

DRAFT REPORT

Study Title

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
EXTENDED POST- EXPOSURE INCUBATION PERIOD(S) AND OPTIONAL
HISTOLOGY**

Product Identity

BS

Author

Hans A. Raabe, M.S.
Toxicologist

Study Completion Date

Pending Final Report

Testing Facility

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

Laboratory Project Number

06AH39.350053

Laboratory Project ID

4610

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.

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.
Toxicologist

Date: _____

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QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Extended Post-Exposure Incubation Period(s) and Optional Histology

Study Number: 06AH39.350053

Study Director: Hans A. Raabe, M.S.

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	18-Oct-06	18-Oct-06	18-Oct-06
Initial Opacity Measurement	17-Oct-06	24-Oct-06	24-Oct-06
Draft Report and Data	10-Jan-07	10-Jan-07	15-Jan-07

Final Report

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
EXTENDED POST- EXPOSURE INCUBATION PERIOD(S) AND OPTIONAL
HISTOLOGY**

Study Director:

Hans A. Raabe, M.S.
Toxicologist

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.
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Matthew Hyder, B.S.

STUDY REPORT

Study Title: Bovine Corneal Opacity and Permeability Assay with Extended Post-Exposure Incubation Period(s) 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 *
06AH39	BS	white cream	11/30/06	10/11/06	room temperature

* Protected from exposure to light

Study Initiation Date: October 13, 2006

Experimental Start Date: October 17, 2006

Experimental End Date: (Pending Final Histology Report)

Study Completion Date: (Pending Final Report)

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 and antibiotics (50 µg/ml gentamicin and 1.0 µg/ml of amphotericin B (fungizone) (Complete MEM)

Minimum Essential Media (EMEM) with phenol red containing 1% FBS and 2mM

L-glutamine and antibiotics (50 µg/ml gentamicin and 1.0 µg/ml of amphotericin B (fungizone) (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 (EM Science or EMD Chemicals Inc.). Initially, the test substances were added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, the test substance was added to 7.5- 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. Treuth & 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 with antibiotics (50 µg/ml gentamicin and 1.0 µg/ml of amphotericin B (fungizone) (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 opacitometer. Nine 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 substance, BS, was 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. Due to its viscous nature, the test substance, BS, was administered directly onto the exposed cornea using a positive displacement pipette. Each treated cornea was completely covered with the test substance. Five corneas were used for each exposure time and designated post-exposure incubation period (ten corneas). The corneas were incubated in the presence of the test substance at $32 \pm 1^\circ\text{C}$ for 10 minutes with post-exposure periods of 4 and 20 hours. The negative control was tested in six corneas at $32 \pm 1^\circ\text{C}$ for 10 minutes, with post-exposure incubation periods of 4 and 20 hours. In addition, three corneas were treated with the negative control for 30 minutes at $32 \pm 1^\circ\text{C}$ with post-exposure incubation period of 1.5 hours. The positive control was tested in three corneas at $32 \pm 1^\circ\text{C}$ for 10 minutes, with a post-exposure incubation period of 2 hours. After the 10 or 30-minute exposure time, the control or test substance treatments were removed and the epithelial side of the corneas was washed at least three times with Complete MEM (with phenol red) to ensure total removal of the control or test substances. The corneas were then given a final rinse with Complete MEM. For the corneas directly exposed to the test substance (without anterior chamber window), the test substance was removed from the treated corneas by rinsing the exposed epithelium of the corneas (special care was taken not to spray the corneas directly) with Complete MEM (with phenol red). The chamber window was returned to the chamber when most or all of the test substance had been removed. The rinsing process continued in the same manner as the positive and negative control corneas. The anterior chamber was refilled with fresh Complete MEM (without phenol red) and an opacity

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. For the positive control, the opacity readings from the negative control corneas treated for 30 minutes was used in the calculation. For the test substance, opacity readings from negative control corneas which were incubated for the same timeframe (4 or 20 hours) were used in the calculations. 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: For the positive control, the average corrected OD₄₉₀ of the negative control corneas treated for 30 minutes was used in the calculation. For the test substance, the average corrected OD₄₉₀ of the negative control corneas incubated for the same timeframe (4 or 20 hours) was used in the calculation.

$$\text{Final Corrected OD}_{490} = (\text{raw OD}_{490} - \text{mean blank OD}_{490}) - \text{average corrected negative control OD}_{490}$$

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:

$$\text{In Vitro Score} = \text{Mean Opacity Value} + (15 \times \text{Mean OD}_{490} \text{ Value})$$

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 39.4 to 64.2), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

The following classification system was established by Sina et. al.(1995) based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of *in vitro* data, these specific classifications 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

Table 1
BCOP Results of the Test Substance

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Post-Exposure Incubation Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score	pH
10/17/06 to 10/18/06	06AH39	BS	Neat	10 minutes	4 hours	220.8	3.818	278.1	14.0
					20 hours	208.7	3.503	261.3	

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
10/17/06	Ethanol	10 minutes	27.3	1.138	44.4

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 except for the 10 minute/20 hour post-exposure incubation group where there was minimal expansion of the collagen matrix immediately subjacent to the epithelium. 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 vacuolation or pyknosis. 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, BS, for ten minutes with a 4 hour post-incubation period exhibited diffusely, epithelial cells which had hypereosinophilic, finely vacuolated cytoplasm (coagulation) and hyperchromatic, vacuolated nuclei. There was diffuse blanching (lack of tissue staining) of the squamous layer and multifocal loss of basal cell adhesion. The epithelium was not viable at the time of fixation. The stroma was similar in thickness to the positive controls. There was mild to moderate expansion of the collagen matrix in the upper 66% of the stroma and marked expansion of the lower stroma adjacent to Descemet's membrane. Keratocyte nuclei were vacuolated throughout the stroma, most severely in the lower stroma adjacent to Descemet's membrane. There was a slight increase in keratocyte cytoplasmic eosinophilia in the upper half of the stroma. The endothelium was generally absent.

Corneas treated with the test substance, BS, for ten minutes with a 20 hour post-incubation period showed diffusely, epithelial cells which had hypereosinophilic, finely vacuolated cytoplasm (coagulation) and hyperchromatic, vacuolated nuclei. There was diffuse blanching (lack of tissue

staining) of the squamous layer and diffuse loss of basal cell adhesion. The epithelium was not viable at the time of fixation. The stroma was slightly thicker than the positive controls. There was moderate expansion of the collagen matrix in the upper 66% of the stroma and marked expansion of the lower stroma adjacent to Descemet's membrane. There was frequent artifactual tearing in the lower stroma due to increased tissue fragility. Keratocyte nuclei were vacuolated throughout the stroma, most severely in the lower stroma adjacent to Descemet's membrane. There was a slight increase in keratocyte cytoplasmic eosinophilia in the upper half of the stroma. The endothelium was absent from all sections.

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 D.

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, BS, were 278.1 (10 minute exposure, 4 hour post-exposure) and 261.3 (10 minute exposure, 20 hour post-exposure). The high *in vitro* scores for BS were attributable to extreme opacity and permeability scores. 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 after a 10 minute exposure, as evidenced by corneas in both the 4 and 20 hour post-exposure incubation groups. Histological evaluation showed full thickness degeneration and a loss of adhesion of the basal layer from the basal lamina as well as moderate to marked expansion of the collagen matrix throughout the stroma, most severe in the lower stroma. Additionally, keratocyte nuclei were vacuolated throughout the stroma, most severely in the lower stroma next to Descemet's membrane and there was a slight increase in keratocyte cytoplasmic eosinophilia in the upper half of the stroma. The endothelium was generally absent. These observations may be associated with the measured increases in fluorescein permeability and opacity and are evidence of loss of corneal barrier function of both the epithelium and the endothelium. The loss of the endothelium in humans is generally regarded as an irreversible catastrophic corneal lesion.

REPORT SUBMITTED BY:

Study Director

Study Completion Date

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.

APPENDIX A

APPENDIX B

Performed on October 17, 2006 to October 18, 2006

Study No. 06AH39.350053

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

<u>TA #</u>	<u>CORNEA #</u>	<u>INITIAL</u>	<u>FINAL</u>	<u>CHANGE</u>	<u>CORRECTED</u>	<u>AVG</u>	<u>STDEV</u>
06AH39	13	5	217	212	212.0		
Neat	17	4	213	209	209.0		
10 minutes	19	4	246	242	242.0		
4-hour post-exp	21	6	231	225	225.0		
	22	2	218	216	216.0	220.8	13.3
06AH39	16	2	228	226	221.3		
Neat	23	4	214	210	205.3		
10 minutes	25	4	229	225	220.3		
20-hour post-exp	26	5	194	189	184.3		
	27	4	221	217	212.3	208.7	15.1
Neg. Control	29	4	5	1	NA		
Sterile DI Water	33	3	3	0	NA		
30 minutes	34	2	3	1	NA	0.7	
2-hour post-exp							
Neg. Control	3	6	5	-1	NA		
Sterile DI Water	4	4	5	1	NA		
10 minutes	5	1	1	0	NA	0.0	
4-hour post-exp							
Neg. Control	7	2	9	7	NA		
Sterile DI Water	9	3	5	2	NA		
10 minutes	11	3	8	5	NA	4.7	
20-hour post-exp							
Pos. Control	35	3	23	20	19.3		
Ethanol	36	3	30	27	26.3		
10 minutes	37	5	42	37	36.3	27.3	8.5
2-hour post-exp							
	*24	4					
	*39	3					
	*40	2					
	*42	4					
	*43	4					
	*44	4					
	*46	4					
	*47	2					
	*50	5					
	*51	5					
	*52	6					
	*53	4					
	*56	5					
	*57	5					
Initial corneal opacity average:		4					

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

Performed on October 17, 2006 to October 18, 2006

Study No. 06AH39.350053

PERMEABILITY SCORE

Neg. Control
Sterile DI Water
30 minutes
2-hour post-exp

Cornea #	OD490
29	0.003
33	0.003
34	0.003

Avg.	0.003

Neg. Control
Sterile DI Water
10 minutes
4-hour post-exp

Cornea #	OD490
3	0.005
4	0.002
5	0.001

Avg.	0.003

06AH39
Neat
10 minutes
4-hour post-exp

Cornea #	OD490	Dilution Factor	Corrected OD490
13	0.849	5	4.242
17	0.771	5	3.852
19	0.593	5	2.962
21	0.684	5	3.417
22	0.924	5	4.617

Avg. =			3.818
STDEV=			0.654

Pos. Control
Ethanol
10 minutes
2-hour post-exp

Cornea #	OD490	Dilution Factor	Corrected OD490
35	1.200	1	1.197
36	1.067	1	1.064
37	1.155	1	1.152

Avg. =			1.138
STDEV=			0.068

Neg. Control
Sterile DI Water
10 minutes
20-hour post-exp

Cornea #	OD490
7	0.026
9	0.019
11	0.037

Avg.	0.027

06AH39
Neat
10 minutes
20-hour post-exp

Cornea #	OD490	Dilution Factor	Corrected OD490
16	0.817	5	4.058
23	0.701	5	3.478
25	0.481	5	2.378
26	0.816	5	4.053
27	0.715	5	3.548

Avg. =			3.503
STDEV=			0.685

Performed on October 17, 2006 to October 18, 2006

Study No. 06AH39.350053

IN VITRO SCORE

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

Test Article	Concentration	Exposure Period	Post-Exp Incubation	Mean Opacity	Mean OD490	In vitro Score
06AH39	Neat	10 minutes	4-hour post-exp	220.8	3.818	278.1
06AH39	Neat	10 minutes	20-hour post-exp	208.7	3.503	261.3
Ethanol	Neat	10 minutes	2-hour post-exp	27.3	1.138	44.4

APPENDIX C

APPENDIX D

DRAFT HISTOPATHOLOGY REPORT

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
EXTENDED POST-EXPOSURE INCUBATION PERIOD(S) AND
OPTIONAL HISTOLOGY**

Testing Facility Study Number 06AH39.350053

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:

January 16, 2007

Bovine Corneal Opacity and Permeability Assay with Extended Post-Exposure Incubation Period(s) and Optional Histology

Study Number: 06AH39.350035

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>
11/03/06	Microtomy	11/06/06	11/06/06
12/04-07/06	Individual Animal Data and Supporting Documentation	12/07/06	12/07/06
12/04-07/06	Draft Histopathology Report	12/07/06	12/07/06

Laura J. 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 06AH39.350053. 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 a test article as measured by the test article's ability to induce opacity and permeability to fluorescein in an isolated bovine cornea. 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 approximately 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 previously 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 5.1.2 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

Negative control corneas were treated with sterile deionized water in three different exposure regimens: 30 minutes with 1.5 hours post incubation time (slides C1228-C1230), 10 minutes with 4 hours post incubation time (slides C1231-C1233), and 10 minutes with 20 hours post incubation time (slides C1234-C1236). Results for all three groups were similar except in the stroma as noted below.

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 (Figures 2, 5, and 8).

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, Figures 4 and 7), except for the 10 minute exposure/20 hour post-exposure incubation group where there was minimal expansion of the collagen matrix immediately subjacent to the epithelium (Figure 10).

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

Cross sections of negative control-treated corneas, showing the general thickness of the whole cornea and stroma, are shown in Figures 3, 6, and 9.

The positive control corneas (slides C1237-C1239), 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 11). 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 12). 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 (Figure 13). 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 vacuolation (Figure 14) or pyknosis. In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 15). 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 #
06AH39 Slides C1240- C1244	BS	10/24	<p>Epithelium: Diffusely, epithelial cells had hypereosinophilic, finely vacuolated cytoplasm (coagulation) and hyperchromatic, vacuolated nuclei (Figure 16). There was diffuse blanching (lack of tissue staining) of the squamous layer and multifocal loss of basal cell adhesion. The epithelium was not viable at the time of fixation.</p> <p>Stroma: The stroma was similar thickness to positive controls (Figure 17). There was mild to moderate expansion of the collagen matrix in the upper 66% of the stroma (Figure 18) and marked expansion of the lower stroma adjacent to Descemet's membrane (Figure 19). Keratocyte nuclei were vacuolated throughout the stroma (Figures 18 and 19), most severely in the lower stroma adjacent to Descemet's membrane. There was a slight increase in keratocyte cytoplasmic eosinophilia in the upper half of the stroma (Figure 20).</p> <p>Endothelium: The endothelium was generally absent from all sections.</p>	16-20
06AH39 Slides C1245- C1249	BS	10/1200	<p>Epithelium: Diffusely, epithelial cells had hypereosinophilic, finely vacuolated cytoplasm (coagulation) and hyperchromatic, vacuolated nuclei (Figure 21). There was diffuse blanching (lack of tissue staining) of the squamous layer and diffuse loss of</p>	21-24

IIVS Number	Sponsor's Designation	Exposure Time (minutes)/ Post-Exposure Incubation (minutes)	Observations	Figure #
			<p>basal cell adhesion. The epithelium was not viable at the time of fixation.</p> <p>Stroma: The stroma was slightly thicker than positive controls (Figure 22). There was moderate expansion of the collagen matrix in the upper 66% of the stroma (Figure 23) and marked expansion of the lower stroma adjacent to Descemet's membrane (Figure 24). There was frequent artifactual tearing in the lower stroma due to increased tissue fragility (Figure 24). Keratocyte nuclei were vacuolated throughout the stroma (Figures 23 and 24), most severely in the lower stroma adjacent to Descemet's membrane. There was a slight increase in keratocyte cytoplasmic eosinophilia in the upper half of the stroma (Figure 23).</p> <p>Endothelium: The endothelium was absent from all sections.</p>	

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/Study Pathologist

 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)

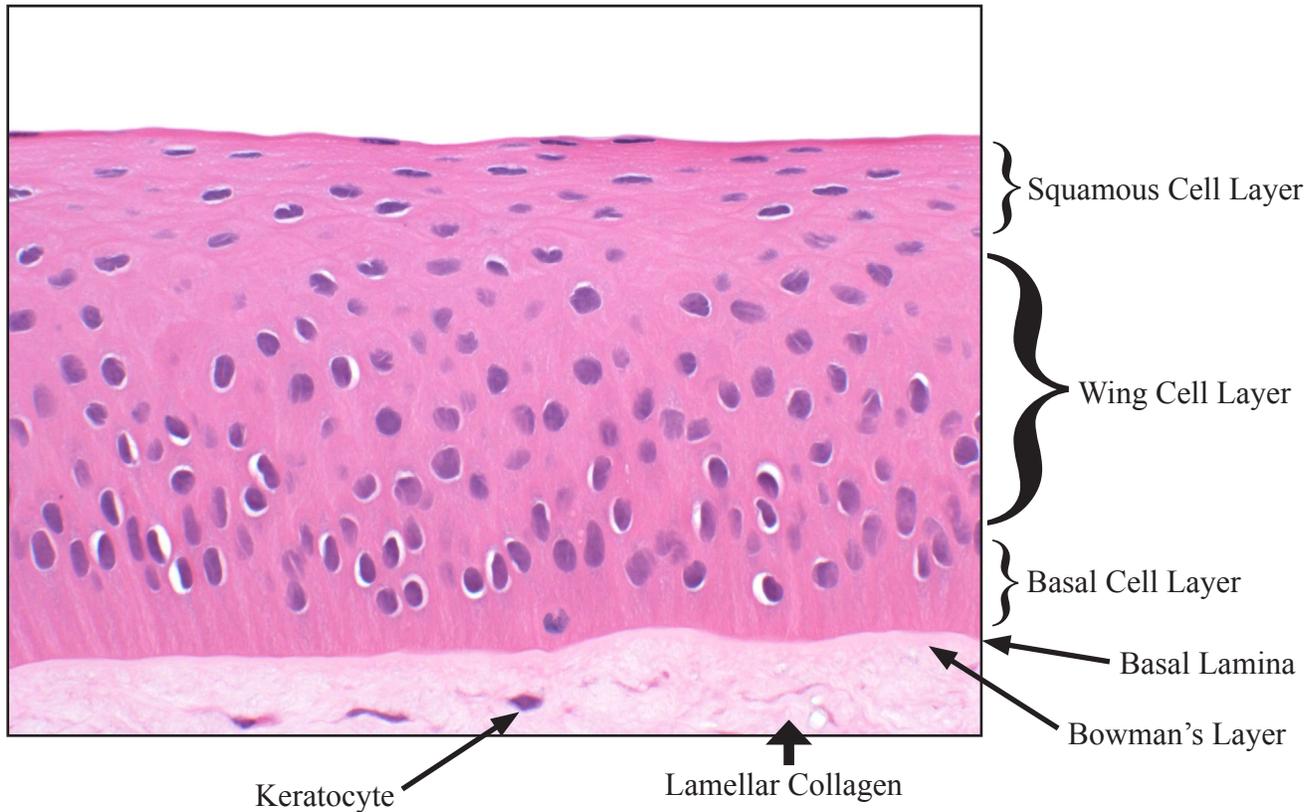


Figure 2. Negative Control (sterile, deionized water, 30 minute exposure/90 minute post incubation) - Epithelium (Slide C1228-1, 40x, H&E)

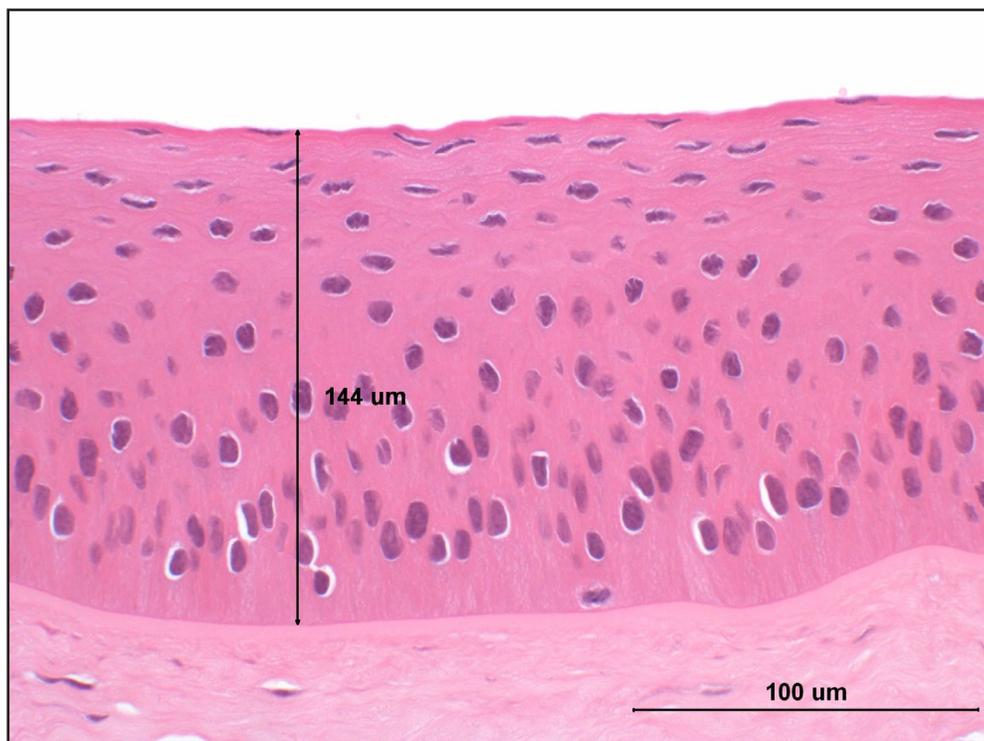


Figure 3. Negative Control (sterile, deionized water, 30 minute exposure/90 minute post incubation) - Full thickness (Slide C1228-1, 4x, H&E)

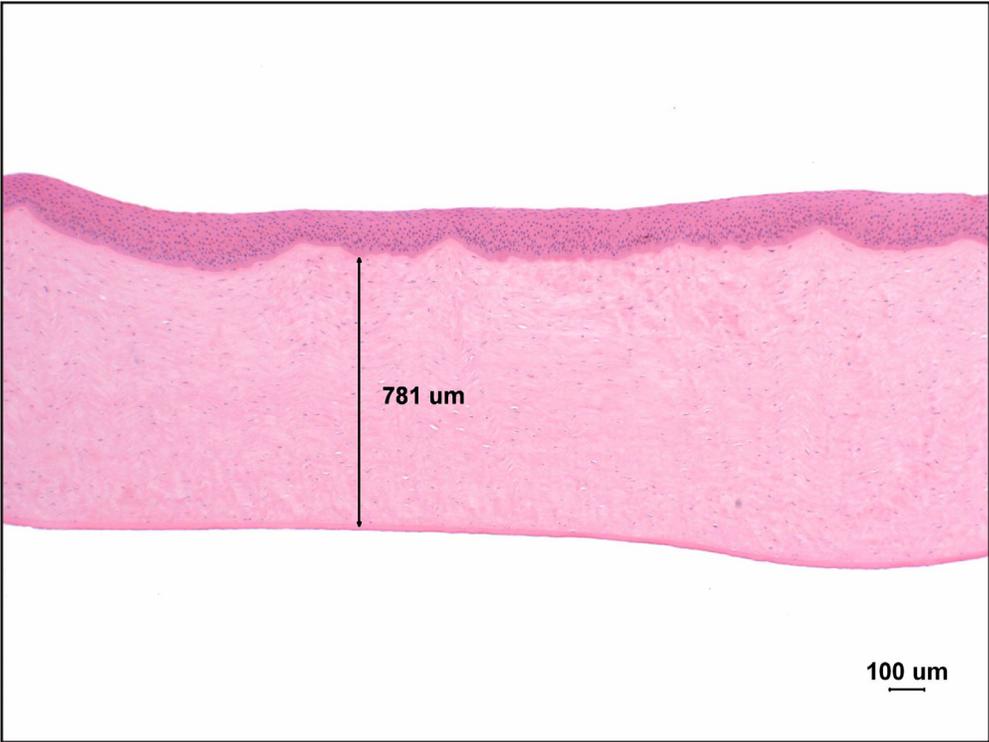


Figure 4. Negative Control (sterile, deionized water, 30 minute exposure/90 minute post incubation) - Stroma directly below Bowman's Layer (Slide C1228-1, 40x, H&E)

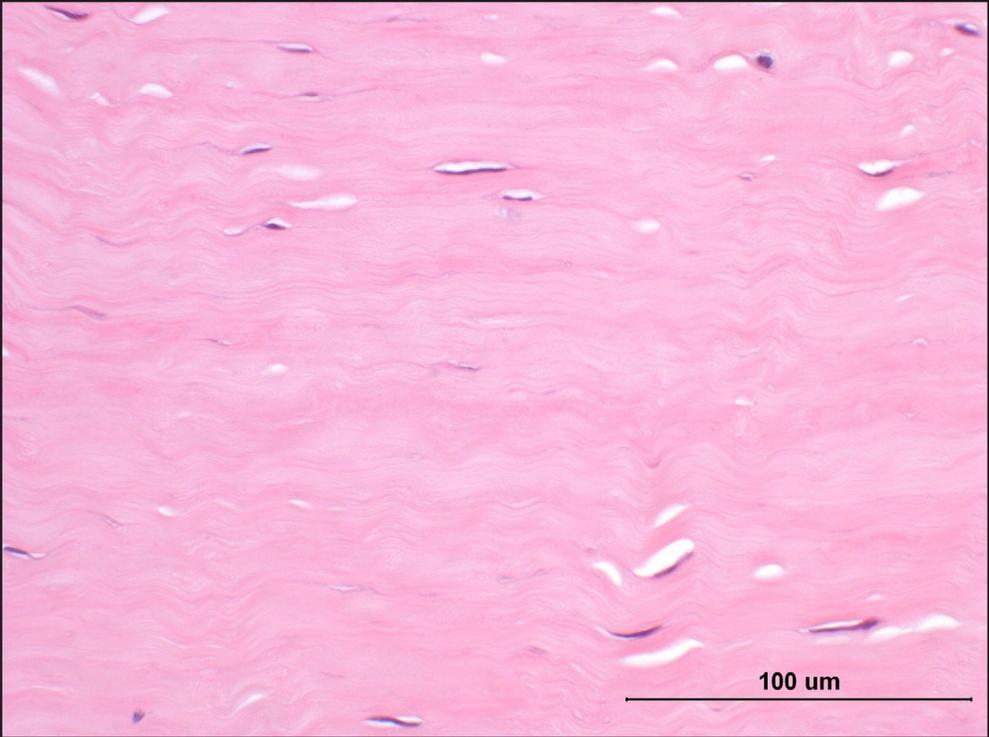


Figure 5. Negative Control (sterile, deionized water, 10 minute exposure/240 minute post incubation) - Epithelium (Slide C1231-1, 40x, H&E)

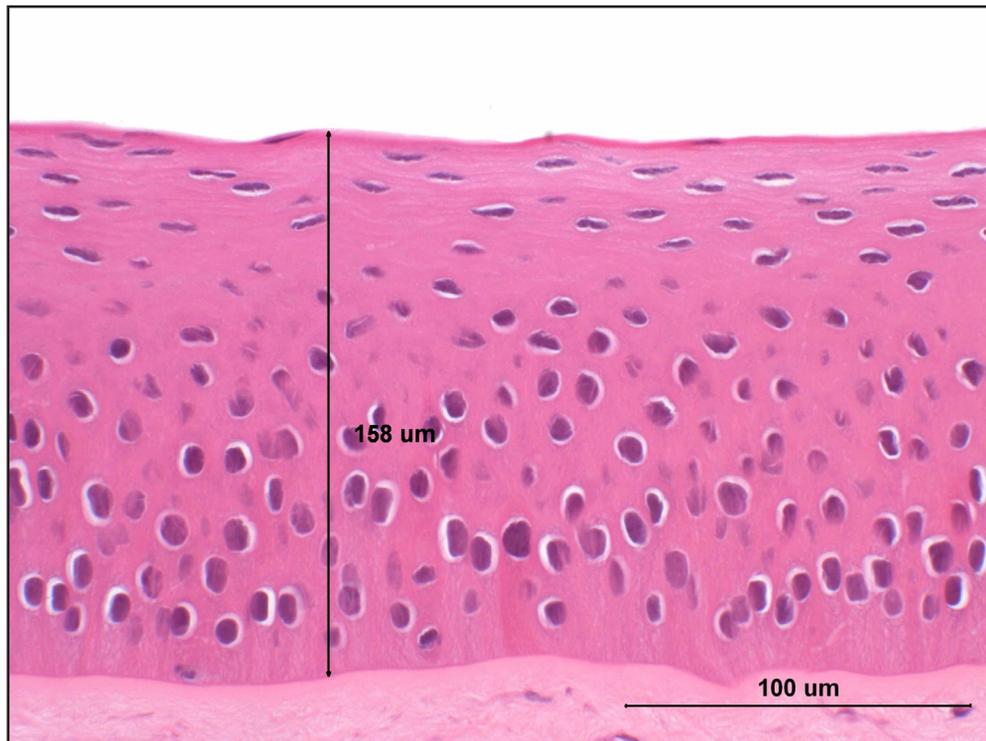


Figure 6. Negative Control (sterile, deionized water, 10 minute exposure/240 minute post incubation) - Full thickness (Slide C1231-1, 4x, H&E)

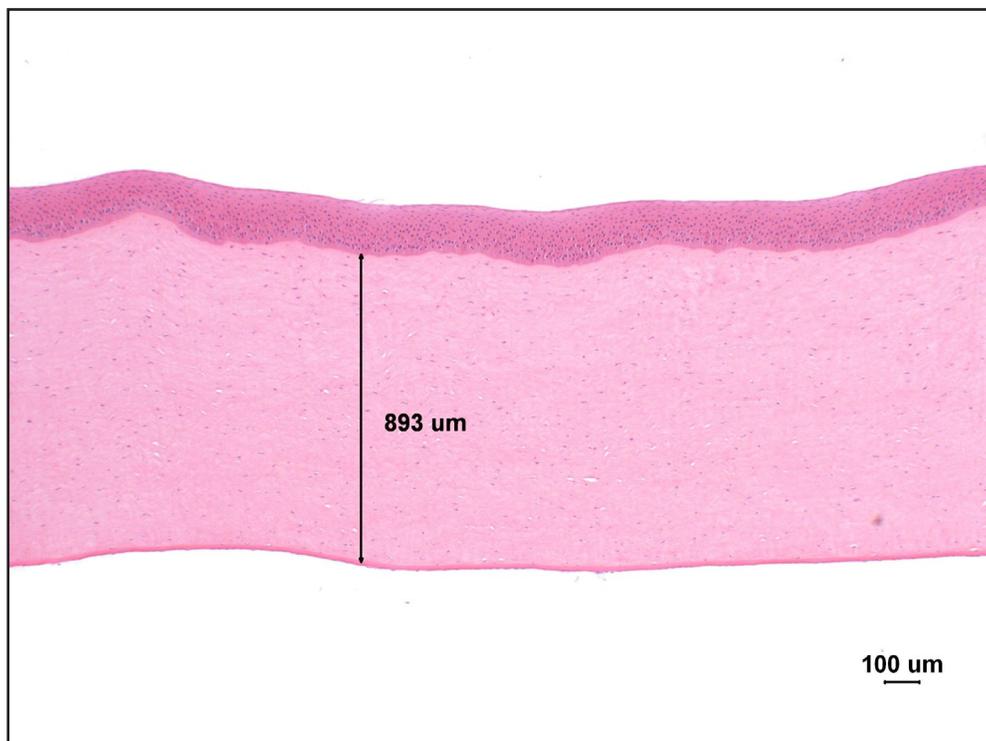


Figure 7. Negative Control (sterile, deionized water, 10 minute exposure/240 minute post incubation) - Stroma directly below Bowman's Layer (Slide C1231-1, 40x, H&E)

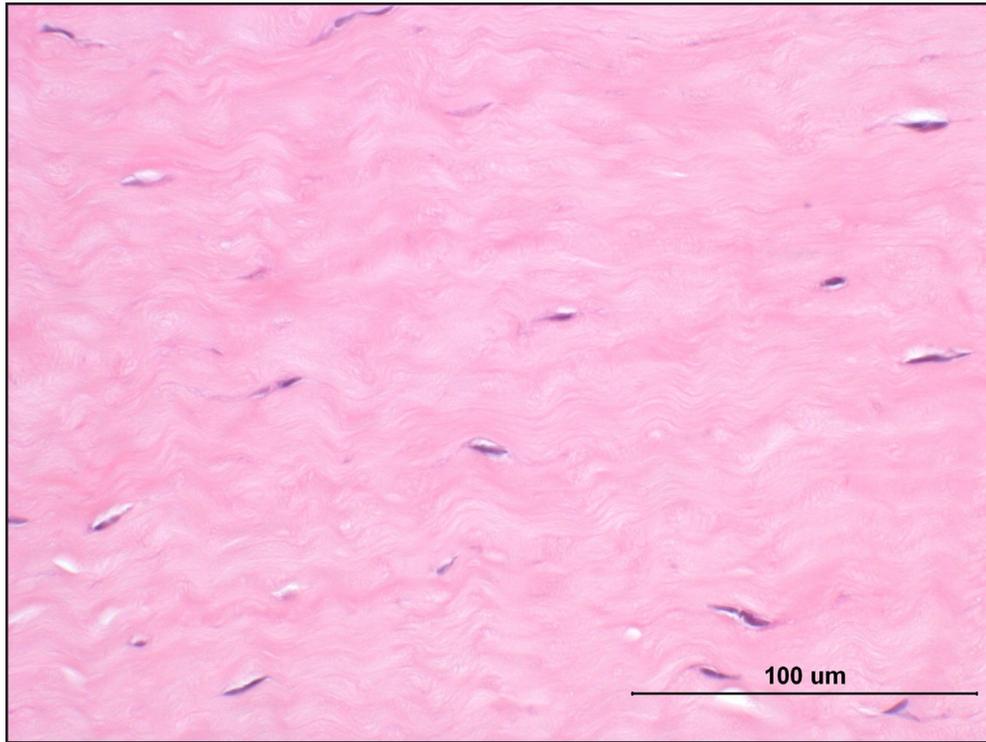


Figure 8. Negative Control (sterile, deionized water, 10 minute exposure/1200 minute post incubation) - Epithelium (Slide C1236-1, 40x, H&E)

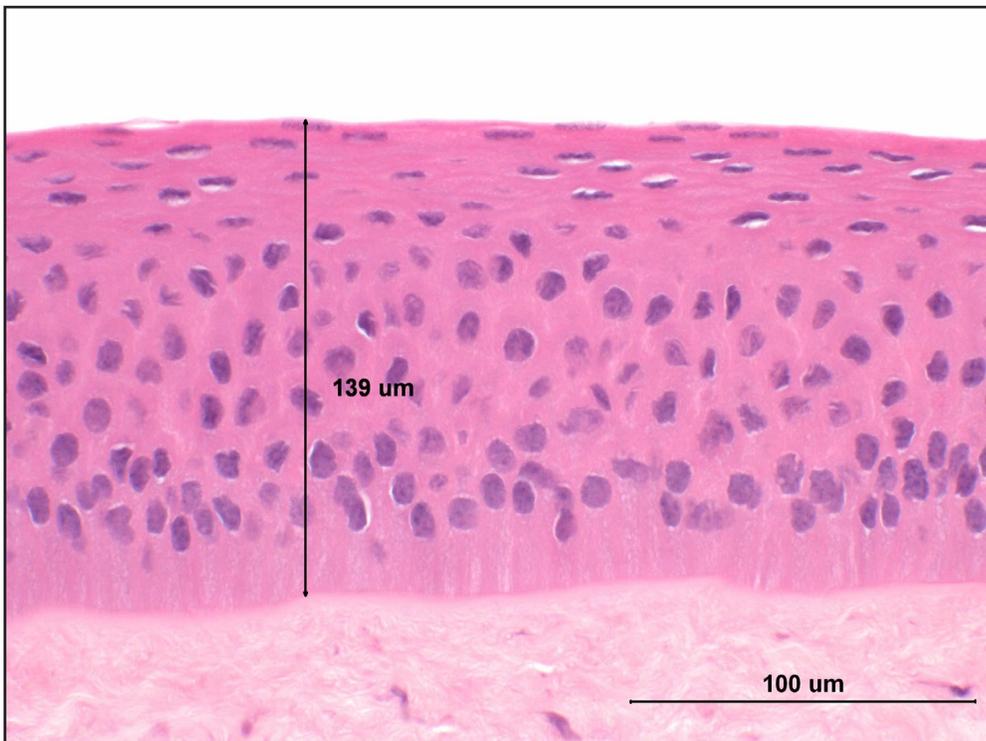


Figure 9. Negative Control (sterile, deionized water, 10 minute exposure/1200 minute post incubation)
- Full thickness (Slide C1236-1, 4x, H&E)

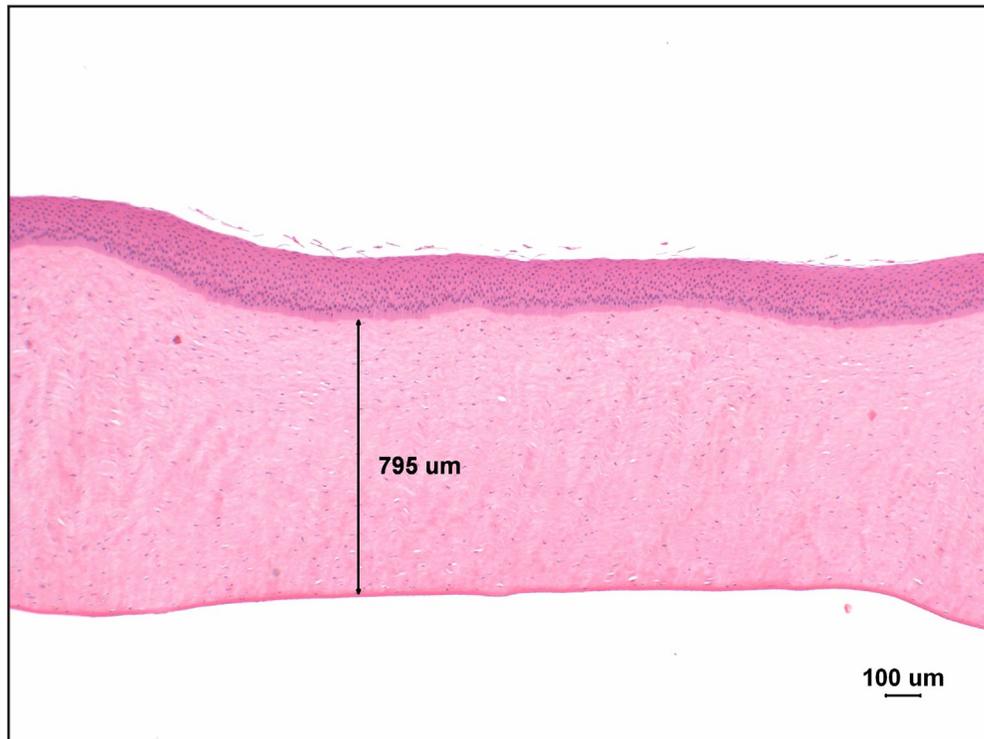


Figure 10. Negative Control (sterile, deionized water, 10 minute exposure/1200 minute post incubation)
- Superficial stroma with expansion of the collagen matrix (→) (Slide C1235-1, 40x, H&E)

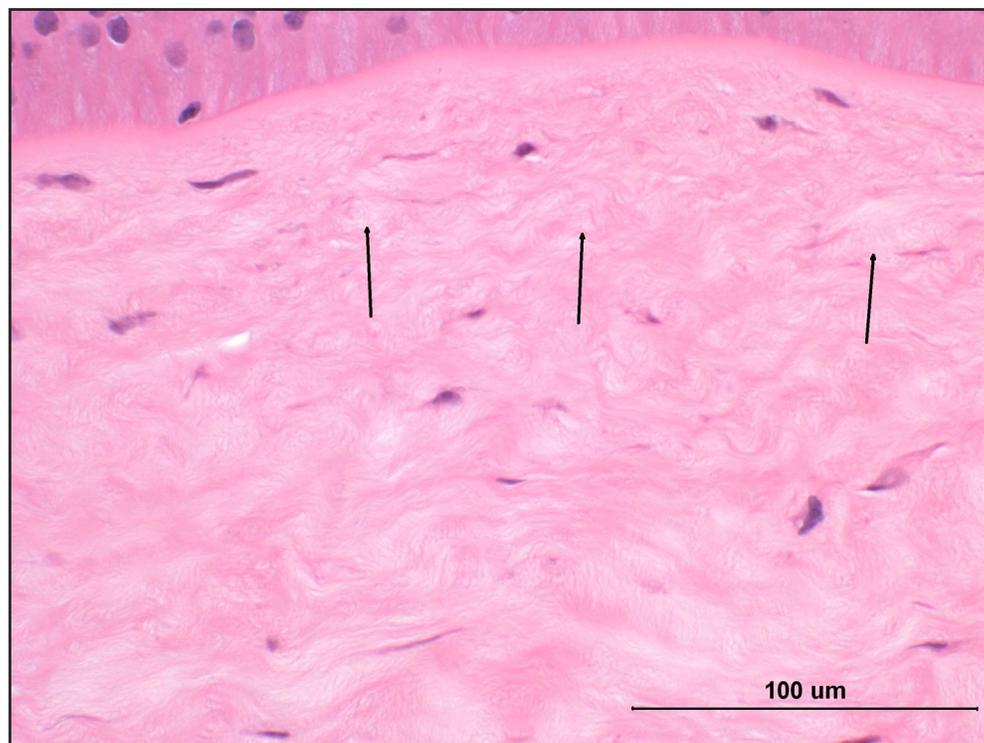


Figure 11. Positive Control, 100% Ethanol (10 minute exposure/120 minute post incubation) - Epithelium with coagulation of the squamous layer (A →) and vacuolation of wing and basal cell nuclei (B →) (Slide C1239-1, 40x, H&E)

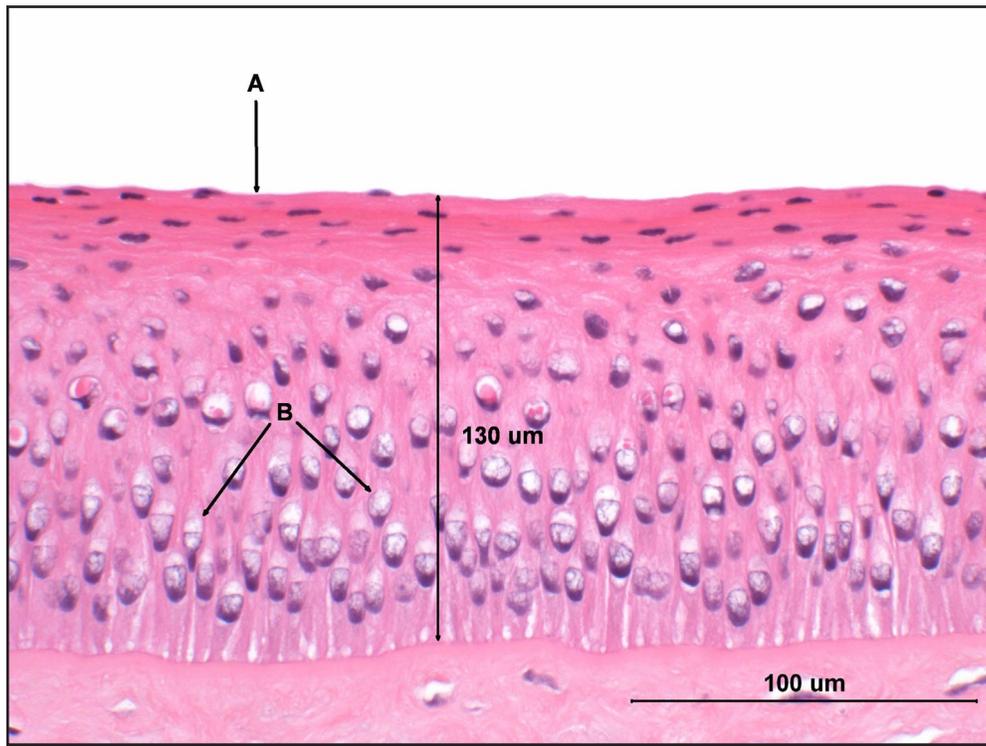


Figure 12. Positive Control, 100% Ethanol (10 minute exposure/120 minute post incubation) - Full thickness (Slide C1237-1, 4x, H&E)

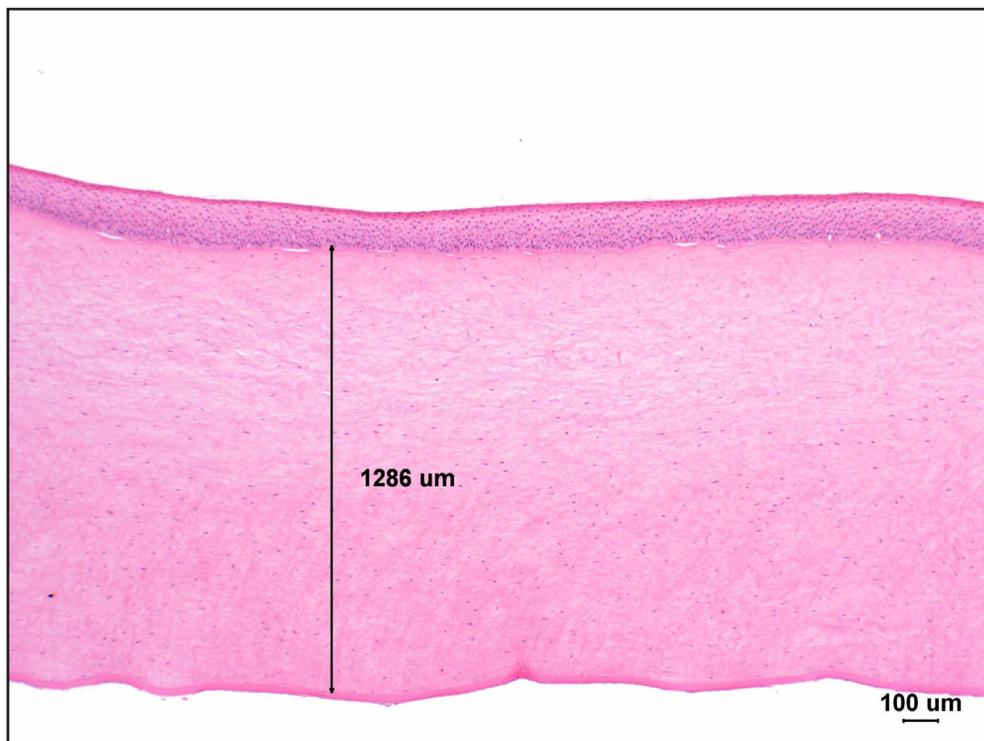


Figure 13. Positive Control, 100% Ethanol (10 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (→) (Slide C1237-1, 40x, H&E)

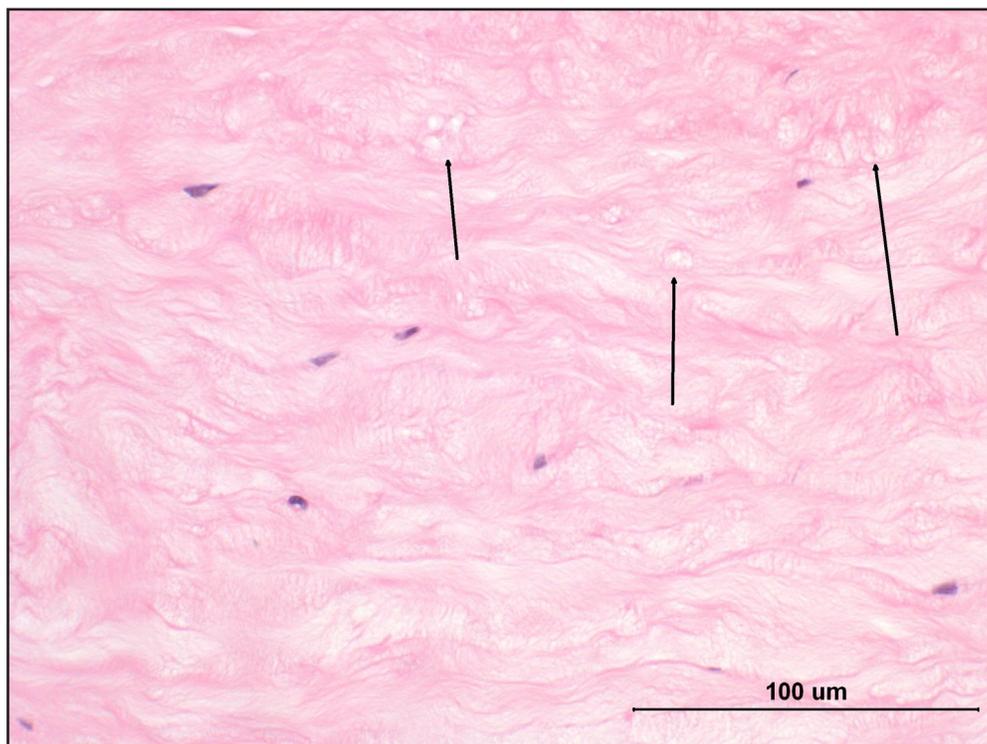


Figure 14. Positive Control, 100% Ethanol (10 minute exposure/120 minute post incubation) - Upper stroma with vacuolated keratocyte nuclei (→) (Slide C1237-1 40x, H&E)

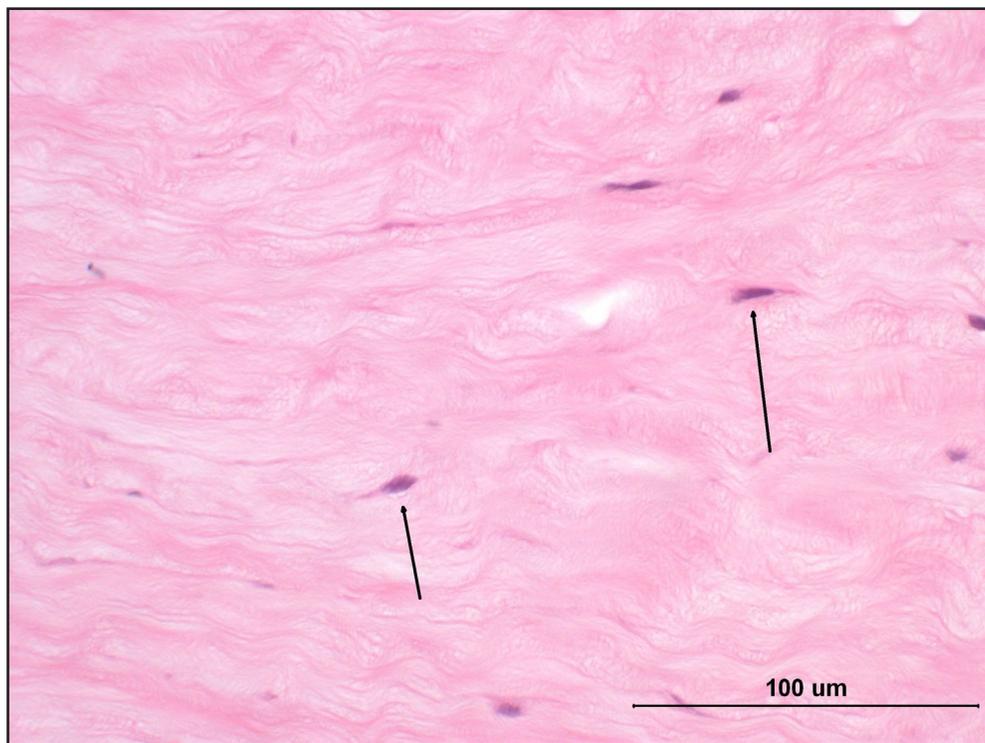


Figure 15. Positive Control, 100% Ethanol (10 minute exposure/120 minute post incubation) - Mid stroma with eosinophilic cytoplasm (—→) (Slide C1238-1, 40x, H&E)

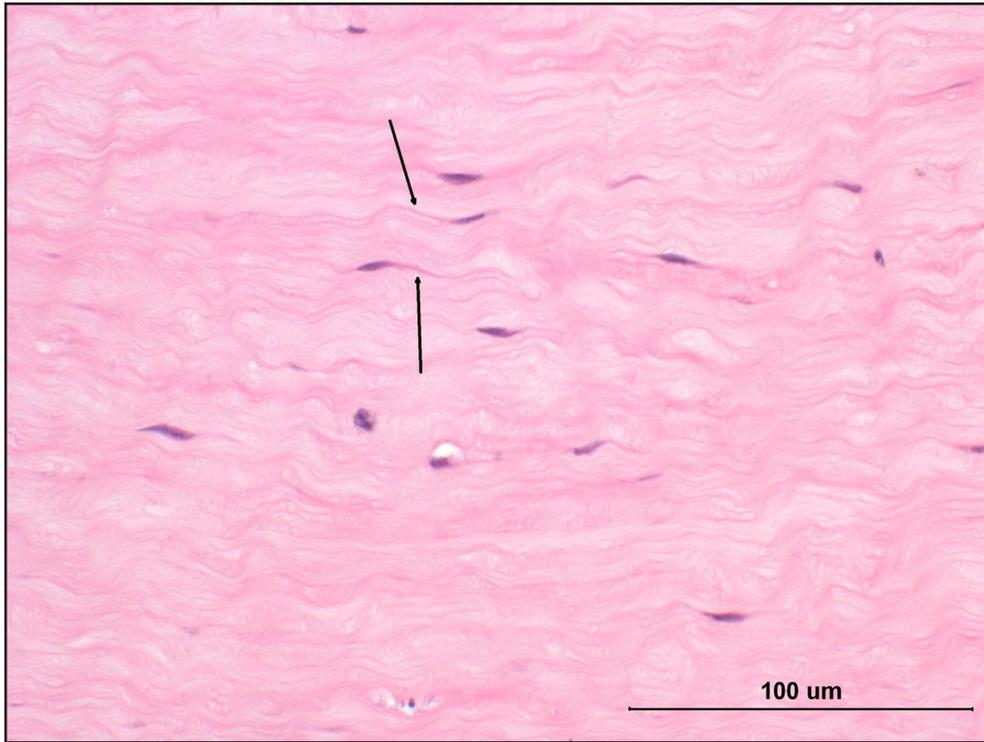


Figure 16. 06AH39, BS (10 minute exposure/240 minute post incubation) - Epithelium with blanched squamous layer, and hyper eosinophilic and vacuolated wing and basal layers (Slide C1241-1, 40x, H&E)

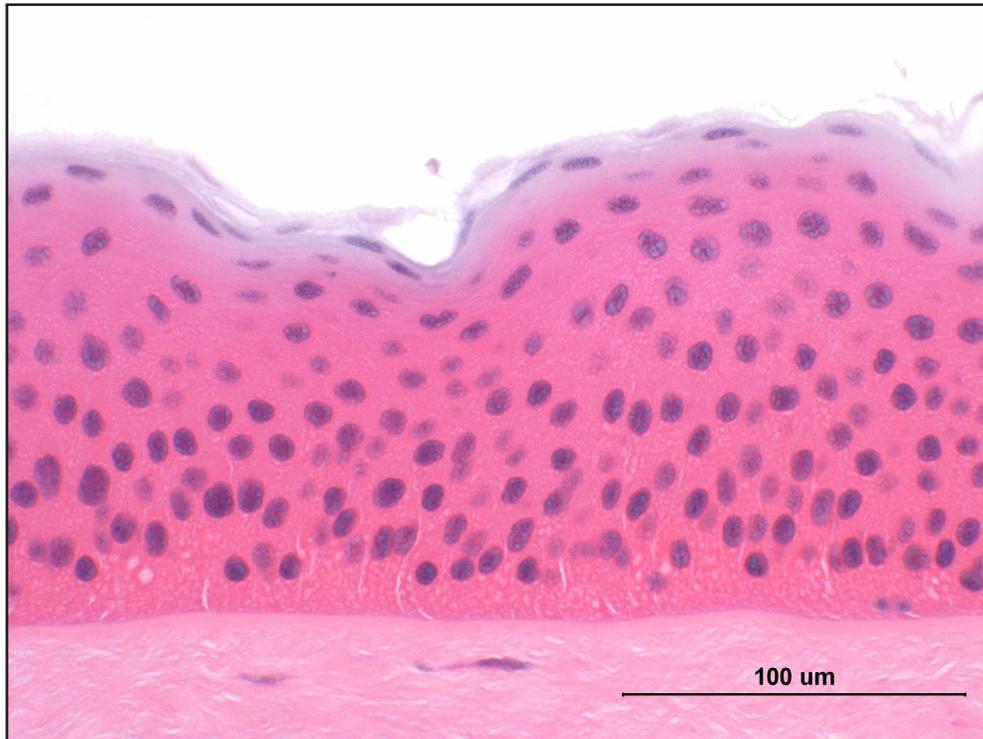


Figure 17. 06AH39, BS (10 minute exposure/240 minute post incubation)- Full thickness (Slide C1241-1, 4x, H&E)

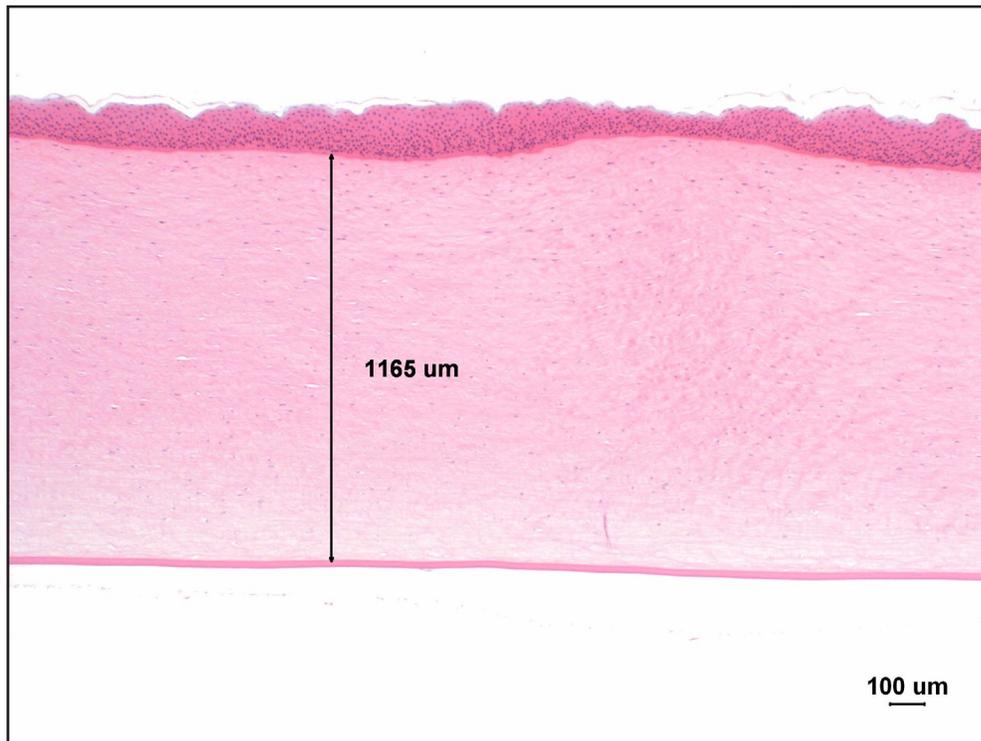


Figure 18. 06AH39, BS (10 minute exposure/240 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →) and vacuolated keratocyte nuclei (B →) (Slide C1241-1, 40x, H&E)

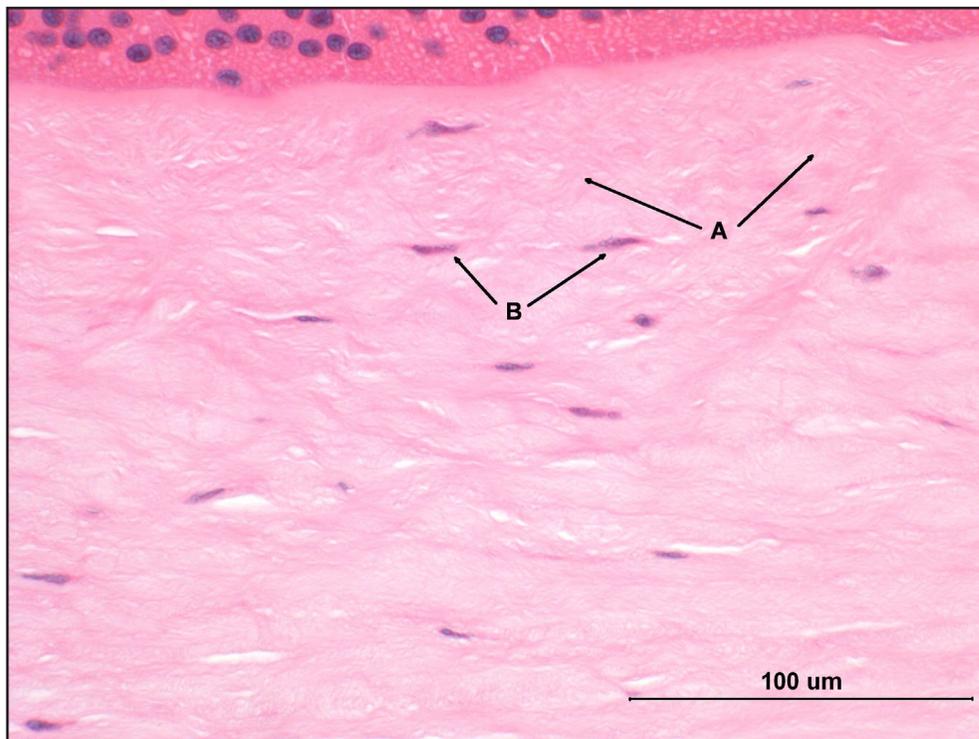


Figure 19. 06AH39, BS (10 minute exposure/240 minute post incubation) - Lower stroma with expansion of the collagen matrix (A →) and vacuolated keratocyte nuclei (B →) (Slide C1241-1, 40x, H&E)

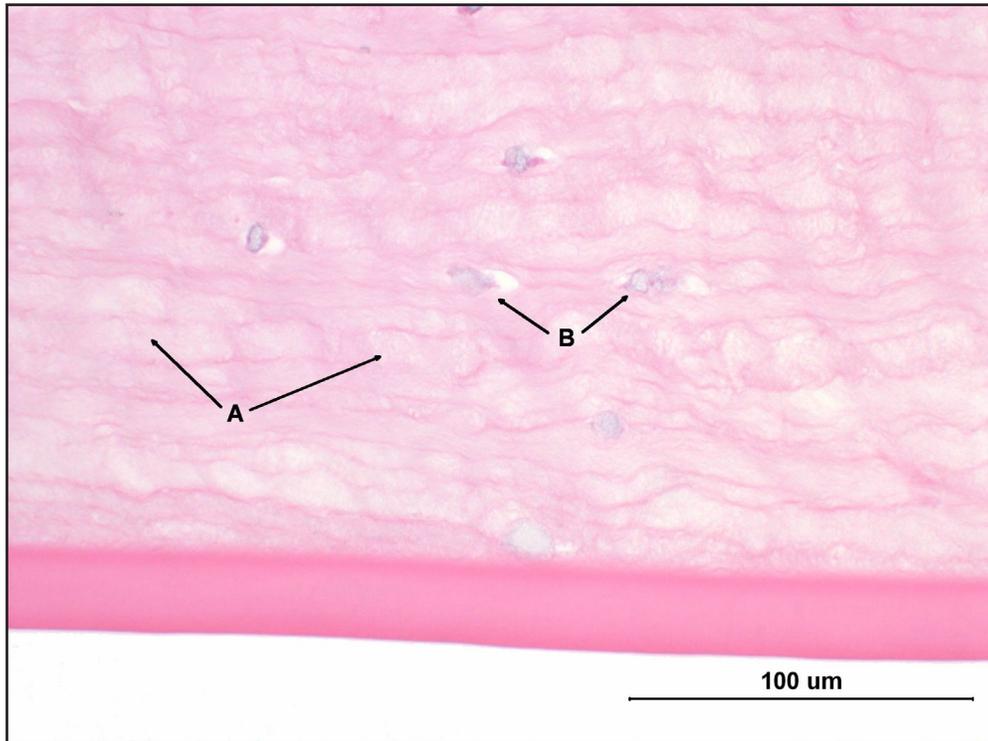


Figure 20. 06AH39, BS (10 minute exposure/240 minute post incubation) - Keratocytes in upper stroma with eosinophilic cytoplasm (→) (Slide C1241-1, 40x, H&E)

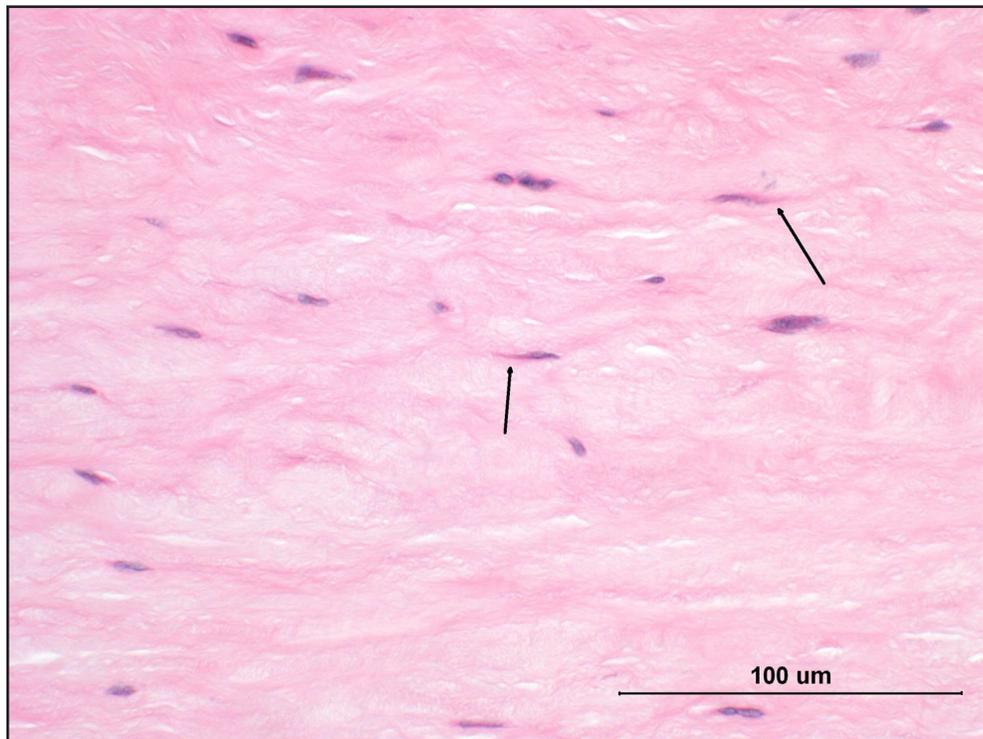


Figure 21. 06AH39, BS (10 minute exposure/1200 minute post incubation) - Epithelium with blanched squamous layer, hypereosinophilic and vacuolated wing and basal layers, and diffuse loss of basal cell adhesion (Slide C1247-1, 40x, H&E)



Figure 22. 06AH39, BS (10 minute exposure/1200 minute post incubation) - Full thickness (Slide C1247-1.1RC, 4x, H&E)

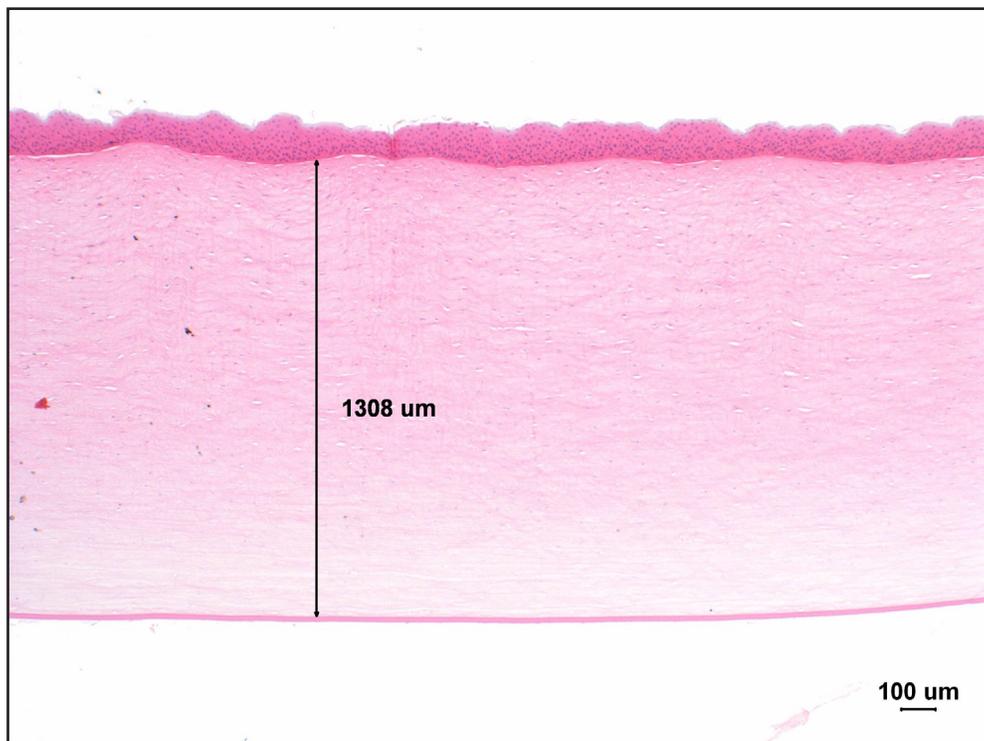


Figure 23. 06AH39, BS (10 minute exposure/1200 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →), vacuolated keratocyte nuclei (B →), and keratocytes with eosinophilic cytoplasm (C →) (Slide C1247-1.1RC, 40x, H&E)

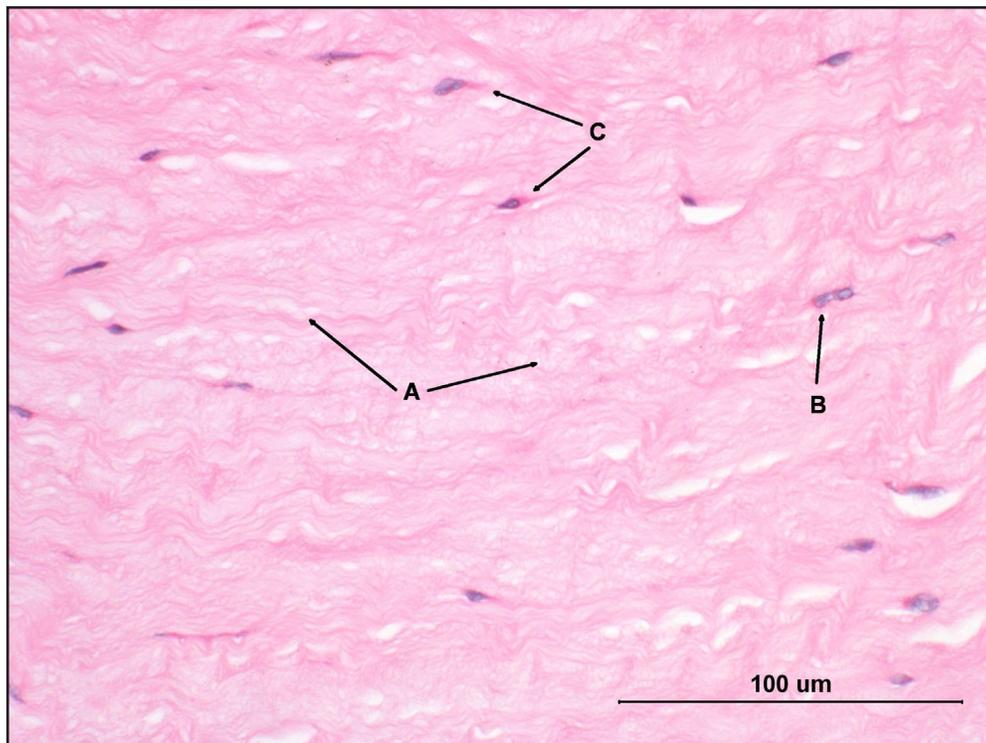


Figure 24. 06AH39, BS (10 minute exposure/1200 minute post incubation) - Lower stroma with expansion of the collagen matrix (A →), vacuolated keratocyte nuclei (B →), and artifactual tearing of the stroma due to tissue fragility (C →) (Slide C1247-1.1RC, 40x, H&E)

