMEABILITY SCORE

**Neg. Control
 Sterile, DI water
 10 minutes**

Cornea #	OD490
1	0.002
4	0.006
8	0.000

Avg.	0.003

**06AA45
 Neat
 3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
14	0.519	5	2.592
15	0.430	5	2.147
17	0.549	5	2.742

Avg. =			2.494
STDEV =			0.309

**06AA46
 Neat
 3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
24	0.434	5	2.167
25	0.437	5	2.182
26	0.422	5	2.107

Avg. =			2.152
STDEV =			0.040

**Pos. Control
 Ethanol
 10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
10	1.223	1	1.220
11	0.845	1	0.842
12	1.301	1	1.298

Avg. =			1.120
STDEV =			0.244

**06AA45
 Neat
 10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
19	0.955	5	4.772
21	1.042	5	5.207
22	1.014	5	5.067

Avg. =			5.016
STDEV =			0.222

**06AA46
 Neat
 10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
28	0.523	5	2.612
29	0.700	5	3.497
33	0.480	5	2.397

Avg. =			2.836
STDEV =			0.583

Performed on February 7, 2006

Study No. 06AA45-AA46.350064

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
06AA45	Neat	3 minutes	4.3	2.494	41.7
06AA45	Neat	10 minutes	6.7	5.016	81.9
06AA46	Neat	3 minutes	32.7	2.152	65.0
06AA46	Neat	10 minutes	32.3	2.836	74.9
Ethanol	Neat	10 minutes	29.3	1.120	46.1

APPENDIX C

FINAL HISTOPATHOLOGY REPORT

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Testing Facility Study Number 06AA45-AA46.350064

HISTOLOGY SITE:

Charles River Laboratories,
Pathology Associates (PAI) - Maryland
15 Worman's Mill Court, Suite I
Frederick, MD 21701

TESTING FACILITY:

Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

SPONSOR:

February 23, 2007

Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 06AA45-AA46.350064

QUALITY ASSURANCE STATEMENT

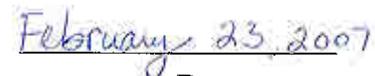
This histopathology project has been inspected and audited by the PAI Quality Assurance Unit (QAU) as required by the Good Laboratory Practice (GLP) regulations promulgated by the U.S. Environmental Protection Agency (EPA). The histopathology report is an accurate reflection of the recorded data. The following table is a record of the inspections/audits performed and reported by the QAU.

<u>Date of Inspection</u>	<u>Phase Inspected</u>	<u>Date Findings Reported to Study Pathologist/PAI Management</u>	<u>Date Findings Reported to Study Director/Study Director Management</u>
11/10/06	Individual Animal Data	11/10/06	11/10/06
11/10/06	Draft Histopathology Report	11/10/06	11/10/06
02/23/07	Final Histopathology Report	02/23/07	02/23/07



Laura Henry

Associate Quality Assurance Auditor



Date

1.0 Introduction

1.1 Overview

This report presents the pathology results of bovine corneas exposed to test and control substances for Institute for In Vitro Sciences, Inc (IIVS) Study Number 06AA45-AA46.350064. All test procedures and tissue harvests were performed at IIVS under the direction of Janet Luczak, M.G.A., Study Director. Histopathology was completed at Charles River Laboratories, Pathology Associates (PAI) - Maryland by Stewart B. Jacobson, DVM, DACVP.

1.2 Objective

The objective of this study was to evaluate the potential ocular irritancy/toxicity of the test article as measured by test article-induced changes in opacity, permeability to fluorescein, and tissue architecture in isolated bovine corneas. This report addresses the histopathology portion of the bovine corneal opacity and permeability (BCOP) assay.

2.0 Methods

2.1 Compliance Statement

The portion of this study performed by PAI was conducted under the U.S. EPA's FIFRA Good Laboratory Practice (GLP) regulations. Although digital photomicrographs are included as part of this report, the diagnoses and findings contained herein were obtained using the original histologic preparations, not the images. An electronic copy of this report (PDF format) is included. It is a representation of the pathology report hard copy; however, only the signed hard copy of the pathology report is considered raw data.

2.2 Archives

All pathology data and materials are maintained at Charles River Laboratories, Pathology Associates (PAI) – Maryland during the conduct of the histology portion of the study. Within three months of the issuance of the draft pathology report, or upon completion of the final pathology report, all data and materials generated by Charles River Laboratories, Pathology Associates (PAI) - Maryland, will have been shipped or delivered to the Institute for In Vitro Sciences, Inc. for archiving with the study.

2.3 Tissue Collection

According to the protocol, following treatment, corneas were placed in pre-labeled cassettes in 10% neutral buffered formalin fixative. After 24 hours, the fixed tissues were transferred to PAI for slide preparation. Tissues were processed, bisected, embedded in paraffin, and cut to 5 microns. Slides were stained with hematoxylin and eosin (H&E).

2.4 Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen 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 1 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*¹. 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 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 5). 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*. Much of the available data come from studies on surfactants and show that loss of the bovine corneal epithelium is predictive of some corneal opacity in the rabbit.

Special effort has been made to detect changes in the stromal elements of the corneas. Jester², Maurer^{3,4} 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. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deep injury to the stroma has more serious consequences. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling within the stromal collagen matrix and loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected by the presence of vacuole-like “holes” in the organized collagen matrix. Their appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of this expansion may be seen in Figure 7 where the positive control exposure has induced some stromal swelling. The depth and degree of 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

¹ 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.

² 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.

³ Maurer, JK and Parker, RD. (1996) Light microscopic comparison of surfactant-induced eye irritation in rabbits and rats at three hours and recovery/day 35. **Toxicologic Pathology** 24(4):403-411.

⁴ 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.

(medium) to enter the stroma and produce the collagen matrix expansion (swelling). Viable epithelium or endothelium will not only retard 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 beginning 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 eosinophilia. Harbell and Curren⁵ have reported that mechanical removal of the corneal epithelium leads to marked stromal swelling (marked collagen matrix vacuolization). The keratocytes in the zone of marked swelling undergo changes as well. The nuclei become enlarged and the cytoplasmic staining is very eosinophilic. Thus, when test article-treated corneas show only this type of keratocyte change (with stromal swelling), the change may be the result of the stromal swelling rather than direct action of the test article on the cells.

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

⁵ Harbell, J and Curren, R (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. ALTEX 22 (Special Issue):236.

under Bowman's Layer 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 Bowman's Layer. 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 (Bowman's Layer) 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 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).

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Where little or no damage to the stroma was detected, the area directly under Bowman's Layer was photographed since that is where one would expect to see damage if it had occurred. Images were captured using a Micropublisher 5.0 Digital Camera connected to an Olympus BX45 microscope and processed using the Image-Pro Plus 5.1 image analysis software.

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. In some cases, the treated corneas will have lost most of their epithelium or it has been compromised (i.e., ethanol treatment). In those cases, the stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

2.5 Histopathologic Results

The negative control corneas were treated for 10 minutes with sterile, deionized water (slides B9445-B9447). The negative control-treated epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. 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. Asymmetric clear spaces ("nuclear halos") were occasionally observed around nuclei in the wing and basal cell layers. These were an artifact of tissue processing and unrelated

to treatment. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figure 2).

The stromal elements in the negative control corneas 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. Rarely cells with eosinophilic cytoplasmic staining were observed. Collagen bundles were generally parallel and well-ordered (Stroma just under Bowman's Layer, Figure 3).

The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well-maintained.

The cross section of a negative control-treated cornea, showing the general thickness of the whole cornea and stroma, is shown in Figure 4.

The positive control corneas (slides B9448-B9450), treated for 10 minutes with 100% ethanol, showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei (Figure 5). 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. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 6). In the stroma directly below Bowman's Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate/marked collagen matrix expansion extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 7) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization (Figure 8). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 9). The endothelial cells were generally intact (similar to the negative control-treated corneas).

Table 1 presents the results for the test article evaluated.

Table 1
Histological Observations of the Test Article-Treated Corneas

IIVS Number	Sponsor's Designation	Exposure Time (minutes)/ Post-Exposure Incubation (minutes)	Observations	Figure #
06AA45 Slides B9451- B9453	X	3/120	<p>All corneas were similar histopathologically.</p> <p>Epithelium: There was diffuse loss of the squamous and wing layers of the epithelium. Fragmentary remnants of the basal layer were necrotic and not viable (Figure 10).</p> <p>Stroma: The stroma was similar thickness to positive controls (Figure 11). There was marked expansion of the upper 50% of the stroma (Figure 12) and minimal to mild expansion of the lower stroma (full thickness damage). There were moderate increases in keratocytes with pyknotic nuclei in the upper 50% of the stroma (Figure 12) and in keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma (Figure 13).</p> <p>Endothelium: The endothelium was generally intact. Multifocally, there were endothelial cells with enlarged nuclei and vacuolated cytoplasm.</p>	10-13
06AA45 Slides B9454- B9456	X	10/120	<p>All corneas were similar histopathologically.</p> <p>Epithelium: There was complete loss of the epithelium and the superficial stroma (Figure 14). The exposed stroma was uniformly blanched (unstained).</p> <p>Stroma: The stroma was similar thickness to positive controls (Figure 15). There was marked expansion of the upper 50% of the stroma (Figure 16) and mild to moderate expansion of</p>	14-17

IIVS Number	Sponsor's Designation	Exposure Time (minutes)/ Post-Exposure Incubation (minutes)	Observations	Figure #
			<p>the lower stroma (full thickness damage). There was a marked increase in keratocytes with pyknotic nuclei in the upper half of the stroma (Figure 16). There were scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma</p> <p>Endothelium: The endothelium was generally intact. Diffusely, endothelial cells had enlarged nuclei and vacuolated cytoplasm. (Figure 17).</p>	
06AA46 Slides B9457- B9459	Y	3/120	<p>All corneas were similar histopathologically.</p> <p>Epithelium: There was diffuse loss of the squamous and upper wing layers and uniform blanching of the exposed surface epithelium (Figure 18). The remaining epithelium was hypereosinophilic (coagulation necrosis), with granular nuclear chromatin, and brightly eosinophilic nucleoli. The epithelium was not viable.</p> <p>Stroma: The stroma was similar thickness to positive controls (Figure 19). There was marked expansion of the upper 50% of the stroma (Figure 20) and mild expansion of the lower stroma (full thickness damage). There was a moderate increase in keratocytes with pyknotic nuclei in the upper half of the stroma (Figure 20). There were scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm in the mid to lower stroma (Figure 21).</p> <p>Endothelium: The endothelium was</p>	18-22

IIVS Number	Sponsor's Designation	Exposure Time (minutes)/ Post-Exposure Incubation (minutes)	Observations	Figure #
			generally intact. Diffusely, endothelial cells had enlarged nuclei and vacuolated cytoplasm. (Figure 22).	
06AA46 Slides B9460-B9462	Y	10/120	<p>All corneas were similar histopathologically.</p> <p>Epithelium: There was diffuse loss of the squamous and wing layers and uniform blanching of the exposed surface epithelium (Figure 23). The remaining epithelium was hypereosinophilic (coagulation necrosis), with granular nuclear chromatin, and brightly eosinophilic nucleoli. The epithelium was not viable.</p> <p>Stroma: The stroma was similar thickness to positive controls (Figure 24). There was marked expansion of the upper 50% of the stroma (Figure 25) and mild to moderate expansion of the lower stroma (full thickness damage). Throughout the stroma there was a moderate increase in keratocytes with pyknotic nuclei (Figure 26) and scattered keratocytes with vacuolated nuclei and/or hypereosinophilic cytoplasm (Figures 25 and 26).</p> <p>Endothelium: The endothelium was generally intact. Diffusely, endothelial cells had enlarged nuclei and vacuolated cytoplasm. (Figure 27).</p>	23-27

The figures displayed on the subsequent pages of this pathology report are representative H&E-stained cross-sections presented at the magnifications indicated in each image caption. A 100 um bar appears on each micrograph for reference. Arrows included in some of the figures identify examples of the lesions noted, however not all lesions in all micrographs are marked. Vertical double-headed arrows mark the limits of the epithelium in the appropriate figures.


Stewart B. Jacobson, DVM DACVP
Principal Investigator

2/23/07
Date

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (H&E)

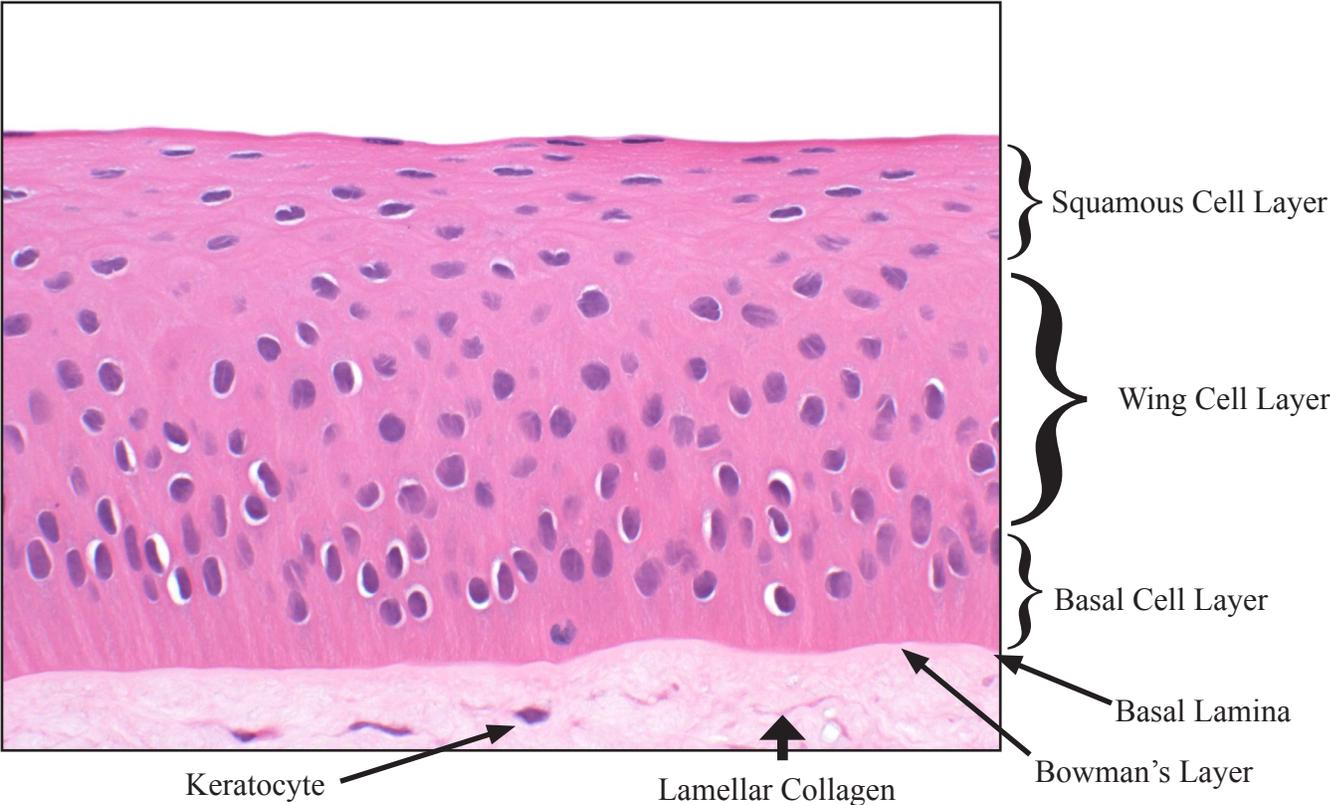


Figure 2. Negative Control (sterile, deionized water, 10 minute exposure/120 minute post incubation) - Epithelium (Slide B9445-1, 40x, H&E)

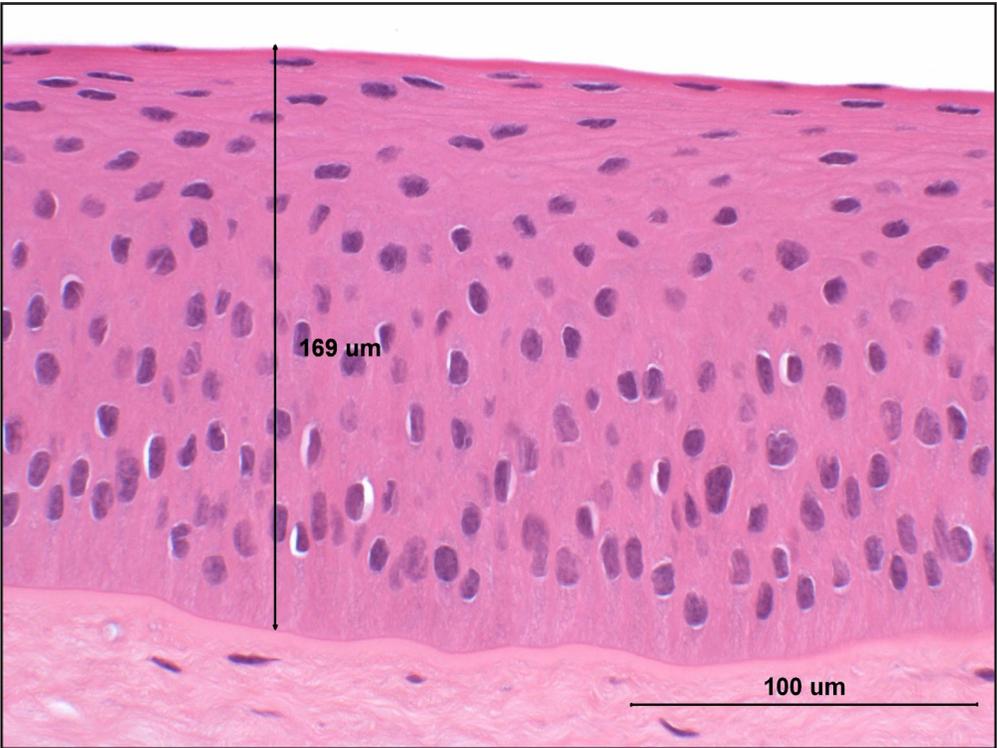


Figure 3. Negative Control (sterile, deionized water, 10 minute exposure/120 minute post incubation)
- Stroma directly below Bowman's Layer (Slide B9446-1, 40x, H&E)

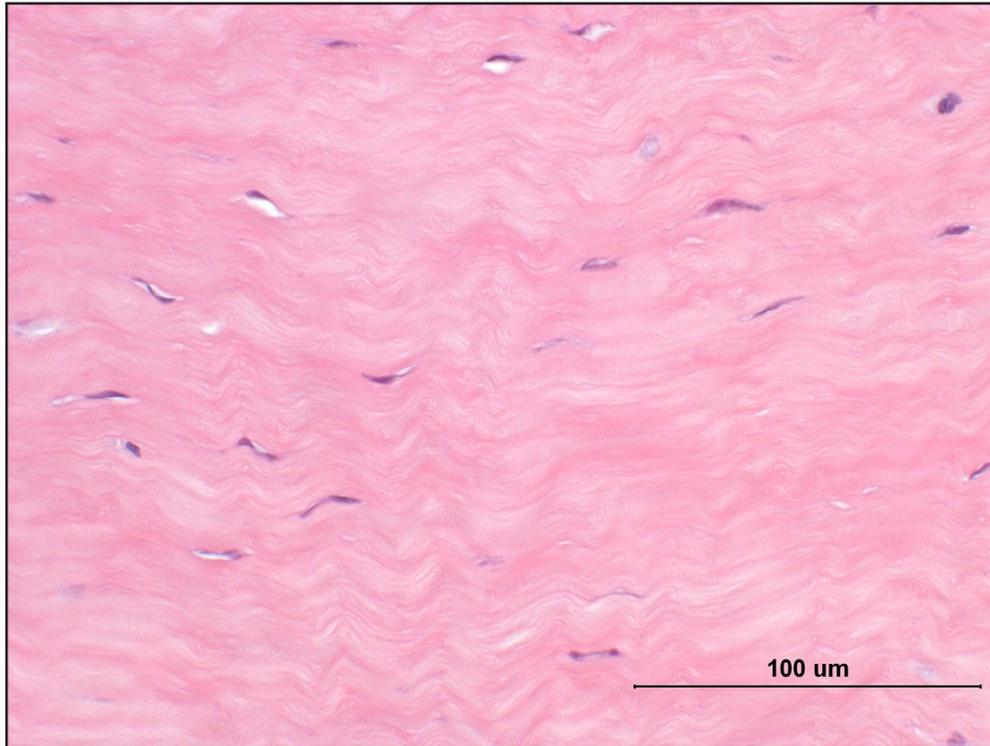


Figure 4. Negative Control (sterile, deionized water, 10 minute exposure/120 minute post incubation)
- Full thickness (Slide B9446-1, 4x, H&E)

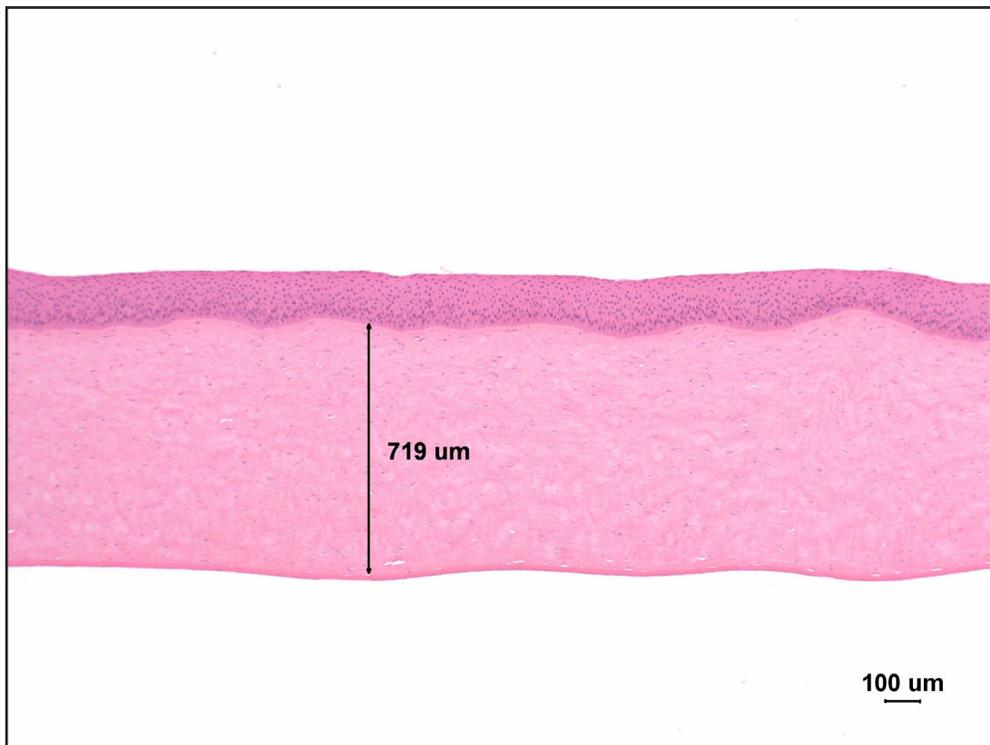


Figure 5. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Epithelium with coagulation of the squamous layer (A →) and vacuolation of wing and basal cells (B →) (Slide B9449-1, 40x, H&E)

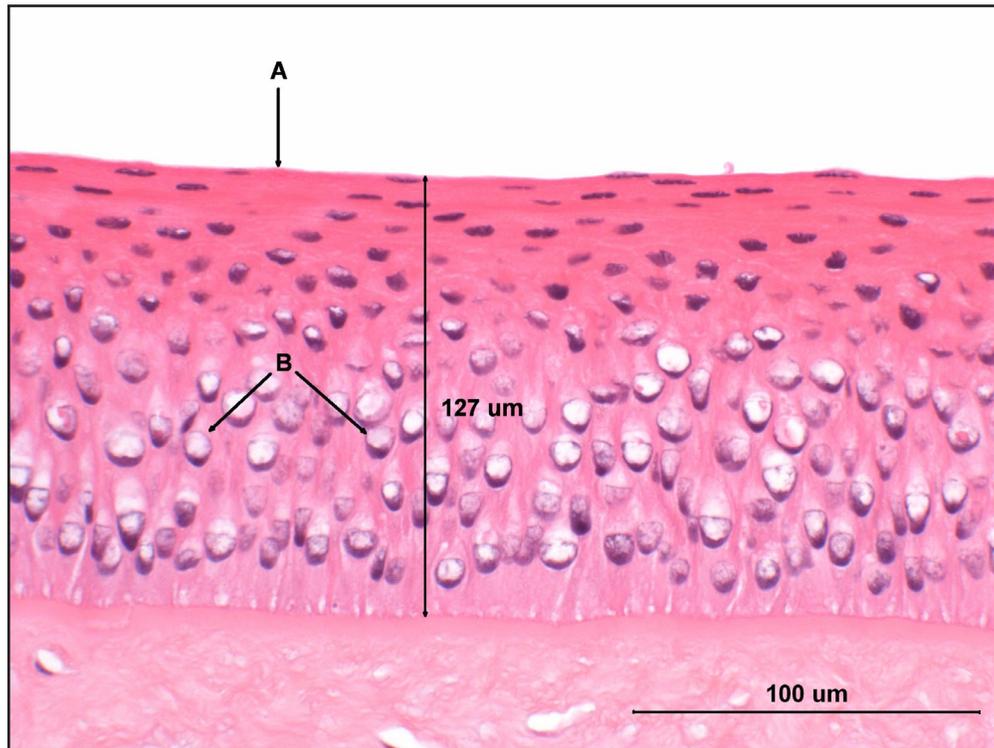


Figure 6. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Full thickness (Slide B9449-1, 4x, H&E)

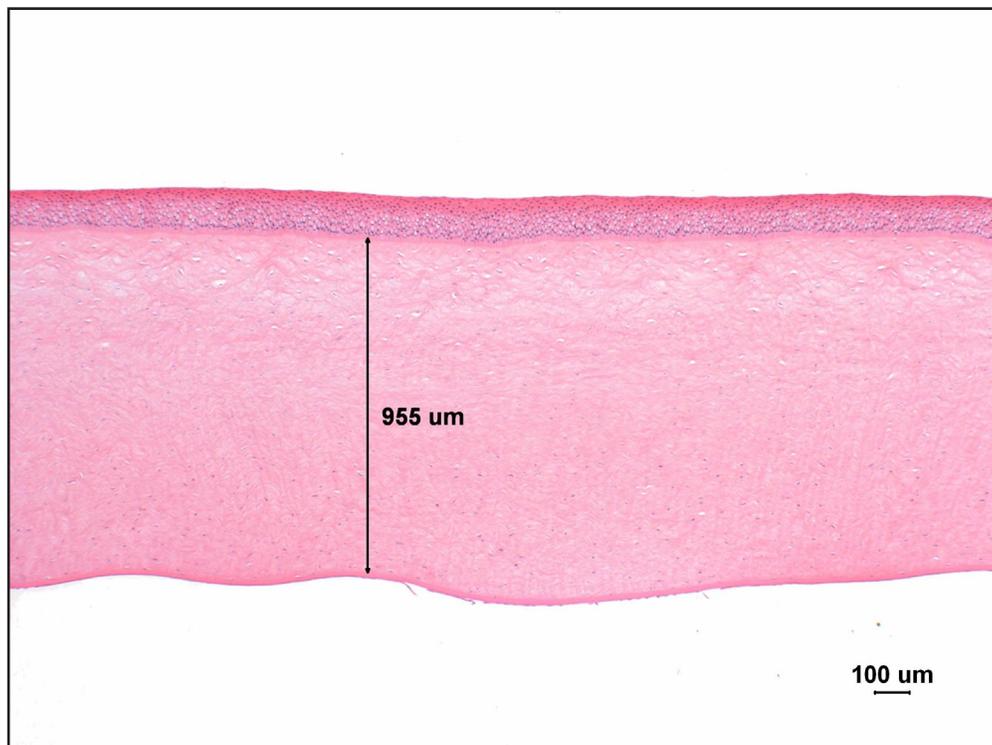


Figure 7. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Upper stroma with vacuolation of the collagen matrix (—>) (Slide B9449-1, 40x, H&E)

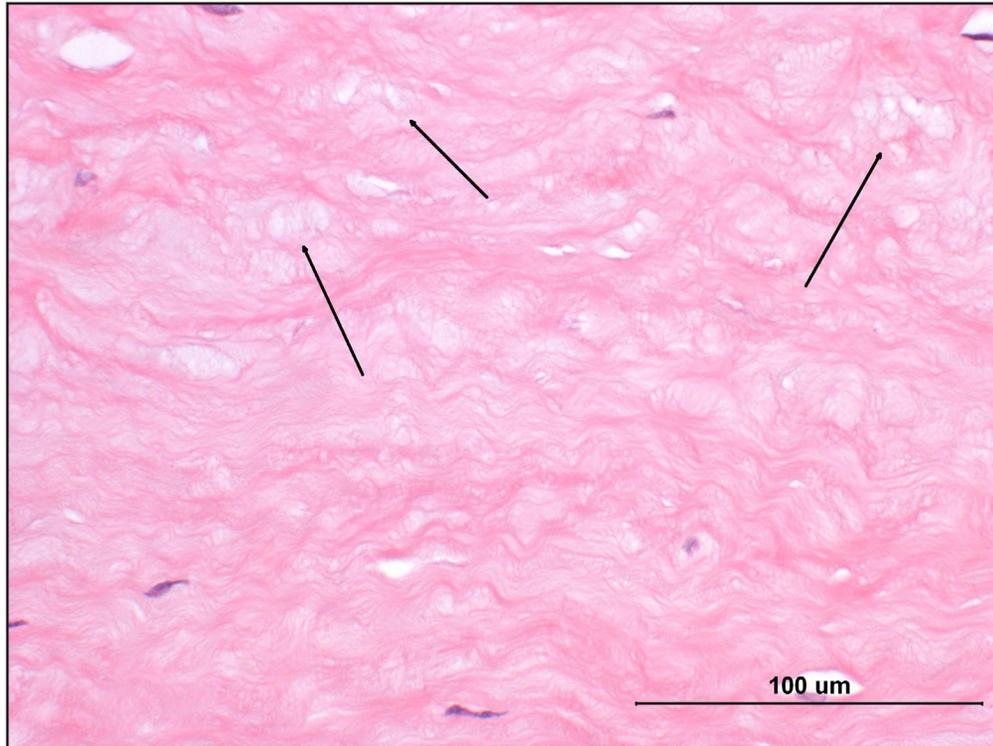


Figure 8. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Upper stroma with pyknotic nuclei (—>) (Slide B9450-1, 40x, H&E)

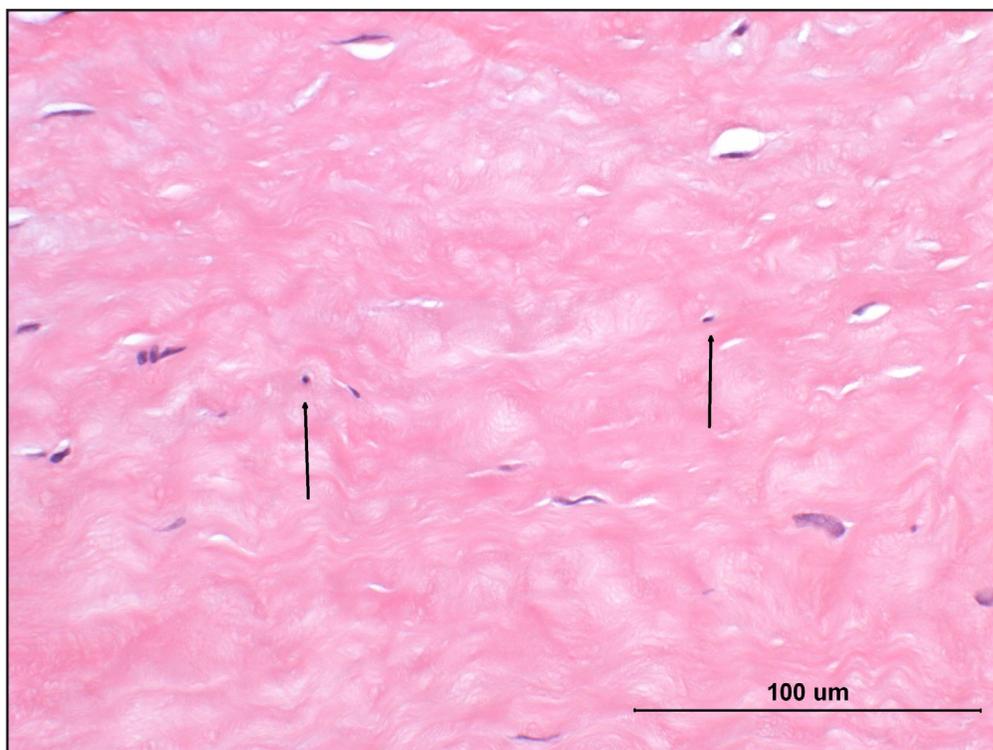


Figure 9. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Mid stroma with cytoplasmic eosinophilia (—→) (Slide B9448-1, 40x, H&E)

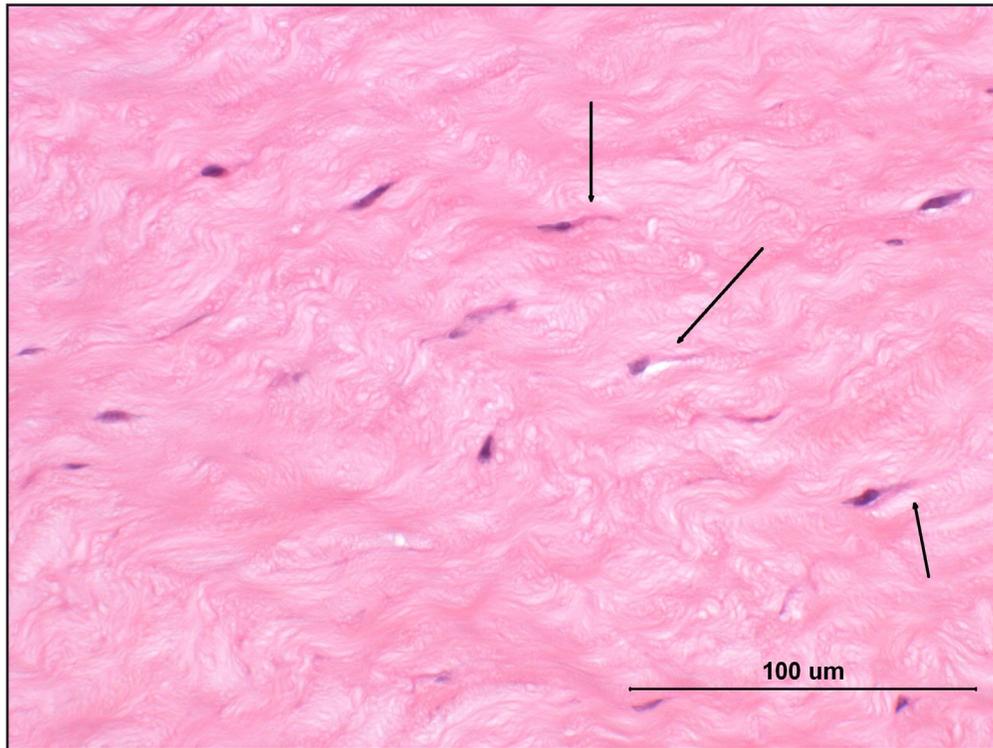


Figure 10. 06AA45, X (3 minute exposure/120 minute post incubation) - Necrotic epithelial remnants (Slide B9451-1.1RC, 40x, H&E)

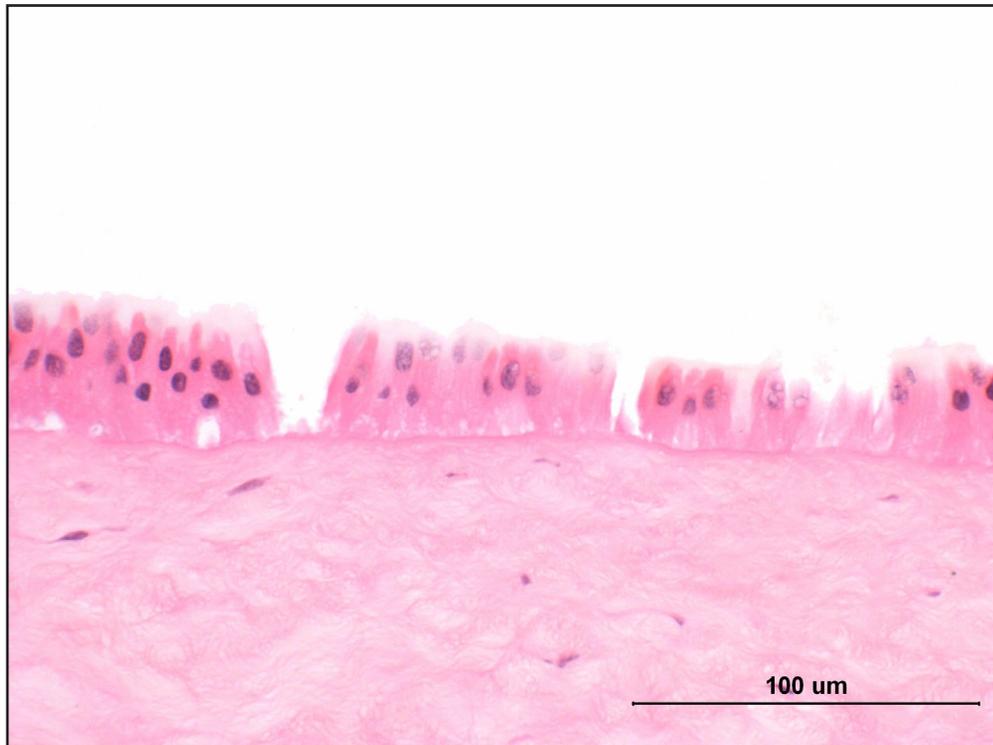


Figure 11. 06AA45, X (3 minute exposure/120 minute post incubation) - Full thickness (Slide B9453-1.1RC, 4x, H&E)

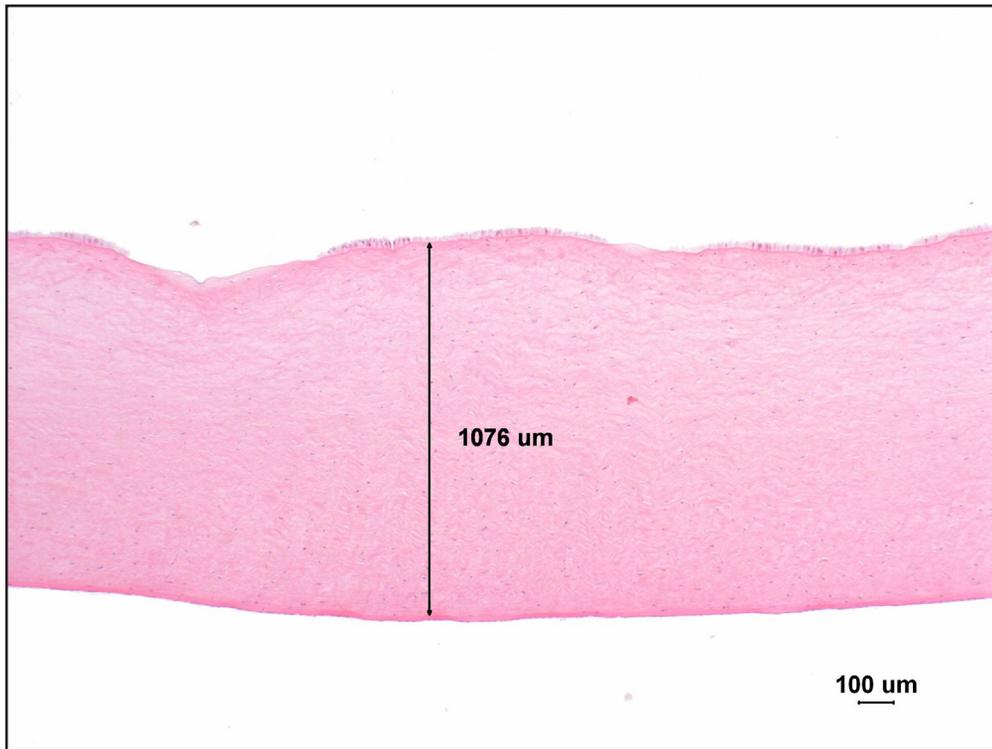


Figure 12. 06AA45, X (3 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →) and pyknotic nuclei (B →) (Slide B9453-1.1RC, 40x, H&E)

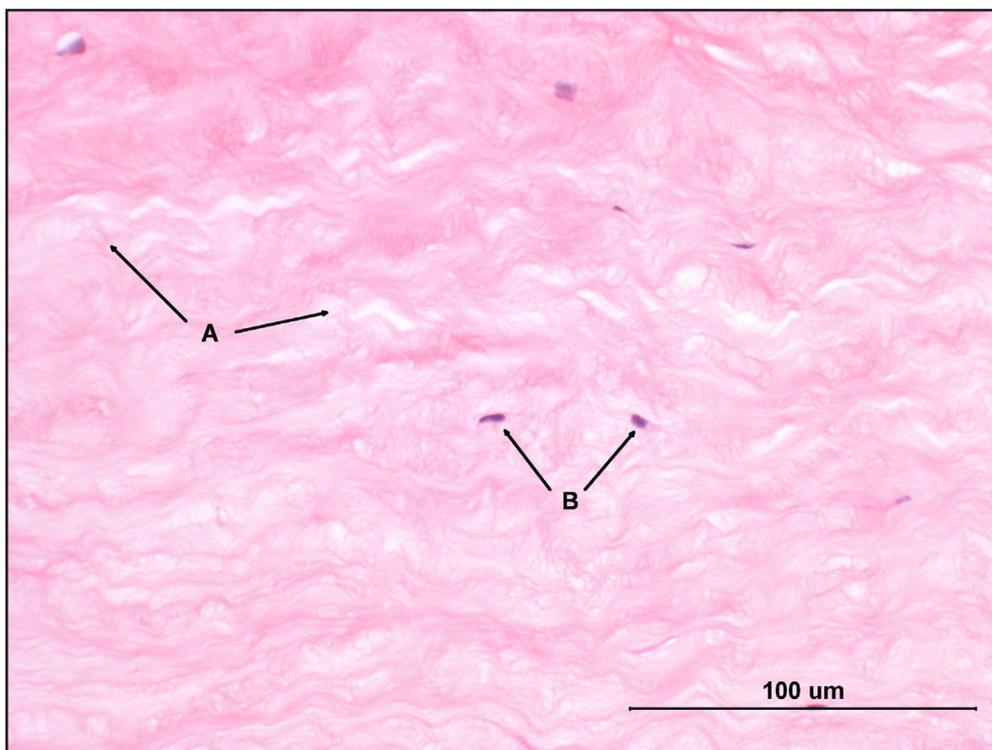


Figure 13. 06AA45, X (3 minute exposure/120 minute post incubation) - Mid stroma with vacuolated keratocyte nuclei (A →) and hypereosinophilic cytoplasm (B →) (Slide B9451-1.1RC, 40x, H&E)

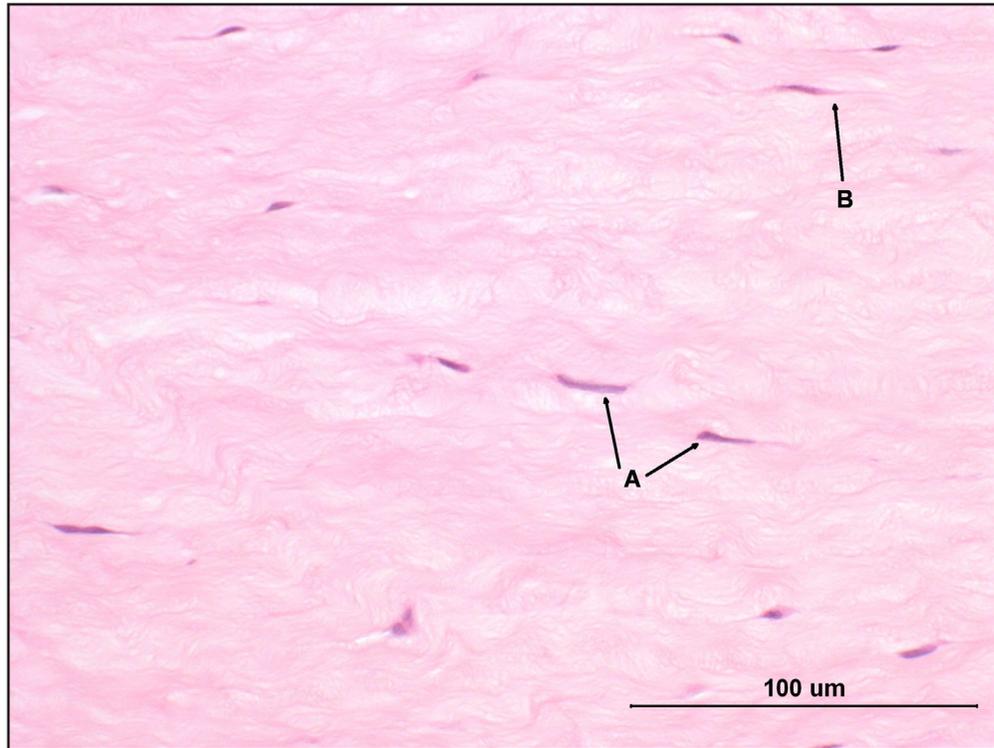


Figure 14. 06AA45, X (10 minute exposure/120 minute post incubation) - Loss of epithelium and superficial stroma (Slide B9455-1.1RC, 40x, H&E)

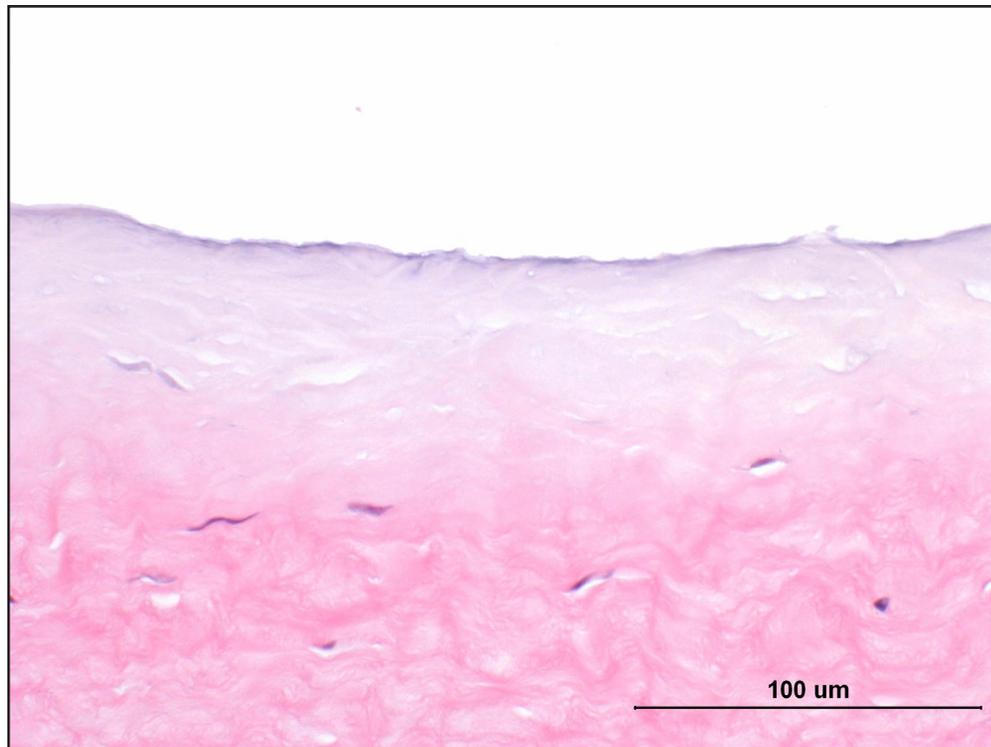


Figure 15. 06AA45, X (10 minute exposure/120 minute post incubation) - Full thickness (Slide B9455-1.1RC, 4x, H&E)

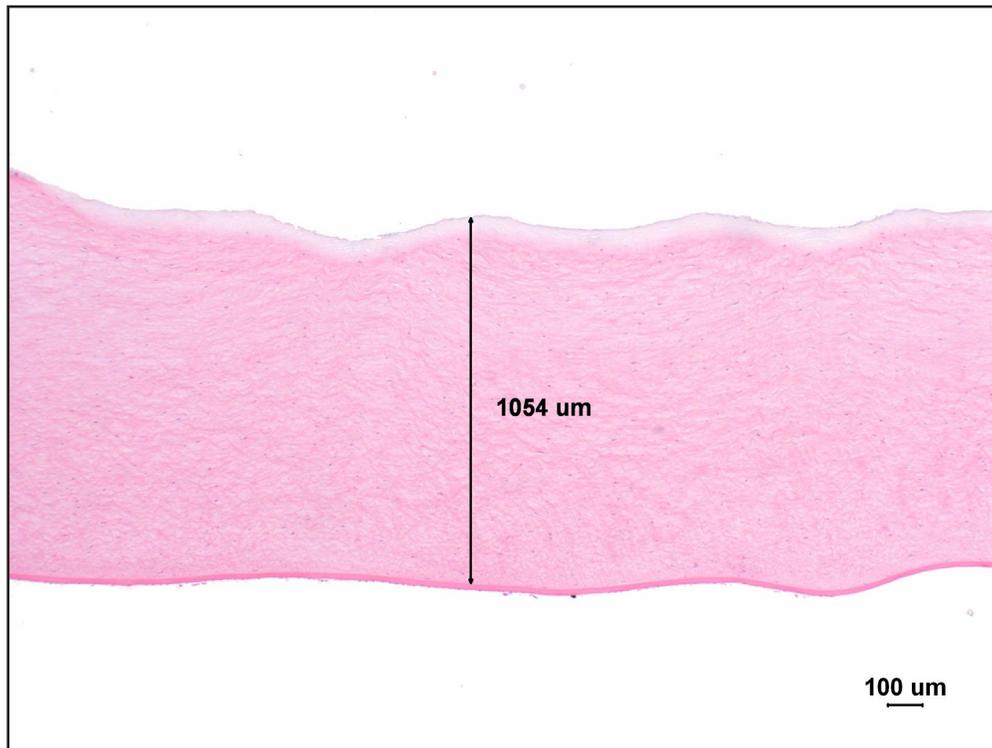


Figure 16. 06AA45, X (10 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →) and pyknotic keratocyte nuclei (B →) (Slide B9455-1.1RC, 40x, H&E)

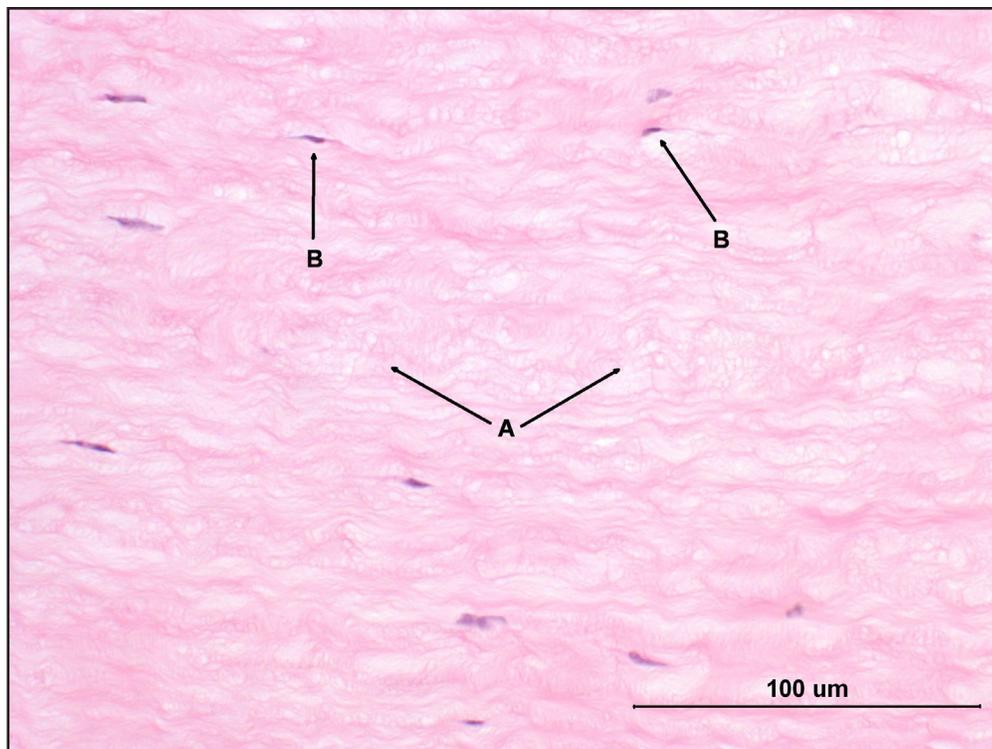


Figure 17. 06AA45, X (10 minute exposure/120 minute post incubation) - Endothelium with enlarged nuclei (—>) and vacuolated cytoplasm (Slide B9455-1.1RC, 40x, H&E)

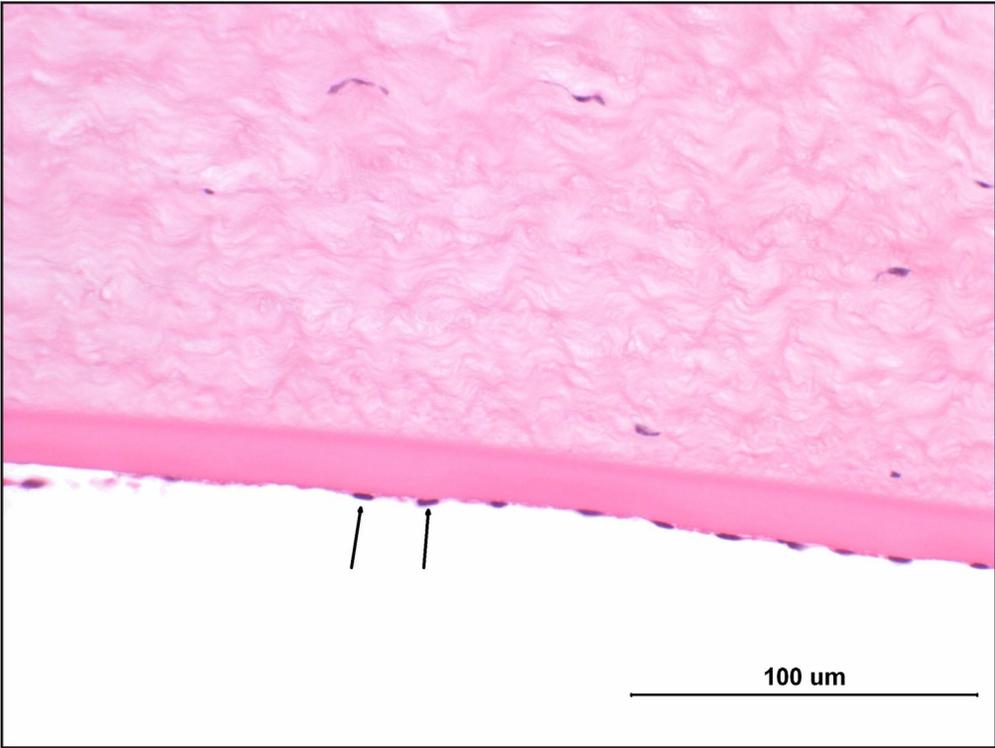


Figure 18. 06AA46, Y (3 minute exposure/120 minute post incubation) - Epithelium with loss of squamous layers, surface blanching, and full thickness necrosis (Slide B9457-1, 40x, H&E)



Figure 19. 06AA46, Y (3 minute exposure/120 minute post incubation) - Full thickness (Slide B9457-1, 4x, H&E)

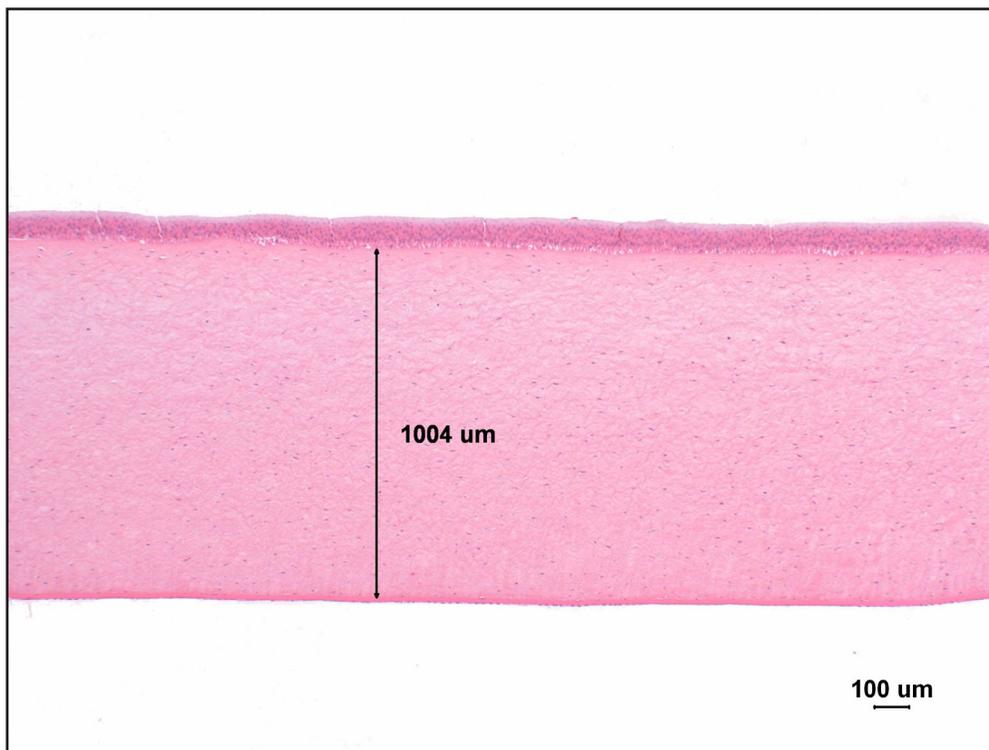


Figure 20. 06AA46, Y (3 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →) and pyknotic keratocyte nuclei (B →) (Slide B9457-1, 40x, H&E)

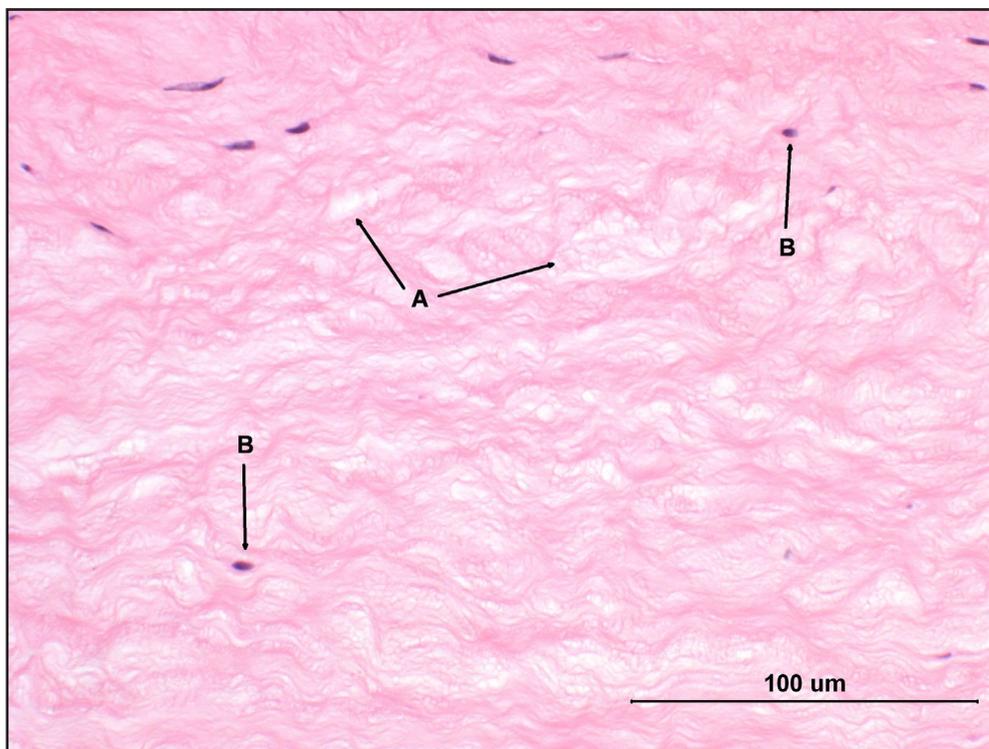


Figure 21. 06AA46, Y (3 minute exposure/120 minute post incubation) - Mid stroma with vacuolated keratocyte nuclei (A →) and hyper eosinophilic cytoplasm (B →) (Slide B9457-1, 40x, H&E)

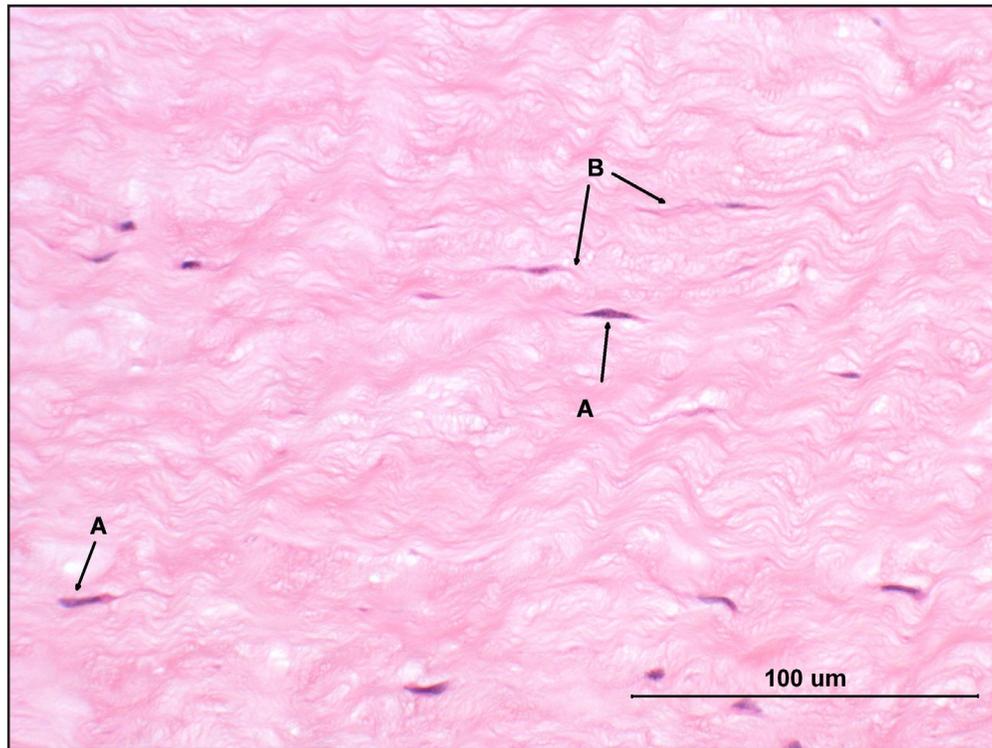


Figure 22. 06AA46, Y (3 minute exposure/120 minute post incubation) - Endothelium with enlarged nuclei and vacuolated cytoplasm (→) (Slide B9457-1, 40x, H&E)

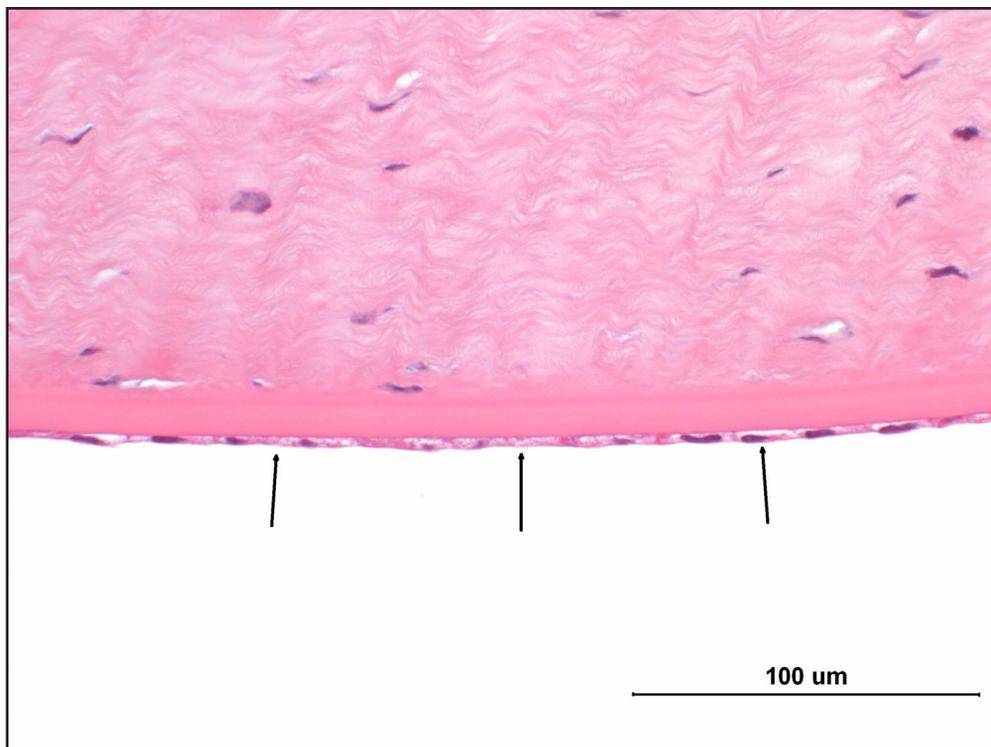


Figure 23. 06AA46, Y (10 minute exposure/120 minute post incubation) - Epithelium with loss of squamous and wing layers, linear surface blanching, and full thickness necrosis (Slide B9462-1.1RC, 40x, H&E)

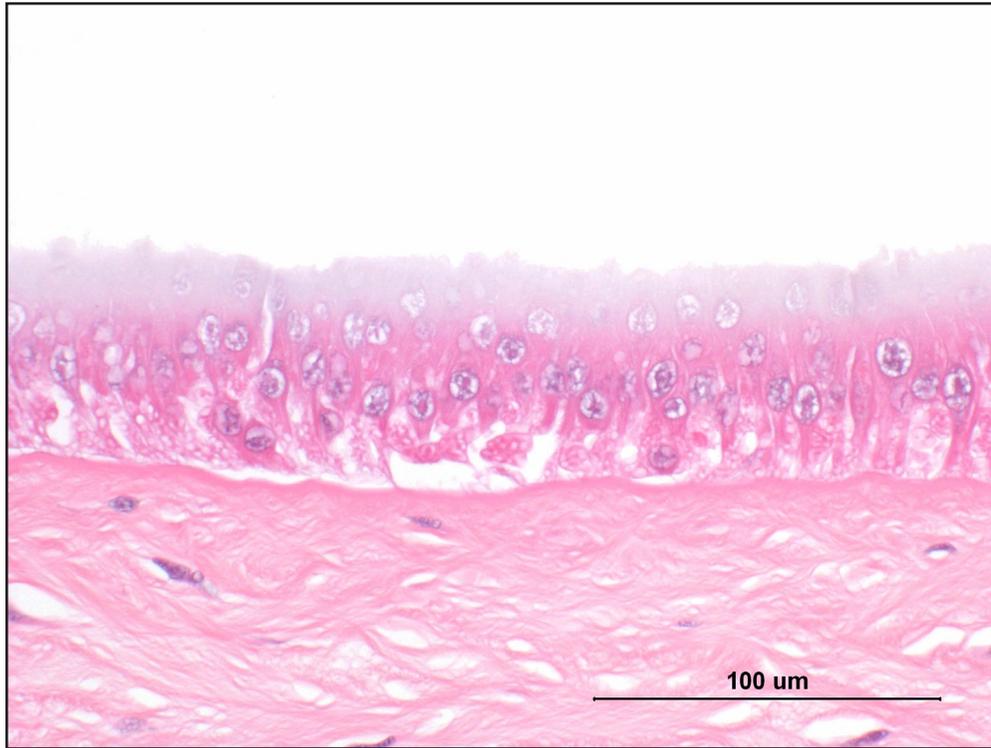


Figure 24. 06AA46, Y (10 minute exposure/120 minute post incubation) - Full thickness (Slide B9461-1.1RC, 4x, H&E)

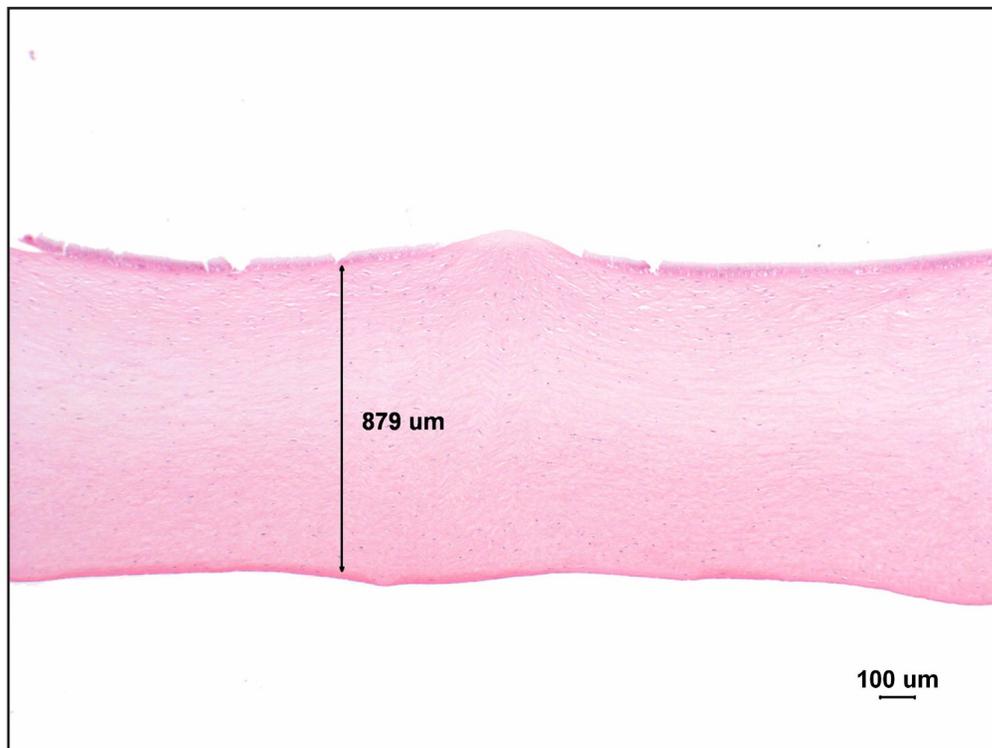


Figure 25. 06AA46, Y (10 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →) and vacuolated keratocyte nuclei (B →) (Slide B9462-1.1RC, 40x, H&E)

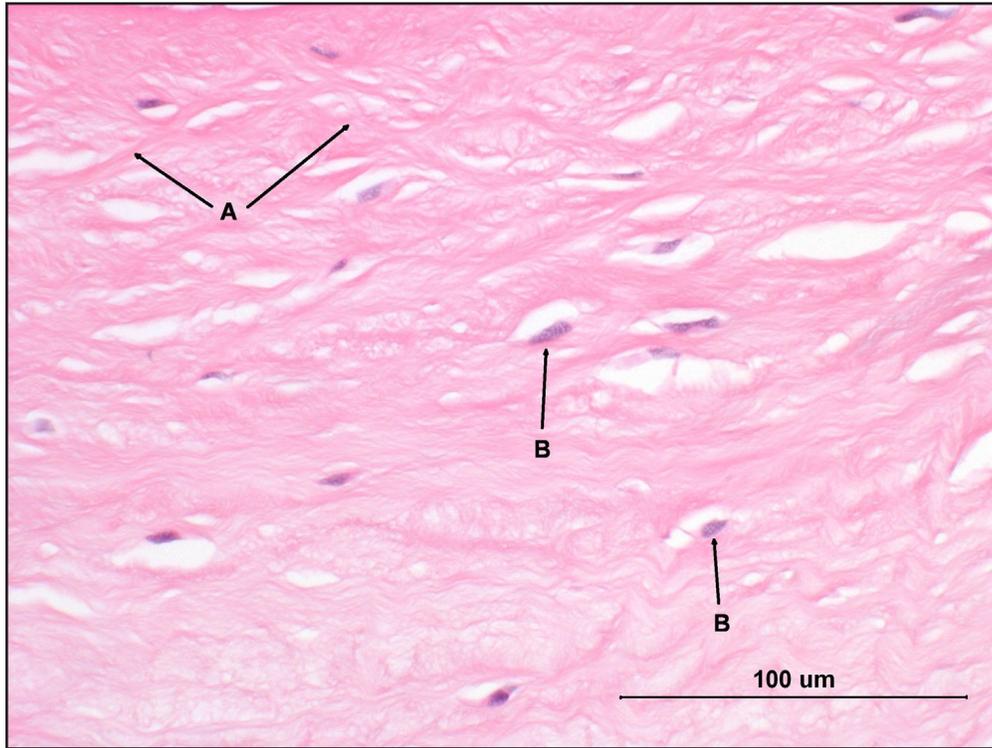


Figure 26. 06AA46, Y (10 minute exposure/120 minute post incubation) - Mid stroma with pyknotic keratocyte nuclei (A →) and hyper eosinophilic cytoplasm (B →) (Slide B9461-1.1RC, 40x, H&E)

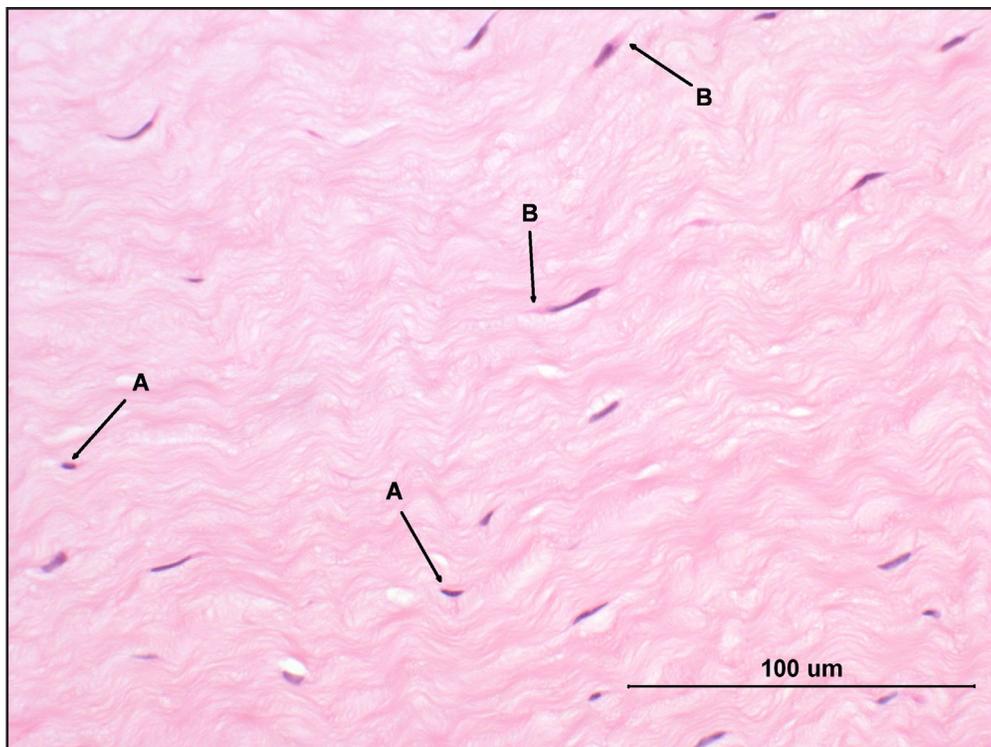


Figure 27. 06AA46, Y (10 minute exposure/120 minute post incubation) - Endothelium with enlarged nuclei and vacuolated cytoplasm (—→) (Slide B9460-1.1RC, 40x, H&E)

