

FINAL REPORT

Study Title

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

Test Substances

G, V, A, I, J, P, R

Reference Substance

H

Authors

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Study Completion Date

December 13, 2005

Performing Laboratory

Institute for In Vitro Sciences, Inc.

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Study Number

05AE36-AE43.350064

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STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity And Permeability Assay With Two Time Exposures and Optional Histology of the test substances, G, V, A, I, H (reference substance), J, P, and R, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test substances have not been determined by the testing facility.

John W. Harbell, Ph.D.
Study Director

Date

QUALITY ASSURANCE STATEMENT

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

Study Number: 05AE36-AE43.350064

Study Director: John Harbell, Ph.D.

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. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice 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-Aug-05	18-Aug-05	31-Aug-05
Removal of the Test Article and Controls (05AE36-AE40)	18-Aug-05	08-Aep-05	19-Aep-05
PAI – Quality Control	19-Oct-05	26-Oct-05	12-Nov-05
PAI – Data Audit	25-Oct-05	26-Oct-05	12-Nov-05
Histology – Negative Control and 05AE37, 3 minutes	23-Nov-05	23-Nov-05	05-Dec-05
Final Report and Data	13-Dec-05	13-Dec-05	13-Dec-05

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

SIGNATURE PAGE

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

Initiation Date: August 17, 2005

Completion Date: December 13, 2005

Sponsor:

Sponsor's Representative:

Testing Facility: Institute for In Vitro Sciences, Inc.
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Laboratory Management: Greg Mun, B.A.

Principle Investigator (slide preparation): David A. Hodge HT (ASCP)
Pathology Associates,
A Charles River Company

Histological Evaluation performed by: John W. Harbell, Ph.D.

TEST SUBSTANCE AND REFERENCE SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions *
05AE36	G	cloudy dark green non-viscous liquid	8/1/05	room temperature
05AE37	V	clear light green non-viscous liquid	8/1/05	room temperature
05AE38	A	cloudy green non-viscous liquid	8/1/05	room temperature
05AE39	I	clear very light blue non-viscous liquid	8/1/05	room temperature
05AE40	H (reference substance)	clear colorless non-viscous liquid	8/1/05	room temperature
05AE41	J	clear blue non-viscous liquid	8/1/05	room temperature
05AE42	P	clear pink non-viscous liquid	8/1/05	room temperature
05AE43	R	clear colorless non-viscous liquid	8/1/05	room temperature

* - Protected from exposure to light

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

INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test substances to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test substances. An *in vitro* score was determined for each of two exposure times tested for each test substance based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas.

The purpose of this study was to evaluate the potential ocular irritancy of the test substances as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. The laboratory phase of this study was conducted from August 18, 2005 to November 25, 2005 at the Institute for In Vitro Sciences, Inc. Three corneas were treated with each test substance at two exposure times of 3 and 10 minutes. Based on changes in corneal opacity and permeability (relative to the control corneas), an *in vitro* score was determined at each exposure time.

MATERIALS AND METHODS

Bovine Eyes

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.

Preparation of Corneas

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 2 mM 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.

Controls

The positive control used in this study was neat ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

Test Substance Preparation

As instructed by the Sponsor, each test substance was administered to the test system without dilution.

Test Substance pH Determination

The pH of each test substance was determined using pH paper (EMD Chemicals Inc./ EM Science). Initially, each test substance was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test substance was added to 5-10 and/or 7.5-14 pH paper with 0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

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. 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.

Method for Testing Liquid or Surfactant Materials

The liquid test substances, G, V, A, I, H, J, P, and R, 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. A set of three corneas was incubated in the presence of each test substance at $32 \pm 1^\circ\text{C}$ for 3 minutes. A second set of three corneas was incubated in the presence of each test substance at $32 \pm 1^\circ\text{C}$ for 10 minutes. A set of three corneas was incubated in the presence of the positive control at $32 \pm 1^\circ\text{C}$ for 10 minutes. A set of three corneas was incubated in the presence of the negative control at $32 \pm 1^\circ\text{C}$ for 10 minutes. Each test substance was tested in at least one valid definitive assay. The reference substance, H, was tested in all assays for comparison. 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 controls or test substances. 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 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 μL 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.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual pre-labeled 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 will be stored up to one year.

Histological Evaluation

The Sponsor elected histological evaluation for the corneas treated with three of the test substances. The fixed corneas were transferred to Pathology Associates, A Charles River Company (Frederick, MD) 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 returned to IIVS for evaluation.

Presentation of Data

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 the test substances 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:

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

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:

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

Criteria for Determination of a Valid Test

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

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test substance. 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 40.1 to 65.0) each 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.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

Opacity measurements were taken immediately after rinsing and after the 2-hour post-exposure incubation. The corneas treated with test substances G and A showed appreciable increases in opacity after the 2-hour post-exposure compared with the values observed immediately after rinsing. Based on the OD₄₉₀ values obtained, there was complete loss (G) and a substantial loss (A) of epithelial barrier integrity in the corneas treated with these test substances.

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.

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score	pH	
8/18/05	05AE36	G	Neat	3 minutes	79.0	3.617	133.3	11.5	
				10 minutes	86.7	3.535	139.7		
	05AE37	V	Neat	3 minutes	0.3	0.214	3.5	10.0	
				10 minutes	5.0	1.050	20.8		
	05AE38	A	Neat	3 minutes	107.3	1.698	132.8	14.0	
				10 minutes	166.0	2.725	206.9		
	05AE39	I	Neat	3 minutes	-0.7	0.023	-0.3	11.5	
				10 minutes	-1.0	0.104	0.6		
	05AE40	H	Neat	3 minutes	0.0	0.117	1.8	12.0	
				10 minutes	0.0	0.613	9.2		
	8/22/05	05AE40	H*	Neat	3 minutes	-0.7	0.225	2.7	12.0
					10 minutes	1.0	0.696	11.4	
05AE41		J	Neat	3 minutes	1.0	0.105	2.6	7.0	
				10 minutes	4.7	0.200	7.7		
05AE42		P	Neat	3 minutes	-0.3	-0.001	-0.3	9.5	
				10 minutes	1.0	0.006	1.1		
05AE43		R	Neat	3 minutes	-0.7	0.002	-0.6	6.0	
				10 minutes	0.0	0.015	0.2		

*- Reference substance, tested in each assay

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
8/18/05	Ethanol	10 minutes	26.0	1.416	47.2
8/22/05	Ethanol	10 minutes	29.3	1.064	45.3

The Sponsor elected to have histological evaluation performed on the corneas treated with three of the test substances. Test substances V and I and the reference substance H, from the assay performed on August 18, 2005, were evaluated. The results of that evaluation are presented below.

Histological Evaluation

Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen in the cornea. This is not surprising as 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 (not from this study). 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 substances appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo*. 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 6). 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 rabbit²

Special effort has been made to detect changes in the stromal elements of the corneas. Jester³, Maurer^{4,5} and others have shown for a range of chemical classes that depth of injury in

² 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, Kuntz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food Chemical Toxicology** 34:79-117.

³ Jester, J.V., Li, H.F., Petroll, W.M., Parker, R.D., Cavanaugh, H.D., Carr, G.J., Smith, B., and Maurer, J.K. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Invest Ophthalmol Vis Sci** 39:922-936.

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 these vacuoles may be seen in Figure 9 where the positive control exposure has induced some stromal swelling. The depth and degree of vacuolization can be indicative of the degree of injury to the cornea and/or penetration of the test substance into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix vacuolization (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 substance penetration or mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test substance exposure tends to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix vacuolization will be located in the deep stroma (just above Descemet’s Membrane). In contrast, test substance-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix vacuolization 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 substance-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

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

⁵ Maurer, J.K., Parker, R.D., and Jester, J.V. (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.

cytoplasmic eosinophilia. Harbell and Curren⁶ 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 substance-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 substance 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 substance to the epithelium, one would expect that exposure to the stroma would progress from the area just 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 vacuolization 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 is actually estimated from the percentage of the stromal cross section that remained undamaged (starting at the posterior border). For example, a cornea reported to show collagen matrix vacuolization to 30% depth would mean that 70% of the cross section of that cornea (starting at Descemet's Membrane) did not show vacuolization. For this report, depth of stromal damage is reported as the percentage of the normal corneal depth (cross-section) involved, starting from the anterior border (Bowman's Layer). It should be clearly understood that the percentage of the stromal depth is only an estimate developed by evaluating several fields in each cornea (where possible). The values are, by necessity, approximations of an average depth to which the lesion extended (e.g., collagen matrix vacuolization). It would be unwise to try to compare small differences in the reported depth. Rather, one should focus on broader bands of 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 prepared using a Spot Insight Digital Camera and Spot 4.0.8 software (Diagnostic Instruments, Inc., Sterling Heights, MI). The color balance of the images was corrected to better represent the colors that would be seen through the microscope.

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 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 a very old animal). If the measured corneal section did not appear to be a

⁶ Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. *ALTEX* 42(Special Issue):236.

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. An example of such a measurement is seen in Figure 4. 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.

Histological Evaluation

The negative control corneas were treated for 10 minutes with sterile, deionized water (slides B8227-B8229). 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. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figures 2 and 3). Some of the nuclei in the epithelium showed clear zones between the nucleus and cytoplasm of the cells. These “halos” are the result of shifting of the nucleus within the section and are an artifact of processing rather than a change in viable tissue.

The stromal elements showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining to narrow, elongated and condensed with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. Rare cells, with eosinophilic cytoplasmic staining, were observed. Collagen bundles were generally parallel and well ordered (Stroma just under Bowman's Layer, Figure 4).

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

A cross section of the negative control showing the general thickness of the whole cornea and stroma is provided in Figure 5.

The positive control corneas (slides B8230-B8232), 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 6). 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 7). In the stroma directly below Bowman's Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate collagen matrix vacuolization extended to 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 8) and the remaining keratocytes showed a moderate increase in the frequency of cells with nuclear vacuolization and abnormal chromatin condensation (Figure 9). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 10). The endothelial cells were generally intact (similar to the negative control-treated

corneas).

Table 3
Histological Evaluation of the Test Substance-Treated Corneas

IIVS Number	Sponsor's Designation	Observations	Figure #
05AE37 Slides B8239- B8241	V, neat, 3- minute exposure, 120- minute post- exposure, 08/18/05	<p>Epithelium: The surface and upper portion of the deeper squamous epithelium were lost in all sections. The deep squamous, wing, and basal cells were intact and essentially the same as the similar cells in the negative control-treated corneas (Figures 11 and 12).</p> <p>Stroma: The test substance-treated corneas were similar in thickness to the negative control-treated corneas (Figure 13). No increased collagen matrix vacuolization or consistent changes in keratocytes were observed (Figure 14, directly below Bowman's Layer).</p> <p>Endothelium: Similar to the endothelium of the negative control-treated corneas.</p>	11 - 14
05AE37 Slides B8242- B8244	V, neat, 10- minute exposure, 120- minute post- exposure, 08/18/05	<p>Epithelium: The squamous and upper wing cell layers were lost in most sections. The wing and basal cells were intact but showed moderate cytoplasmic vacuolization (Figure 15). In some fields, there was also loss of cells through the basal cell layer (Figure 16). Bowman's Layer was intact in all sections.</p> <p>Stroma: The test substance-treated corneas were thicker than the negative control-treated corneas (Figure 17). Moderate collagen matrix vacuolization extended past mid depth. In the upper half of the stroma, the keratocyte nuclei were slightly enlarged and there was an increase in cytoplasmic eosinophilia (Figure 18). Under the zones of complete epithelium cell loss, the stroma showed marked to moderate collagen matrix vacuolization (in the upper third of the stroma) and the keratocytes showed more pronounced nuclear enlargement and cytoplasmic eosinophilia (Figure 19). These keratocyte changes are associated with stromal swelling rather than direct test substance action on the cells.</p> <p>Endothelium: One of the three corneas showed some collagen matrix vacuolization directly above Descemet's Membrane (indicating loss of</p>	15 - 19

IIVS Number	Sponsor's Designation	Observations	Figure #
		endothelial cell function) but the other two corneas did not. Thus, the damage to the one cornea may have been mechanical rather than the result of test substance exposure.	
05AE39 Slides B8251- B8253	I, neat, 3-minute exposure, 120-minute post-exposure, 08/18/05	Epithelium: The test substance-treated epithelium was essentially the same as the negative control-treated epithelium (Figure 20). Stroma: The test substance-treated corneas were similar in thickness to the negative control-treated corneas (Figure 21). No increased collagen matrix vacuolization or consistent keratocyte changes were observed (Figure 22, directly under Bowman's Layer). Endothelium: Similar to the endothelium of the negative control-treated corneas.	20 - 22
05AE39 Slides B8254- B8256	I, neat, 10-exposure, 120-minute post-exposure, 08/18/05	Epithelium: The treated corneas showed a loss of the surface and some of the deeper squamous epithelium. The wing and basal cells were similar to those of the negative control-treated corneas (Figure 23). Stroma: The test substance-treated corneas were similar to or just slightly thicker than the negative control-treated corneas (Figure 24). In the upper quarter of the stroma, there was a slight increase in collagen matrix vacuolization and a very slight increase in the frequency of keratocytes with some cytoplasmic eosinophilia (Figure 25). The deeper stroma was essentially normal. Endothelium: While there was some disruption of the endothelial cell layer, the absence of deep stromal swelling suggests that the damage occurred after fixation.	23 - 25
05AE40 Slides B8257- B8259	H, neat, 3-minute exposure, 120-minute post-exposure, 08/18/05	Epithelium: The treated corneas showed a loss of the surface and some of the deeper squamous epithelium. The wing and basal cells were similar to those of the negative control-treated corneas (Figure 26). Stroma: The test substance-treated corneas were similar to or just slightly thicker than the negative control-treated corneas (Figure 27). In the upper quarter of the stroma, there was a slight increase in collagen matrix vacuolization and a very slight increase in keratocytes with some	26 - 28

IIVS Number	Sponsor's Designation	Observations	Figure #
		cytoplasmic eosinophilia (Figure 28). The deeper stroma was essentially normal. Endothelium: Similar to the endothelium of the negative control-treated corneas.	
05AE40 Slides B8260- B8262	H, neat, 10-minute exposure, 120-minute post-exposure, 08/18/05	<p>Epithelium: The squamous cell layer was lost in all fields. In the majority of fields, the upper wing cells were lost or damaged and there was an increase in cytoplasmic vacuolization in the remaining wing and basal cells (Figures 29 and 30). In a minority of the fields, the damage penetrated into or through the basal cells (Figure 31). Bowman's Layer was intact in all sections.</p> <p>Stroma: The test substance-treated corneas were slightly thicker than the negative control-treated corneas (Figure 32). Under areas of intact basal cells, moderate collagen matrix extended to mid depth. Under areas of full epithelial loss, marked vacuolization extended through the upper quarter of the stroma and moderate vacuolization extended past mid depth. Of particular note were the keratocyte changes. There was a marked increase in the frequency of keratocytes with abnormal chromatin condensation or full nuclear pyknosis in the upper two thirds of the stroma (Figures 33 and 34). These changes were observed under zones of both more intact epithelium and full epithelial cell loss. This histological picture is suggestive selective action on the keratocytes consistent with a reactive chemistry.</p> <p>Endothelium: While the cell layer was damaged in a minority of the fields, there was very limited stromal vacuolization directly above Descemet's Membrane suggesting that much of the damage may have occurred late in the incubation or after fixation.</p>	29 - 34

The figures displayed on the subsequent pages of this report are representative hematoxylin and eosin-stained cross-sections presented at the indicated magnification. The black bar, on each micrograph, represents 100 μm . Arrows from the text to the figures are intended to show examples of the lesions mentioned. Not all lesions are marked. The vertical double-headed arrows mark the limits of the epithelium in the appropriate figures.

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (magnification 290x)

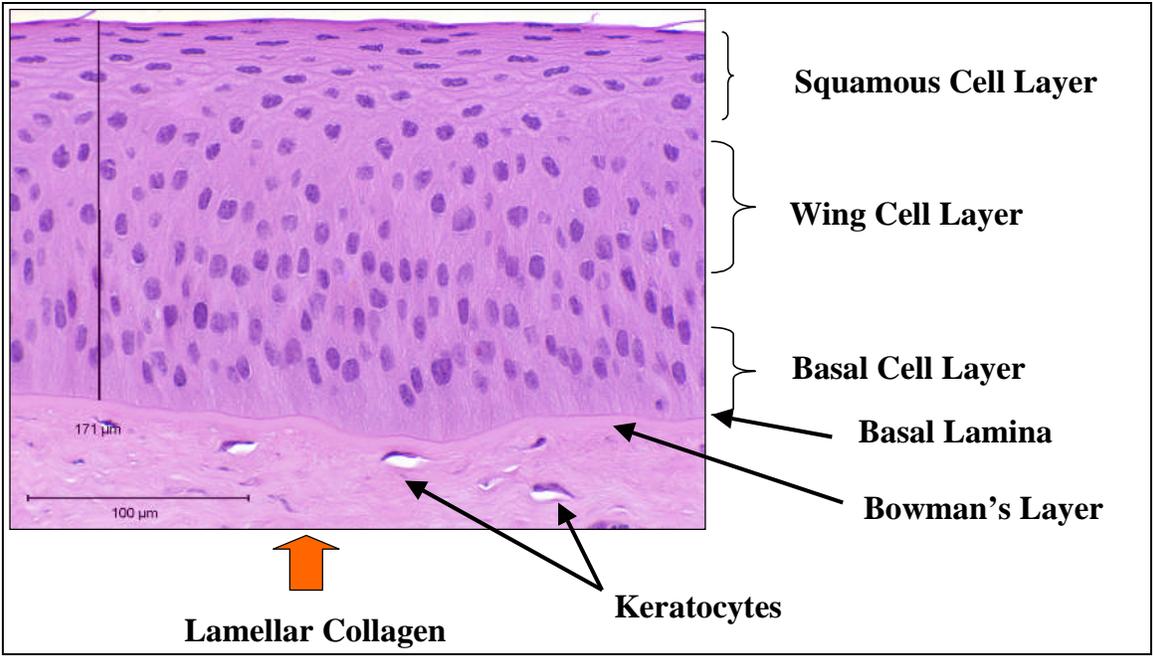


Figure 2. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium (magnification 237x)

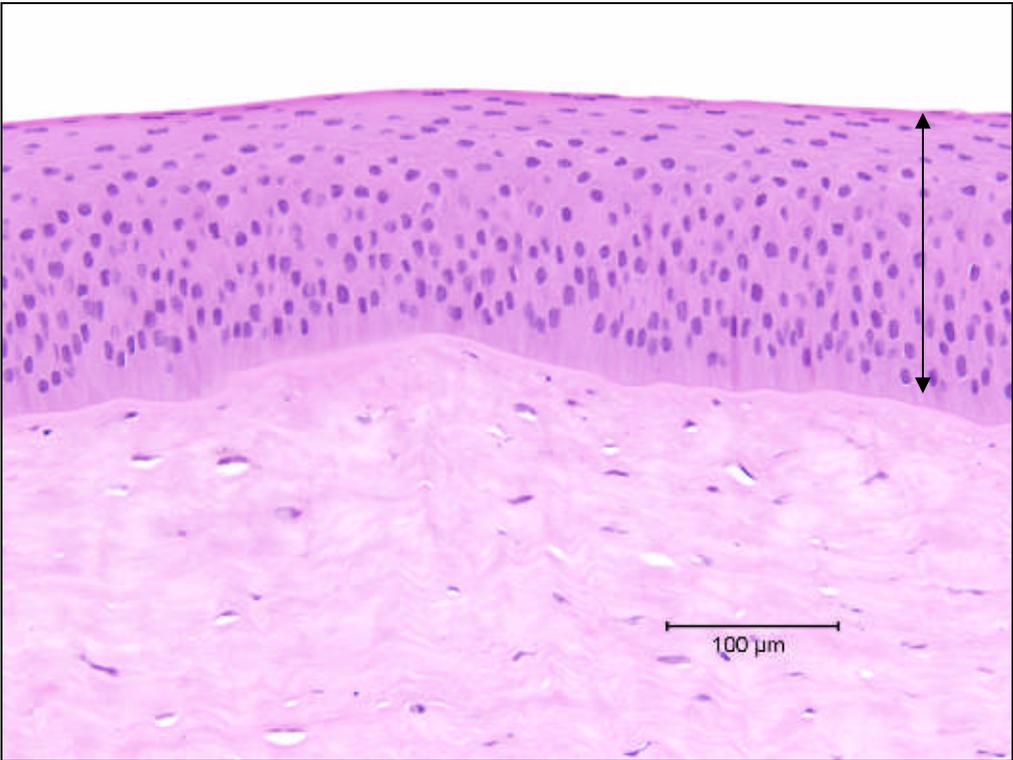


Figure 3. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium (magnification 475x)

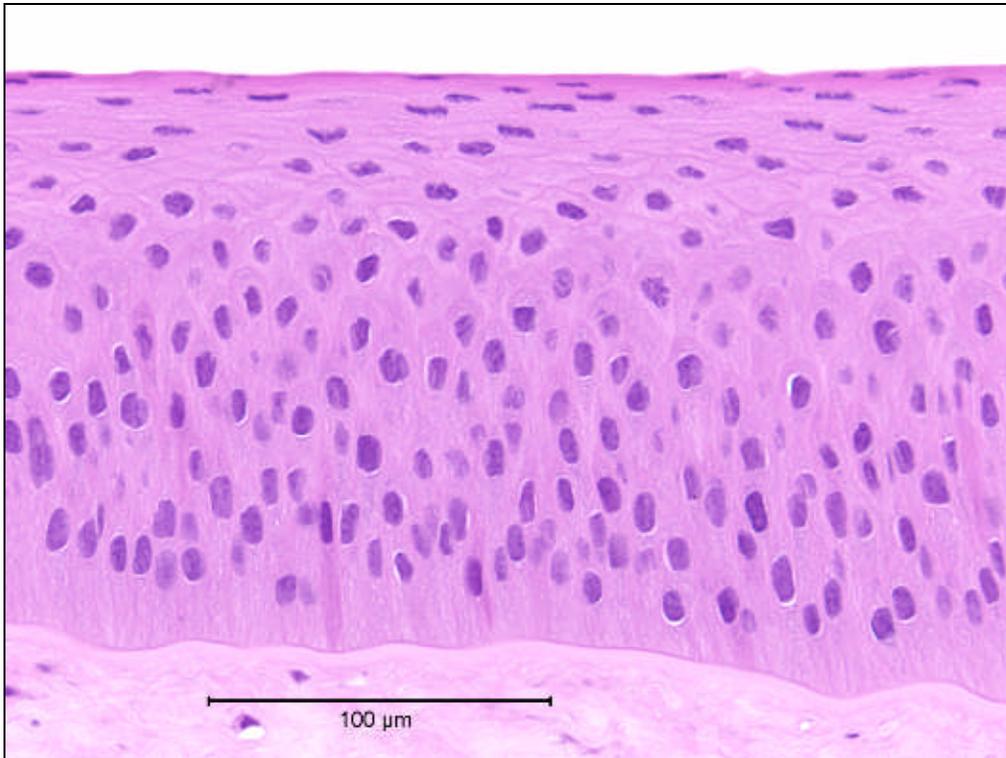


Figure 4. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma directly below Bowman's Layer (magnification 475x)

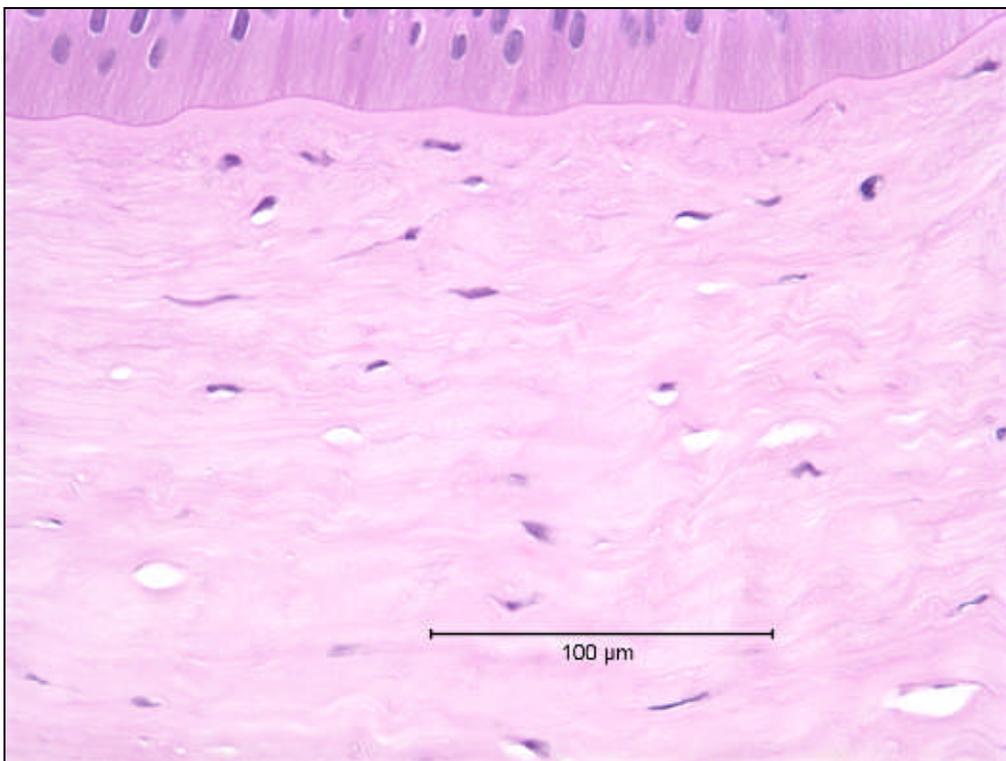


Figure 5. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

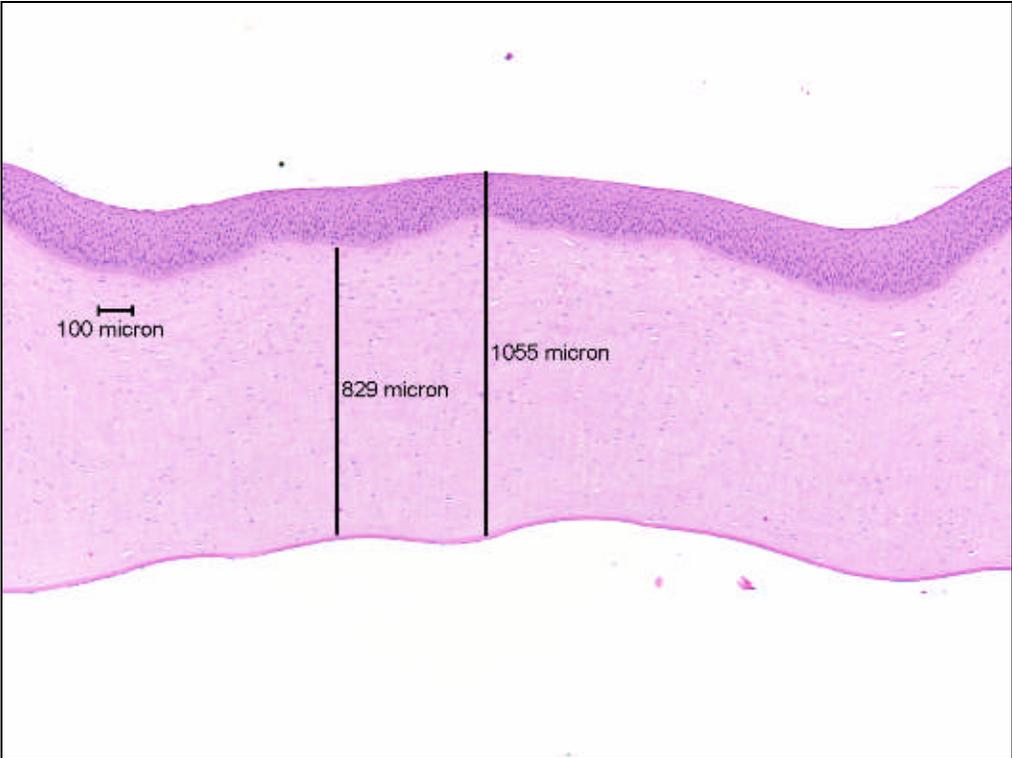


Figure 6. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium (probably not viable at the time of fixation) (magnification 237x)

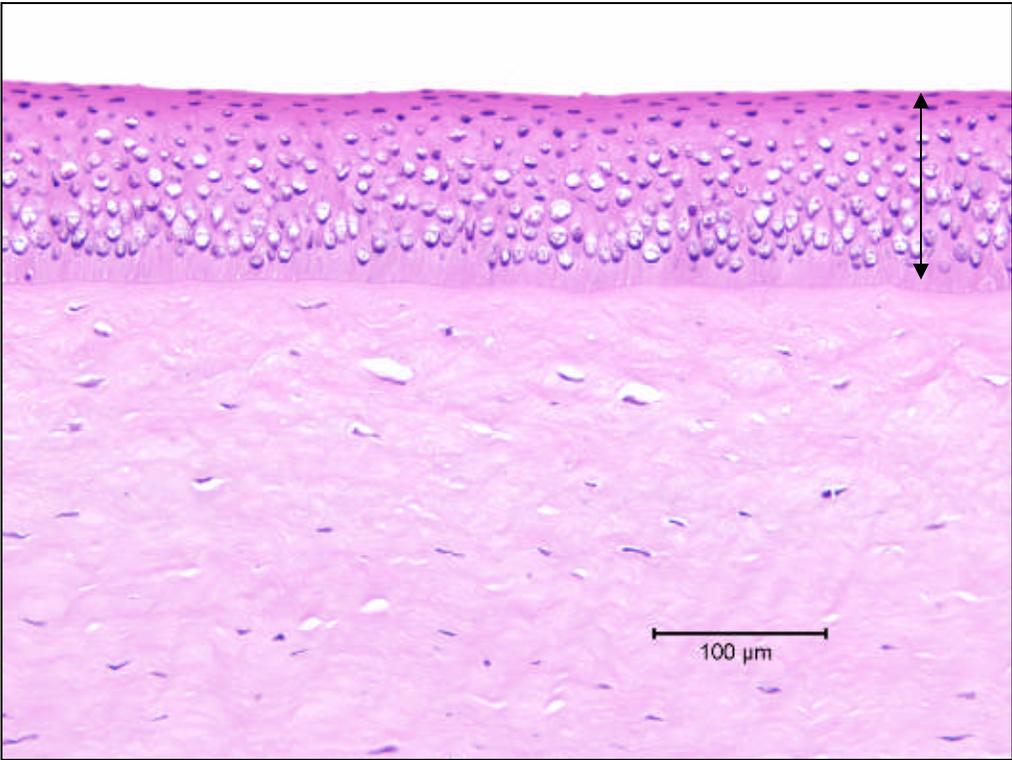


Figure 7. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

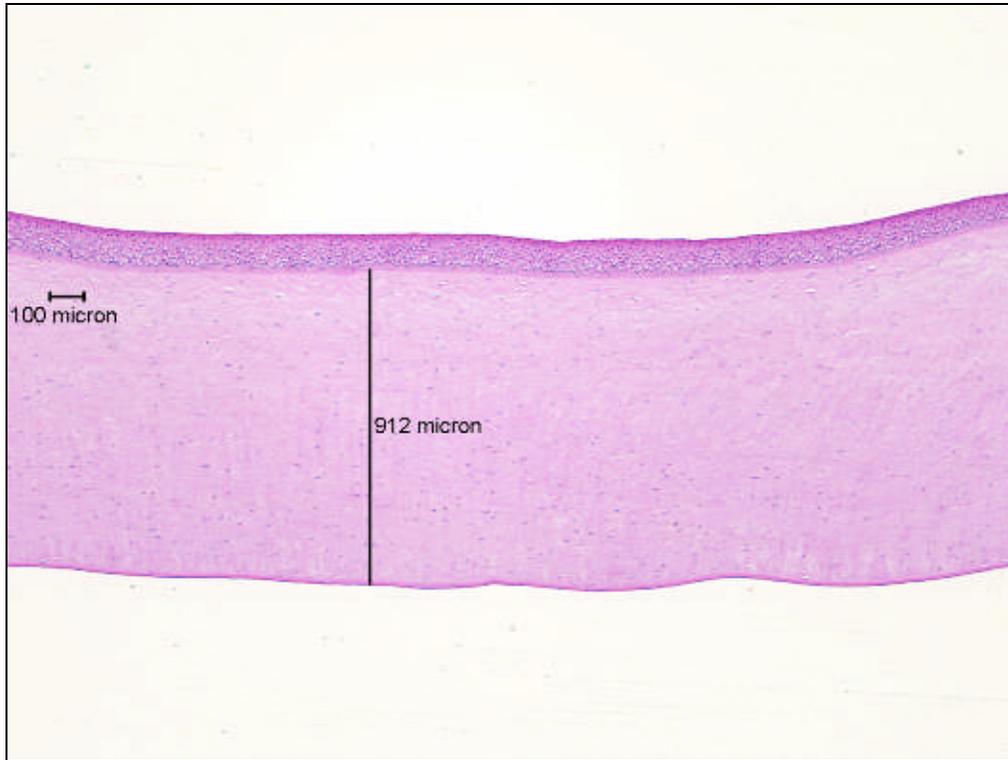


Figure 8. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (08/18/05) - Upper stroma showing hyperchromatic staining in the zone directly below Bowman's Layer and the decrease in the density of viable keratocytes (magnification 237x)

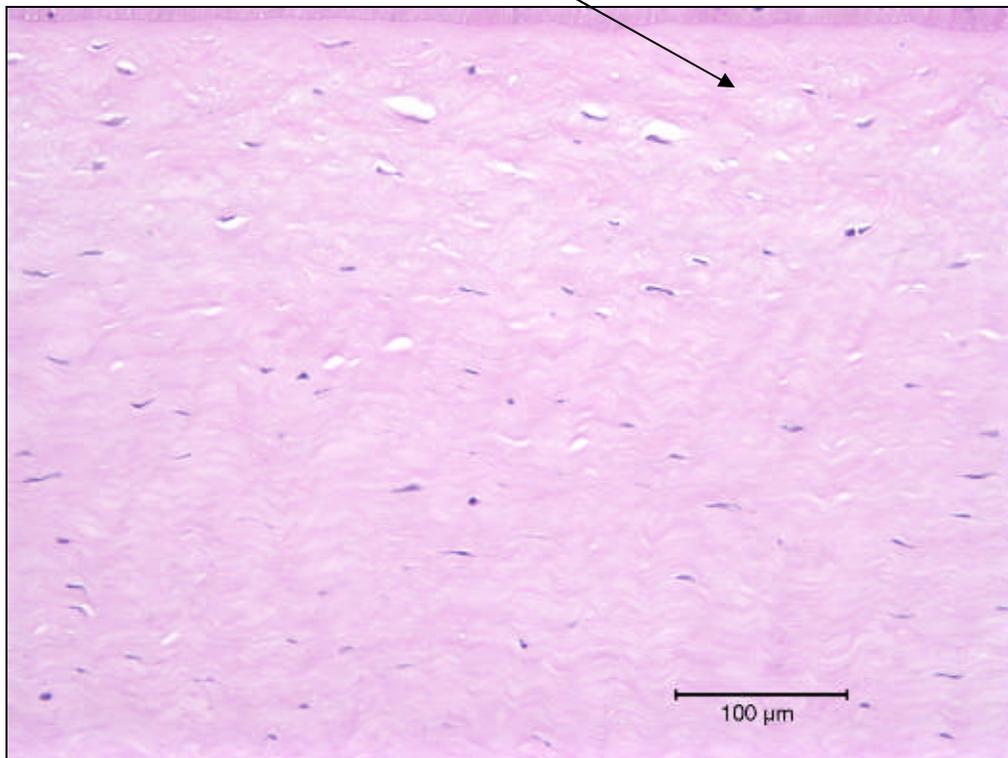


Figure 9. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and an increased frequency of keratocytes with abnormal chromatin condensation (magnification 475x)

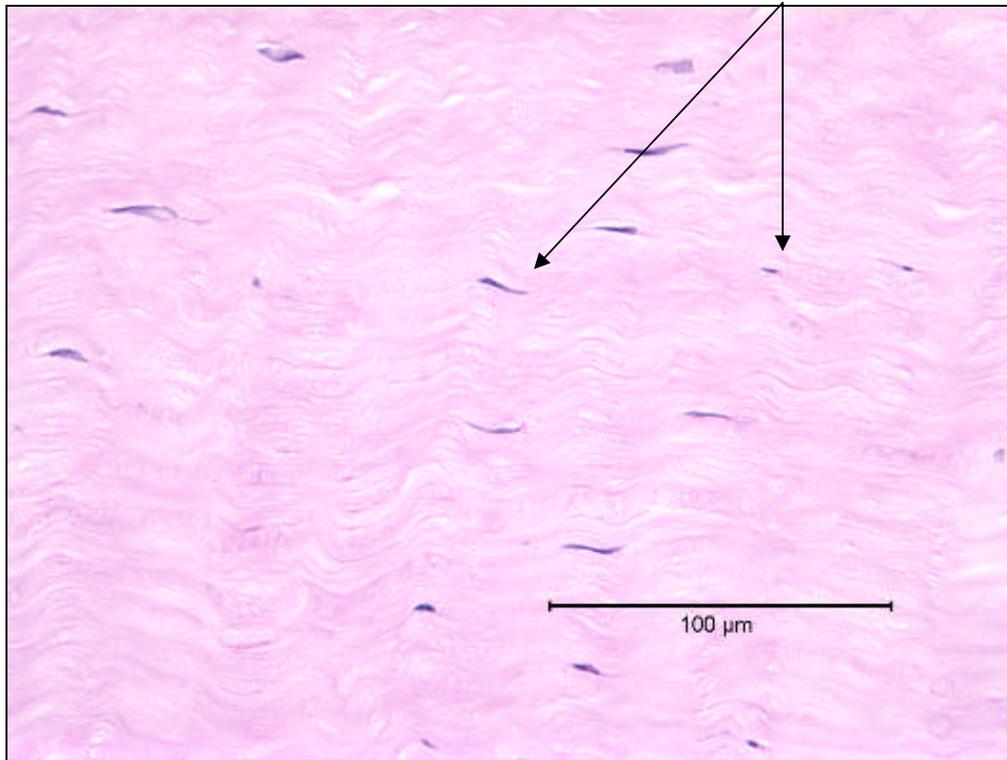


Figure 10. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma just below mid depth showing keratocytes with nuclear enlargement and cytoplasmic eosinophilia (magnification 475x)

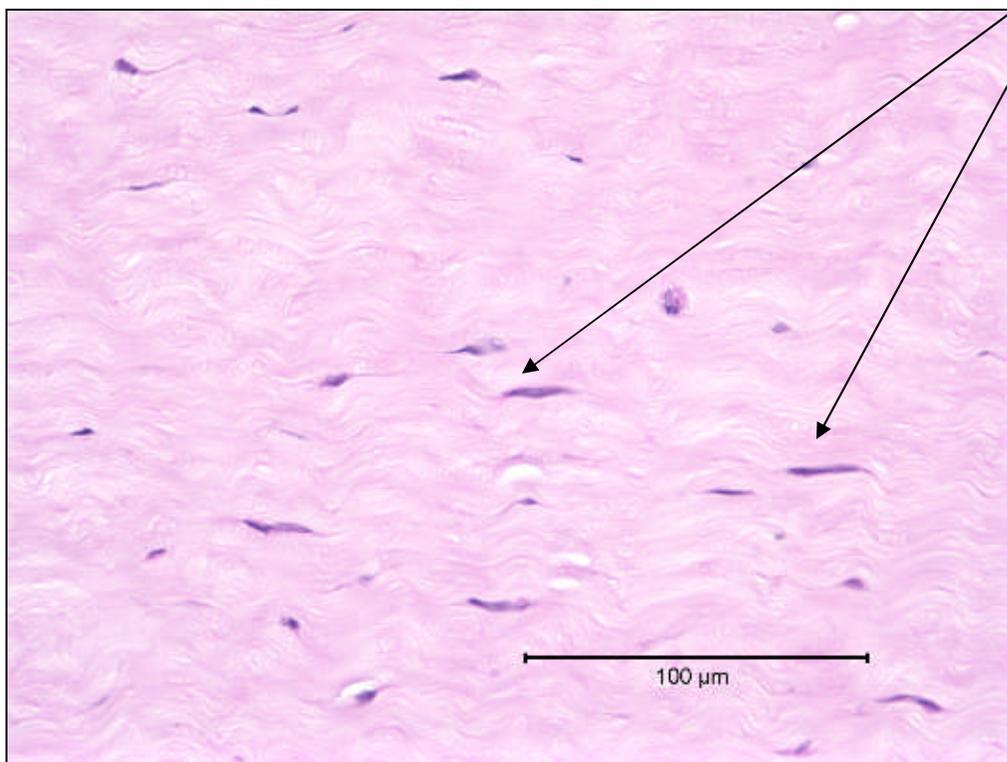


Figure 11. V, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium (magnification 237x)

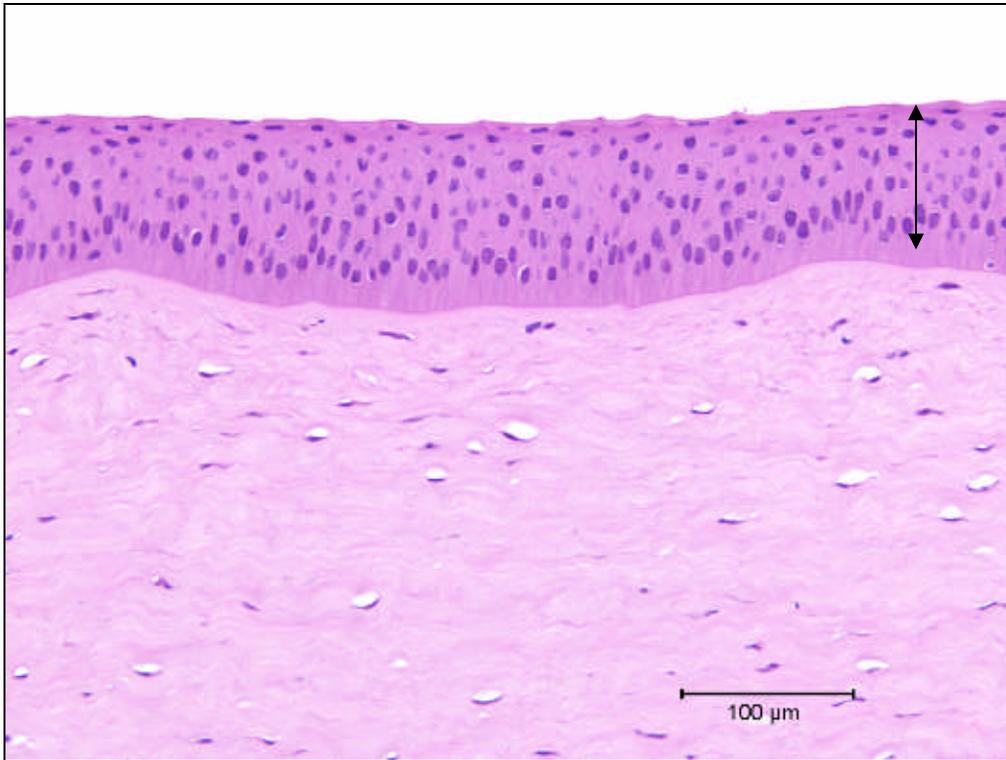


Figure 12. V, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing the loss of the surface squamous epithelium (magnification 475x)

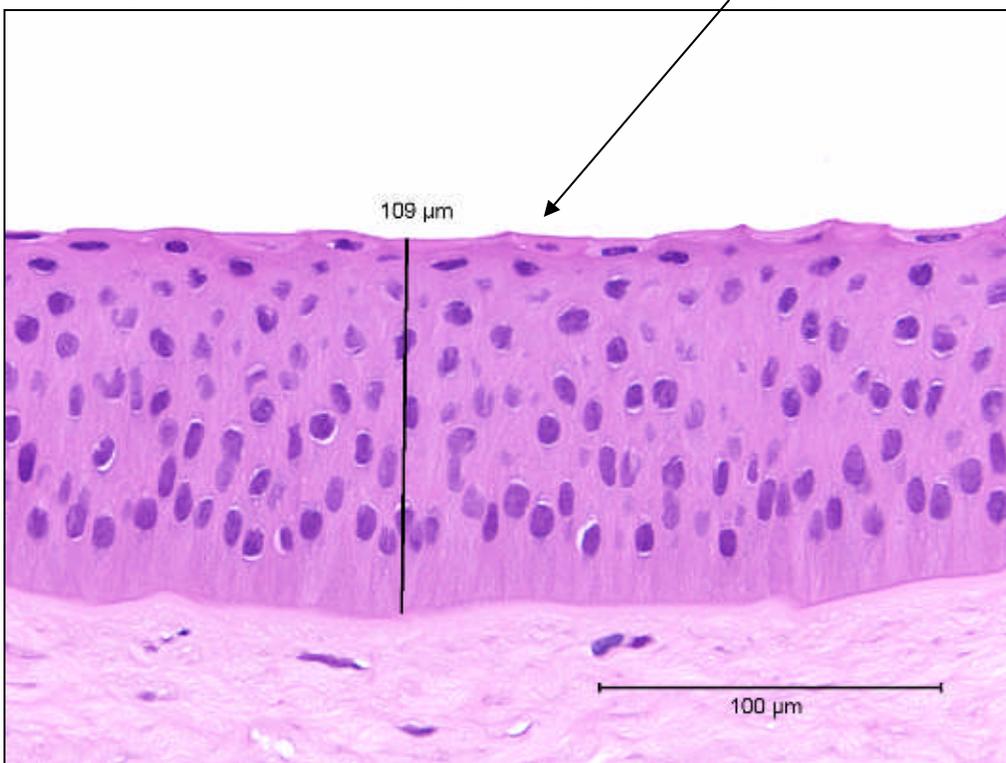


Figure 13. V, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

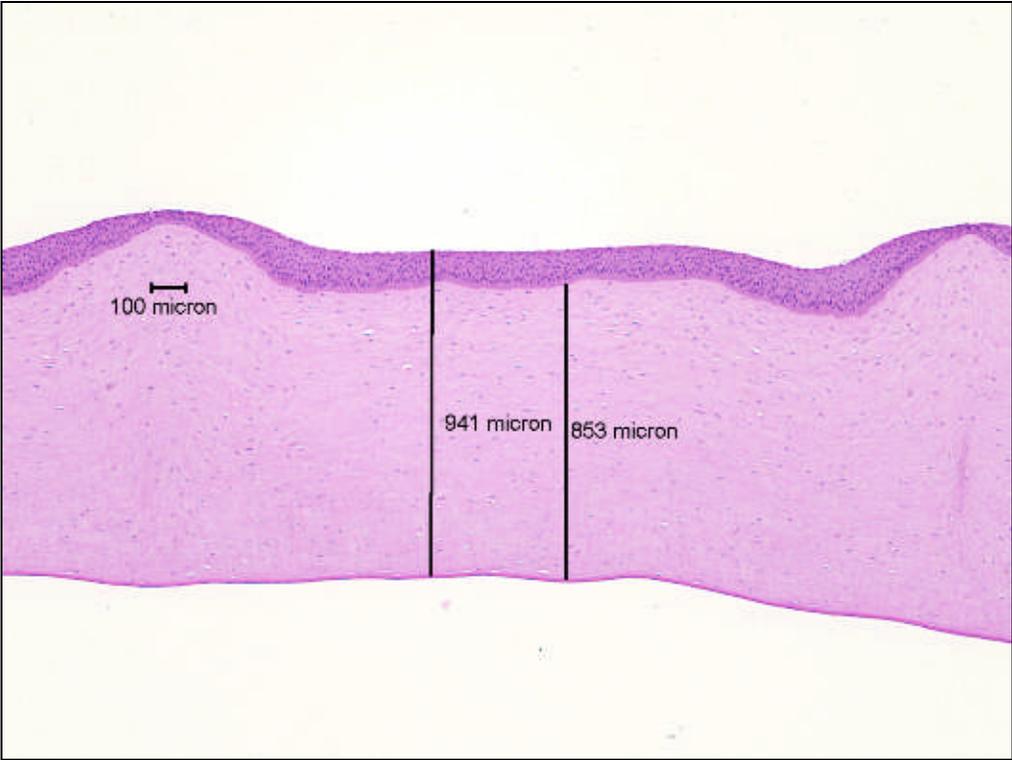


Figure 14. V, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Stroma directly below Bowman's Layer (magnification 475x)

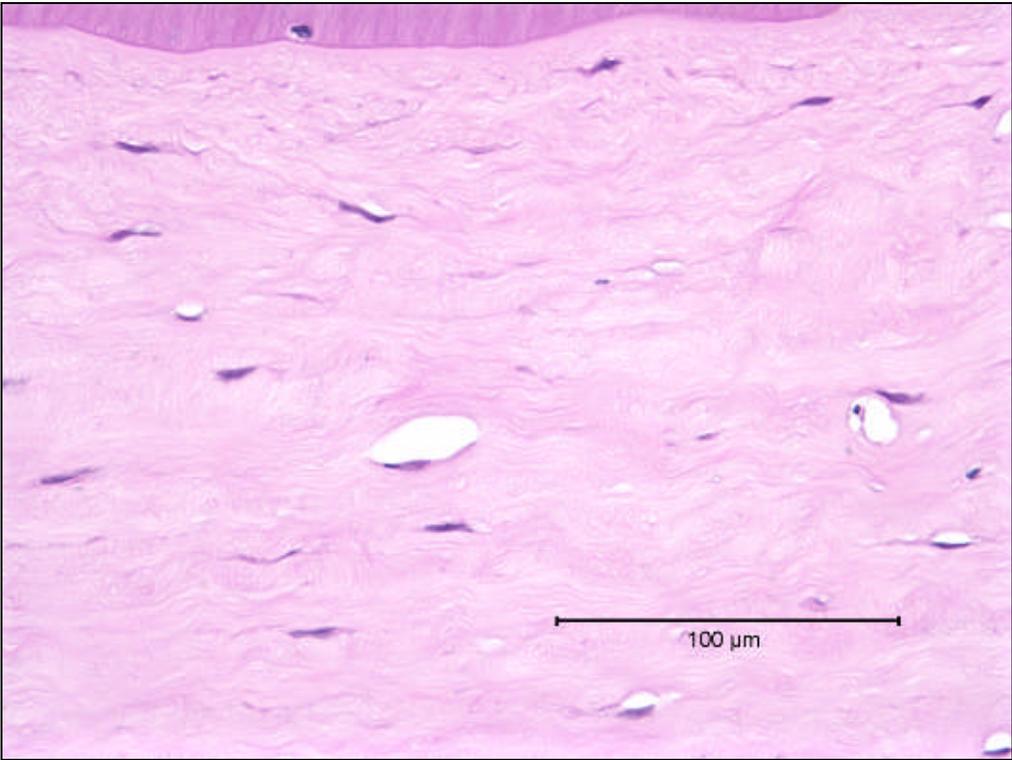


Figure 15. V, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing loss of the squamous and upper wing cell layers (magnification 237x)

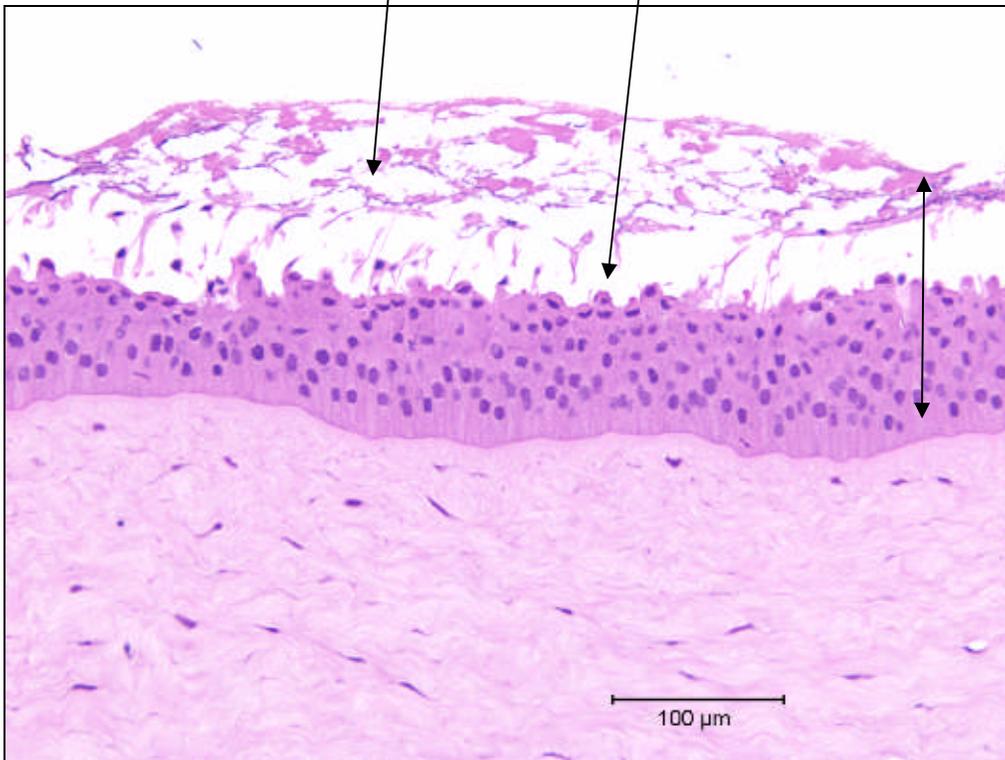


Figure 16. V, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing a lesion through the full epithelium (magnification 237x)

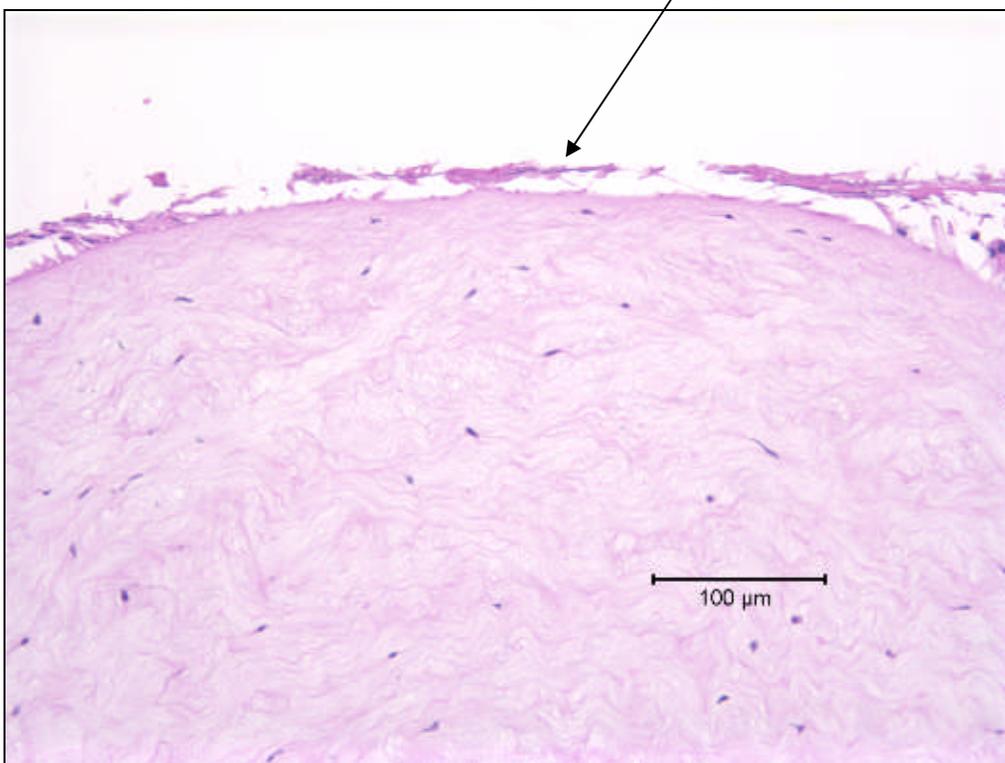


Figure 17. V, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

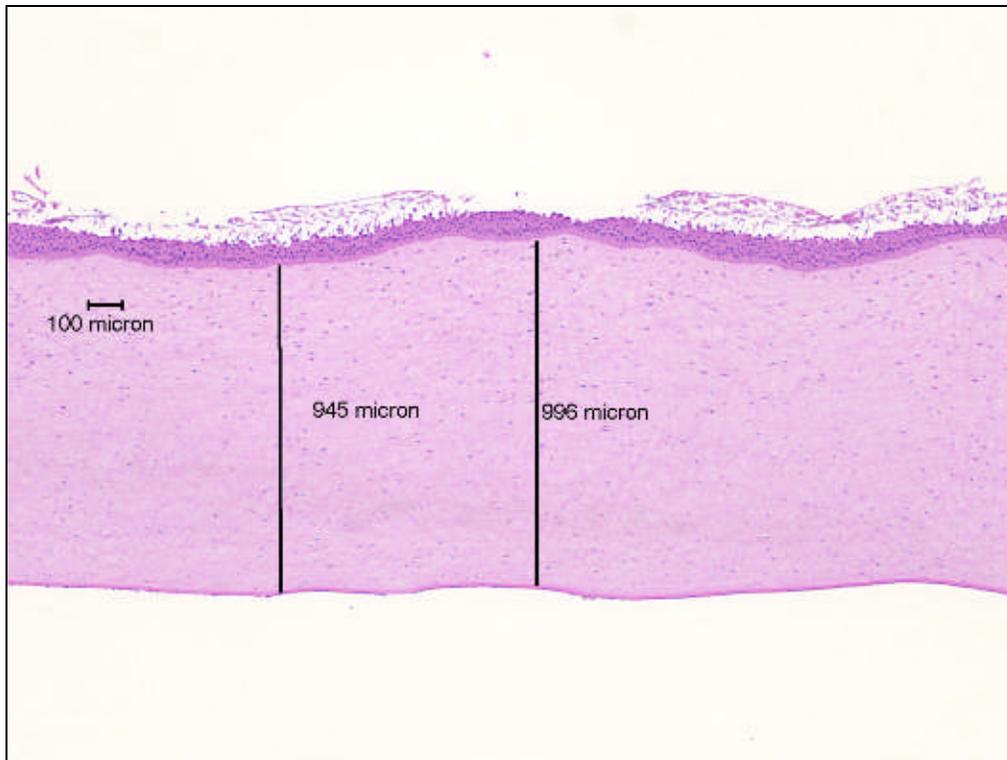


Figure 18. V, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma at 20% depth under a zone of more intact epithelium showing moderate collagen matrix vacuolization and keratocytes with somewhat enlarged nuclei and moderate cytoplasmic eosinophilia (magnification 475x)

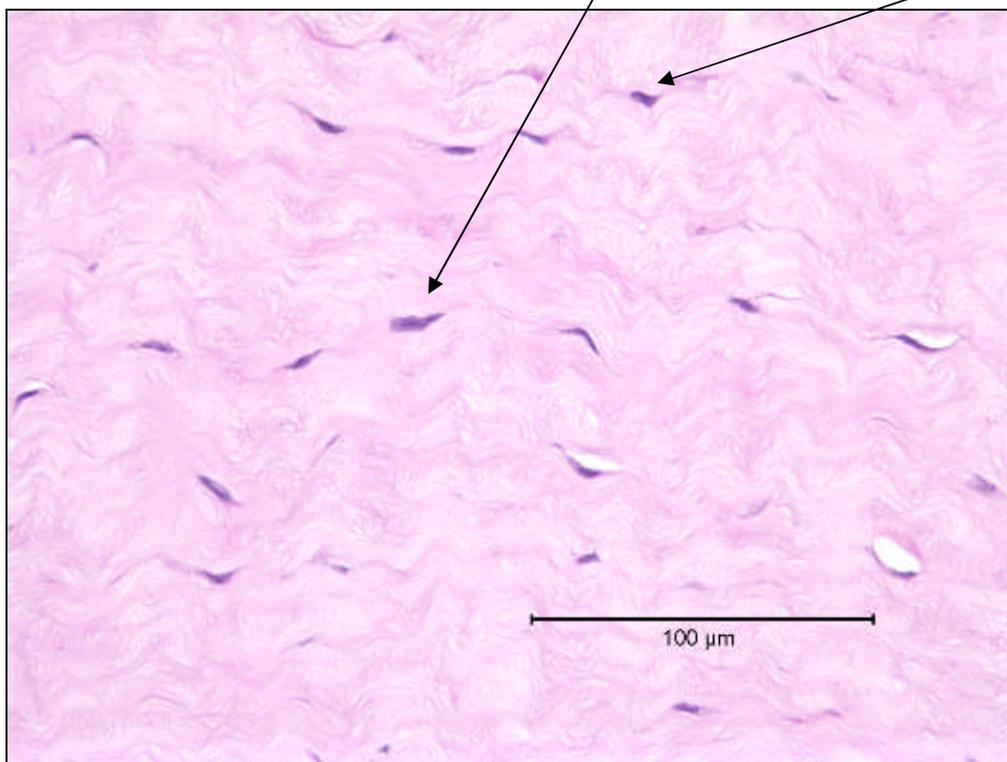


Figure 19. V, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma at 20% depth under a zone of basal cell loss showing more marked collagen matrix vacuolization and keratocytes with enlarged nuclei and cytoplasmic eosinophilia (magnification 475x)

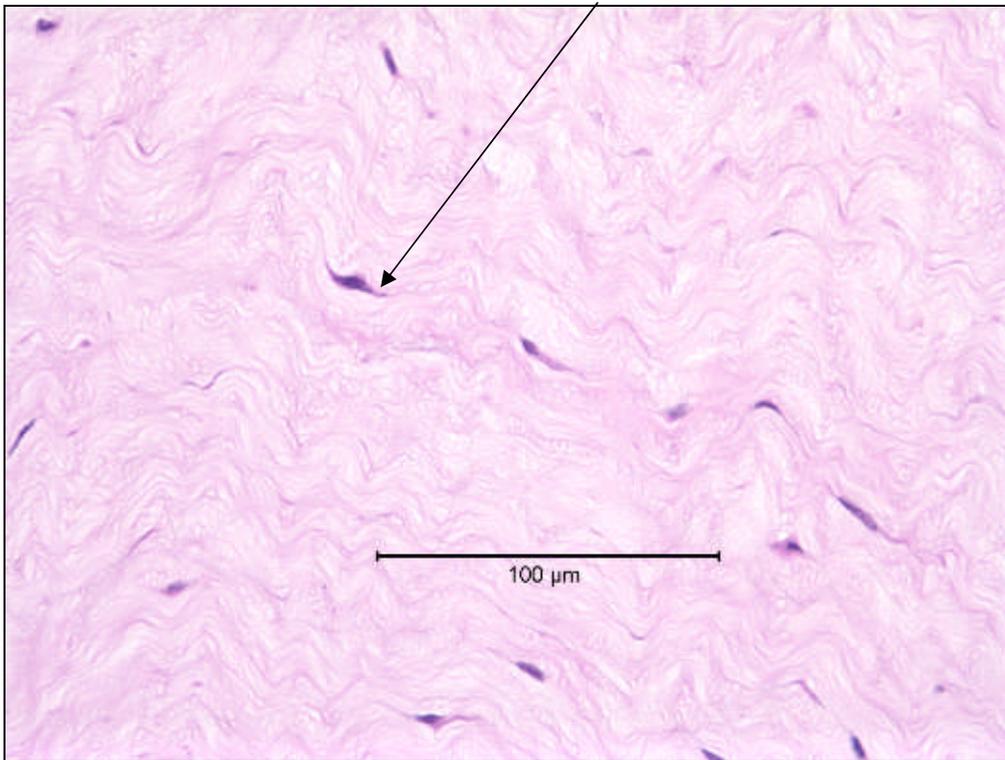


Figure 20. I, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium (magnification 237x)

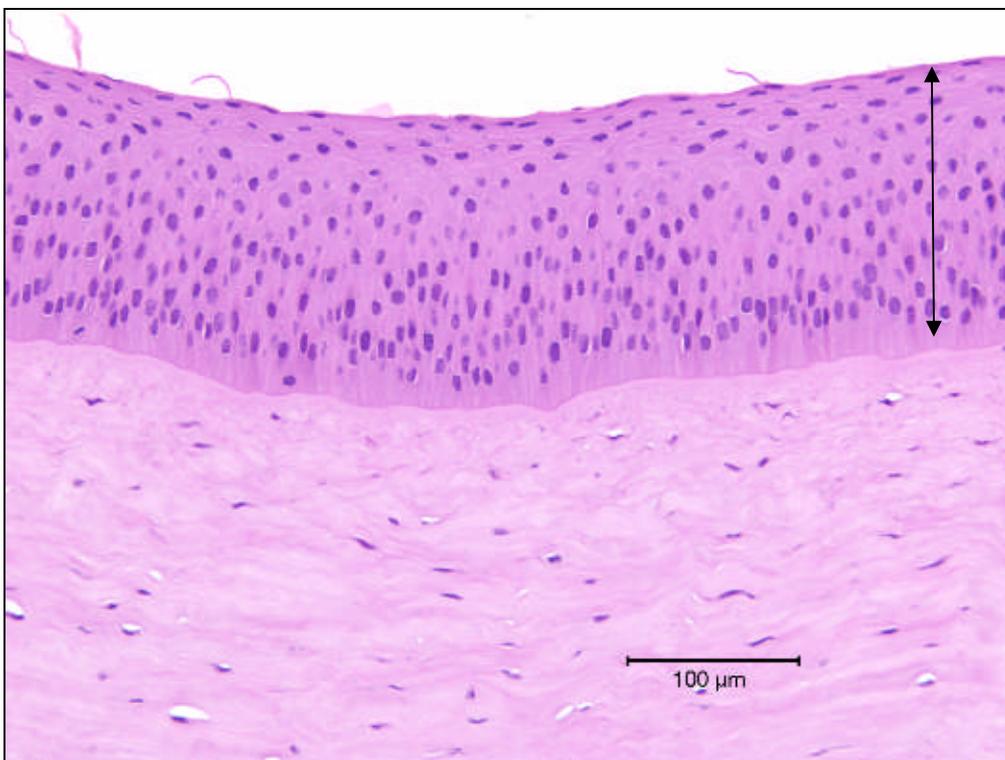


Figure 21. I, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

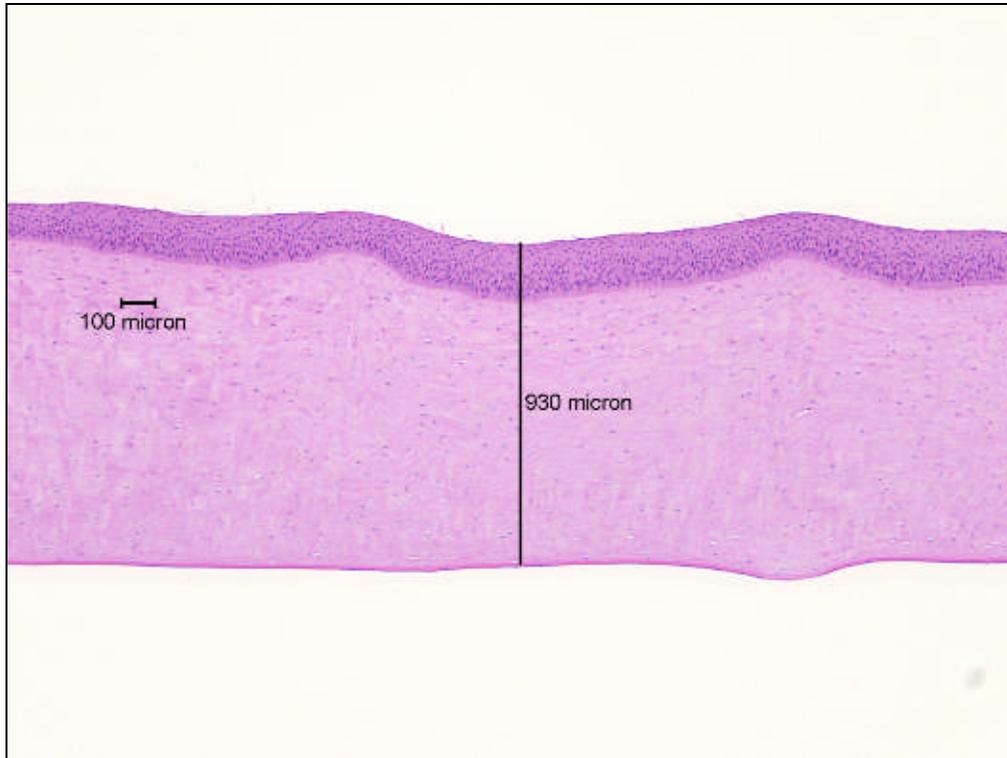


Figure 22. I, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Stroma directly below Bowman's Layer (magnification 475x)

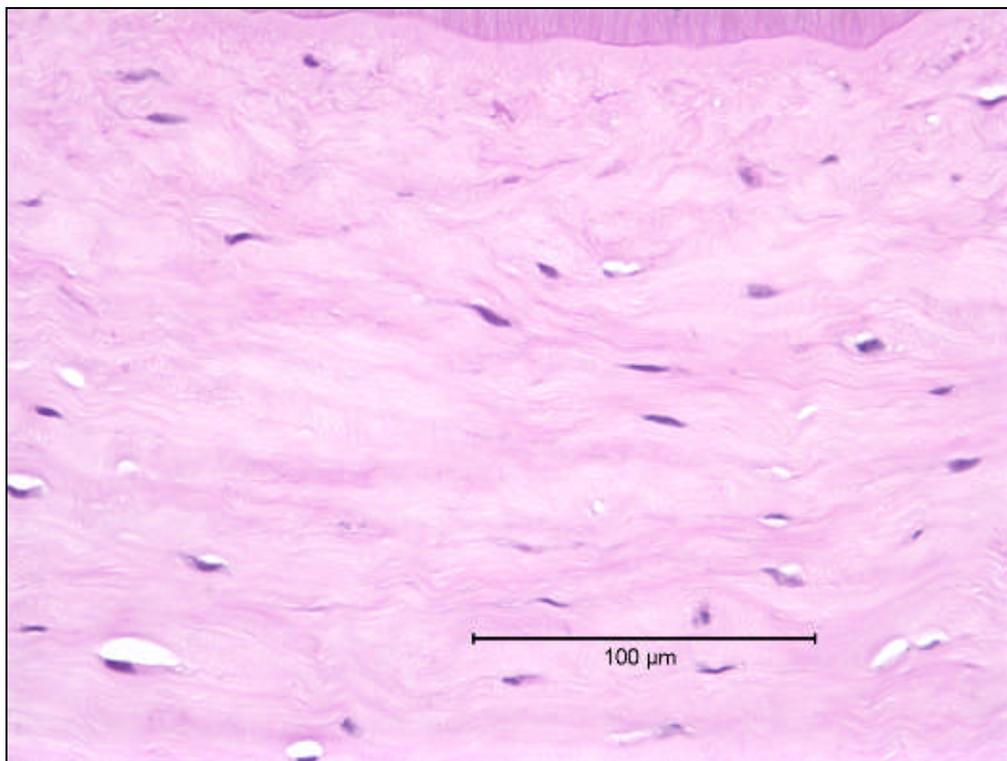


Figure 23. I, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing loss of the surface squamous cells (magnification 237x)

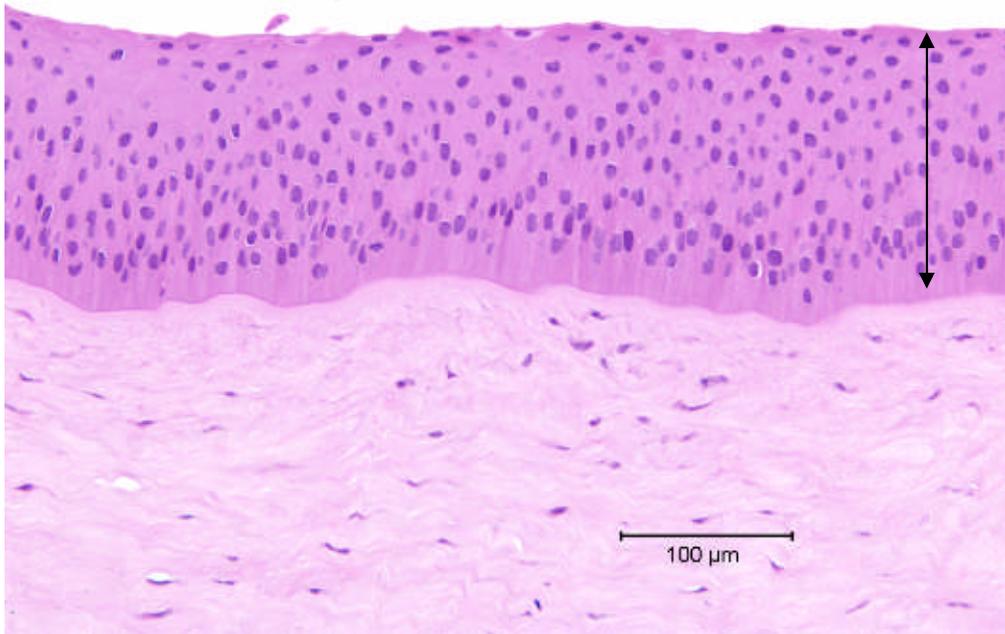


Figure 24. I, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

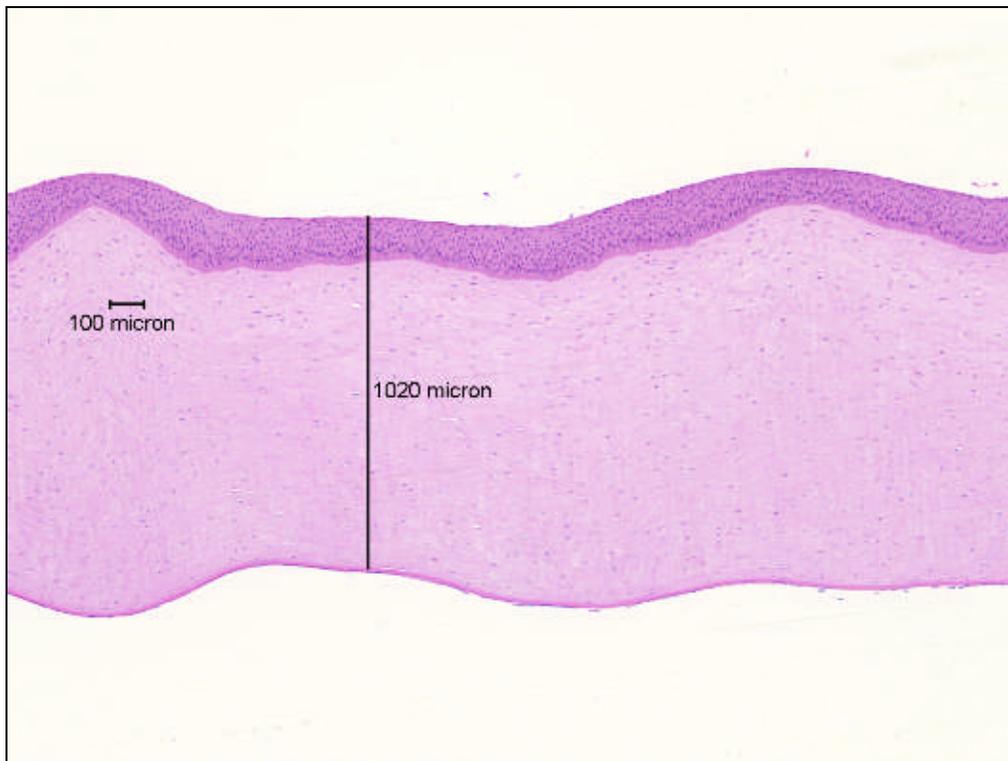


Figure 25. I, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma directly below Bowman's Layer showing slight collagen matrix vacuolization and rare keratocytes with cytoplasmic eosinophilia (magnification 475x)

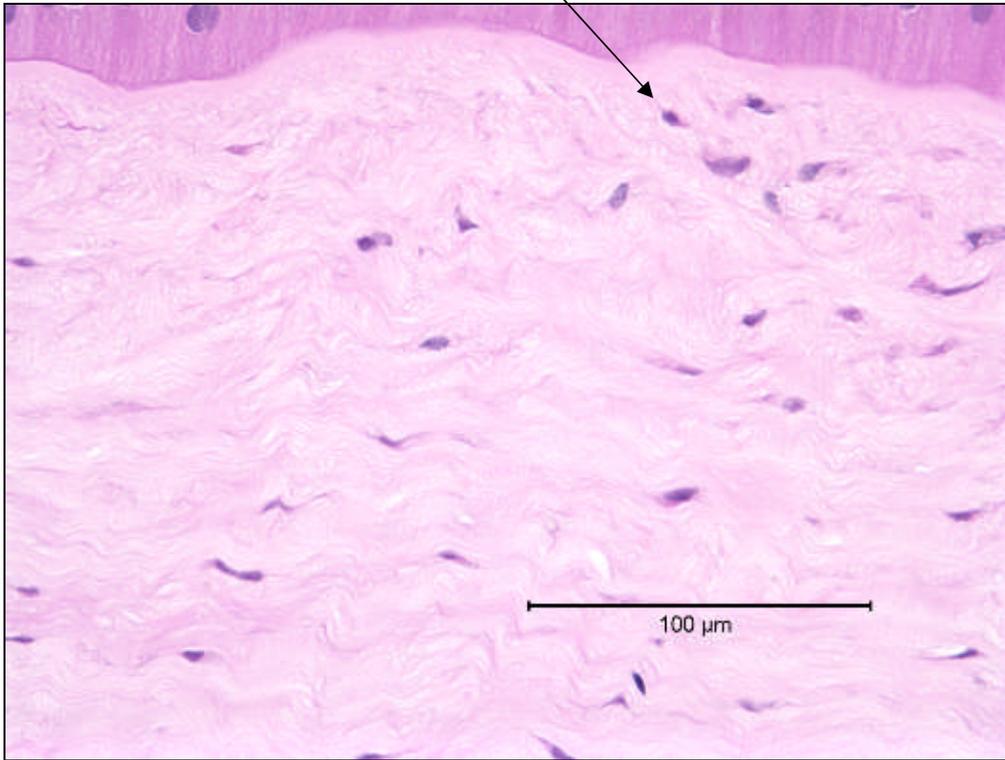


Figure 26. H, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing loss of the surface squamous cells (magnification 237x)

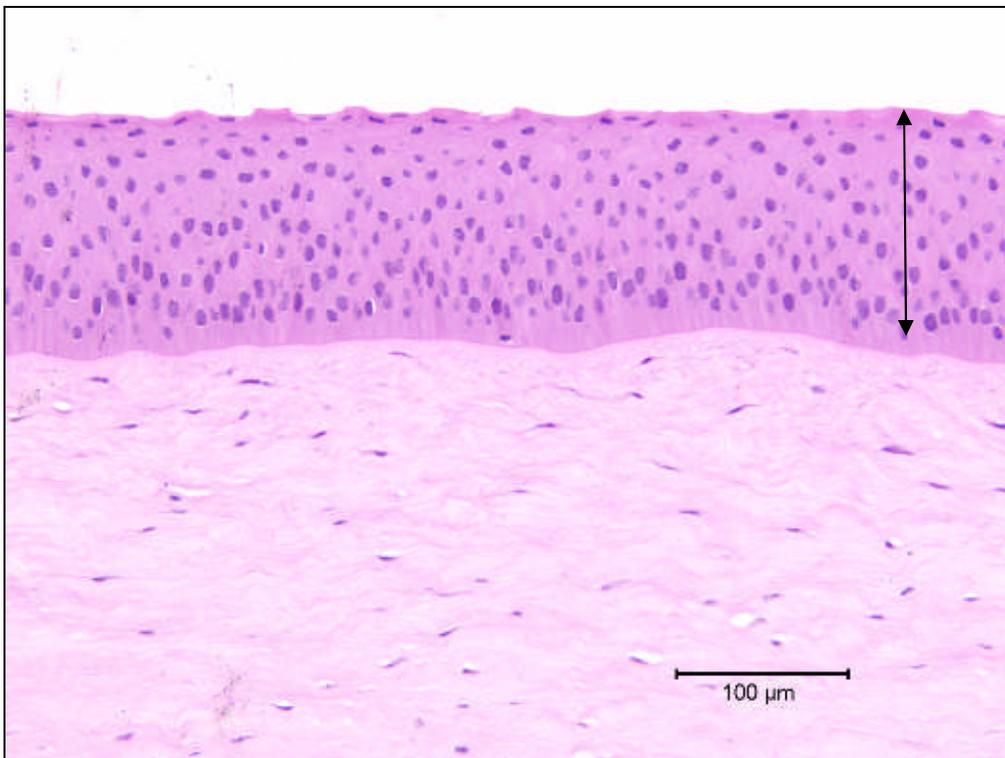


Figure 27. H, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

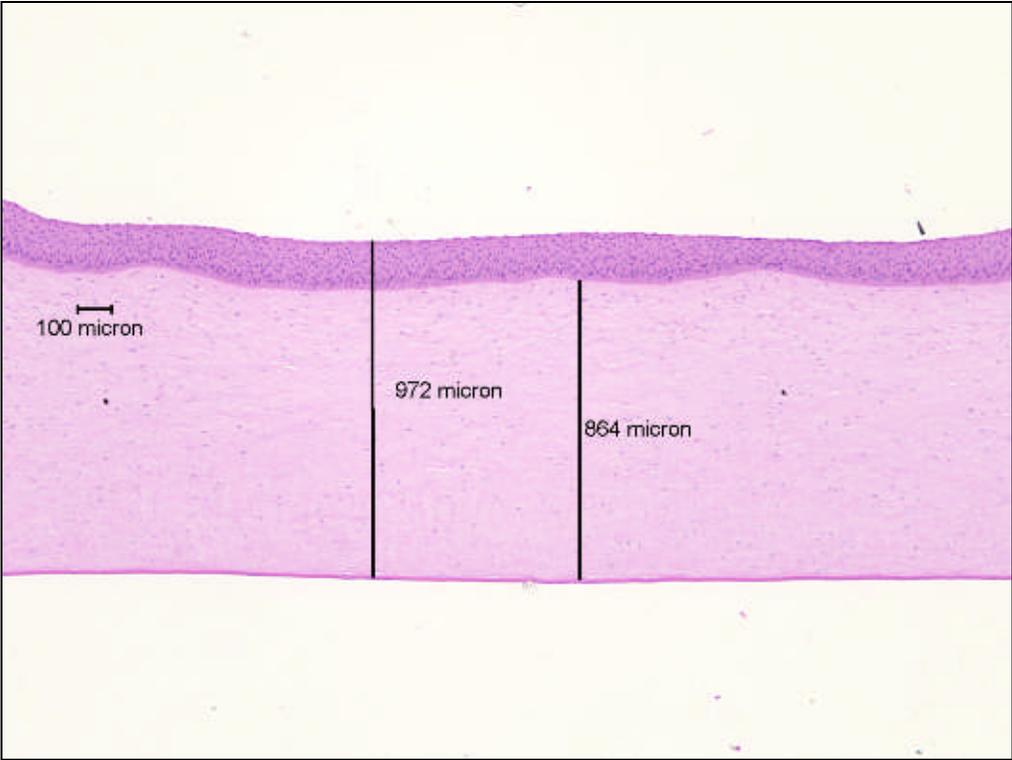


Figure 28. H, neat, 3-minute exposure, 120-minute post-exposure (08/18/05) - Stroma directly below Bowman's Layer showing slight collagen matrix vacuolization and rare keratocytes with cytoplasmic eosinophilia (magnification 475x)

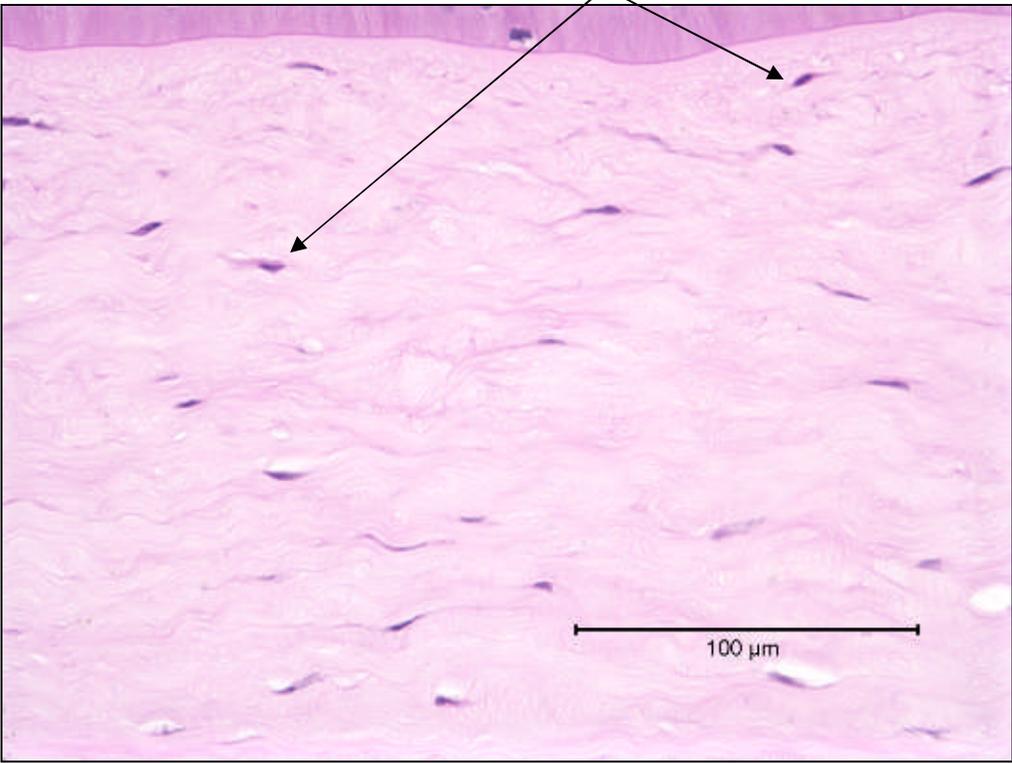


Figure 29. H, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium (overview) (magnification 237x)

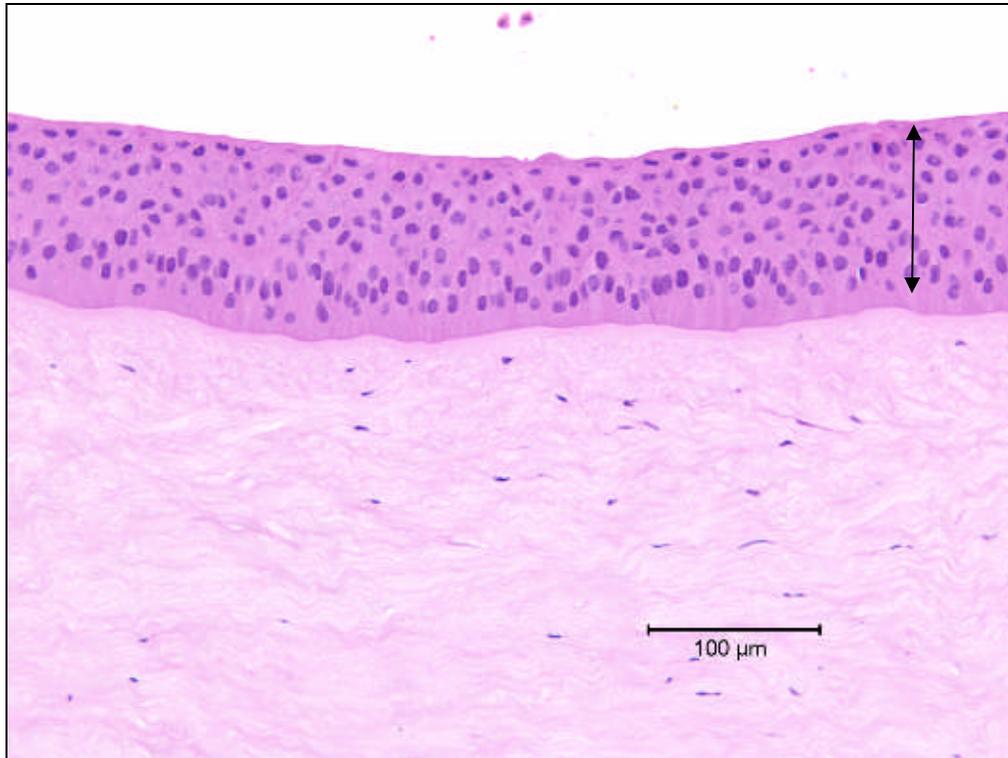


Figure 30. H, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing the loss of the squamous and upper wing cells as well as increased cytoplasmic vacuolization in the remaining cells (magnification 475x)

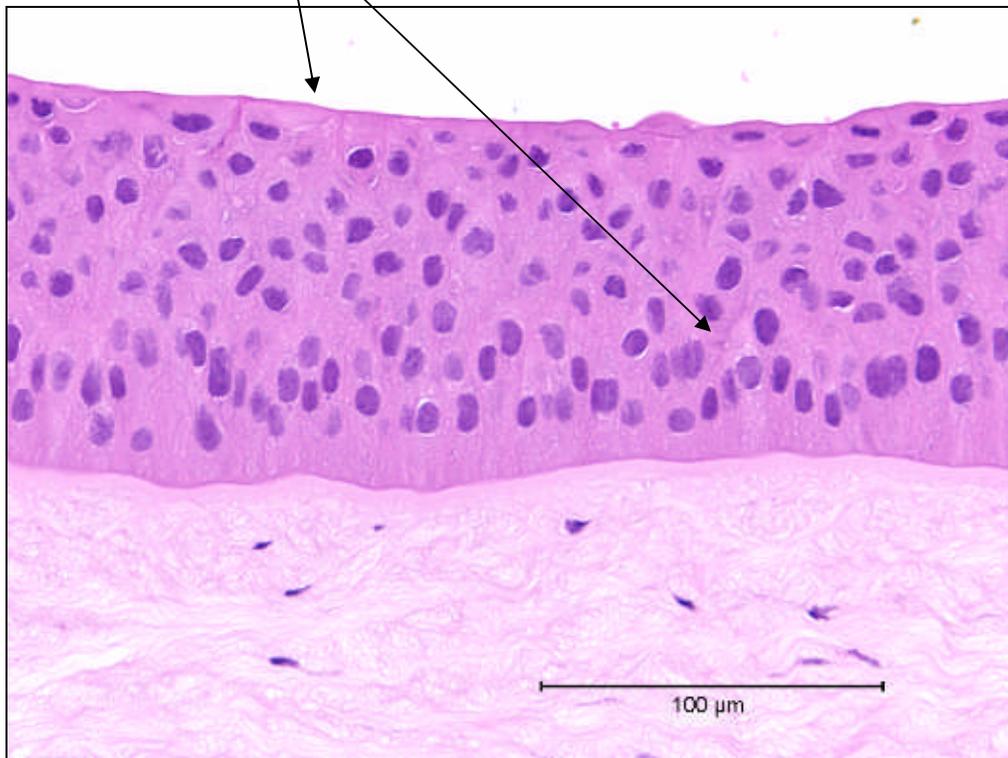


Figure 31. H, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Epithelium showing a full thickness loss (magnification 237x)

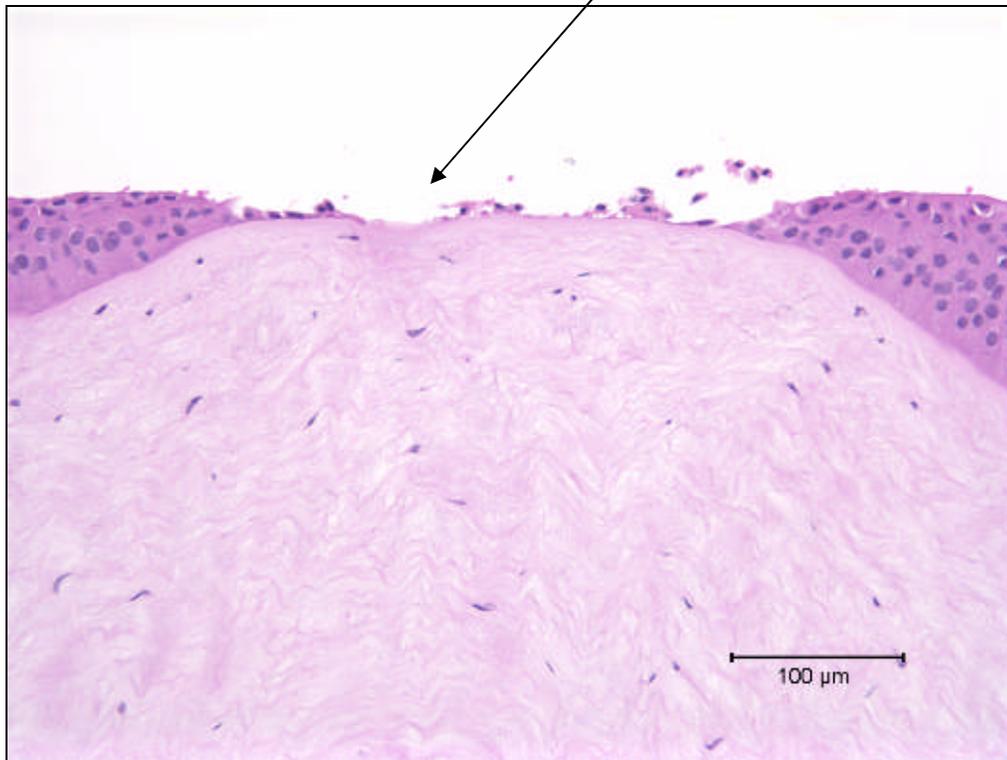


Figure 32. H, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Full thickness (magnification 48x)

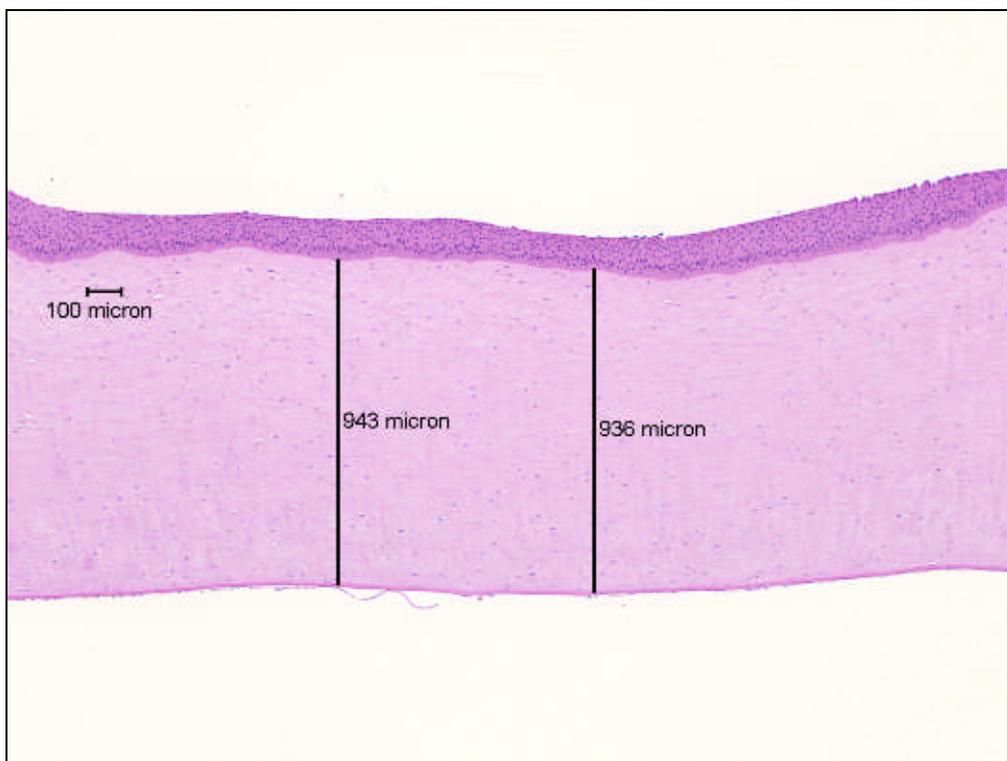


Figure 33. H, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma directly below Bowman's Layer showing moderate collagen matrix vacuolization and a marked increase in keratocytes with abnormal chromatin condensation or nuclear pyknosis (magnification 475x)

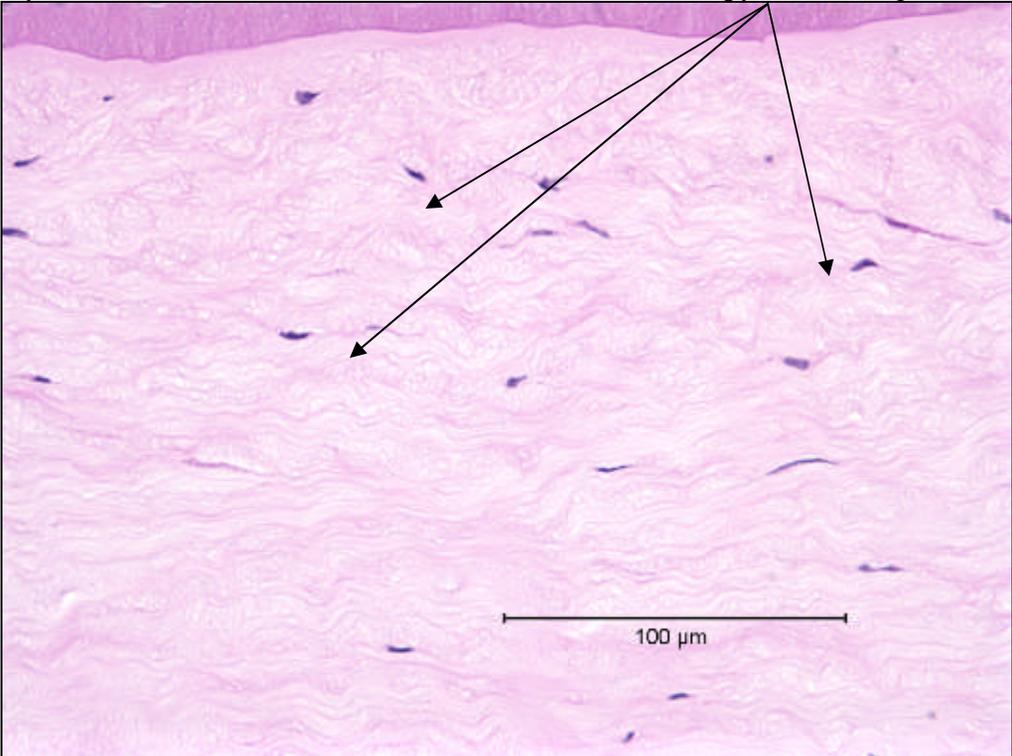
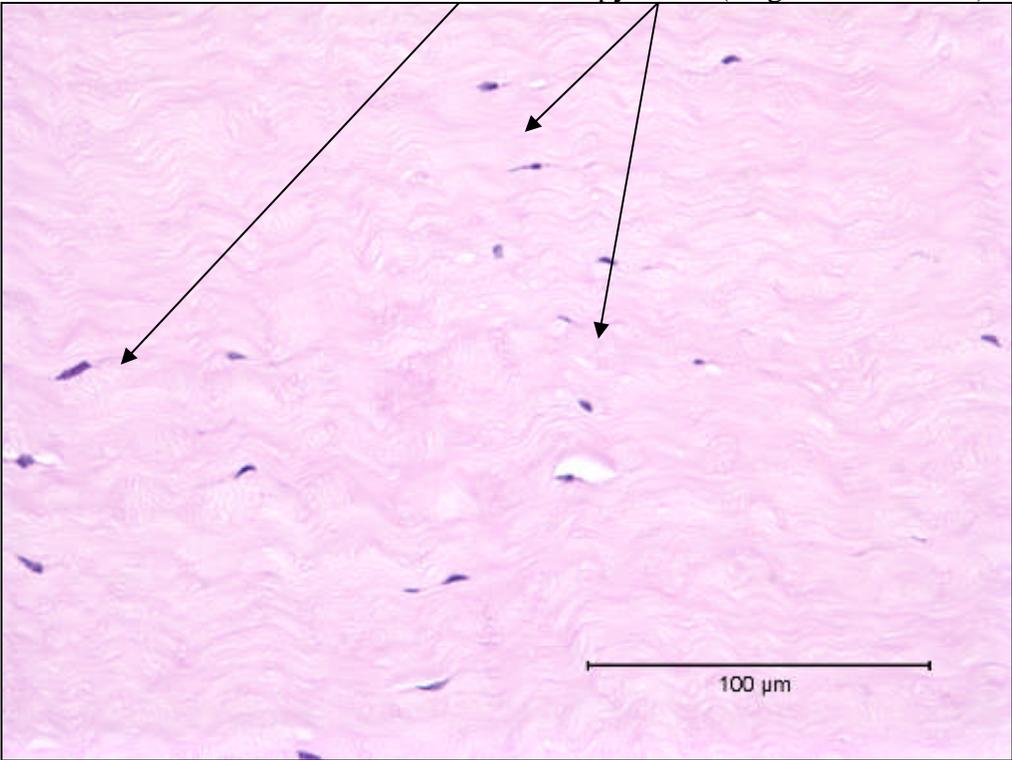


Figure 34. H, neat, 10-minute exposure, 120-minute post-exposure (08/18/05) - Stroma at mid depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with abnormal chromatin condensation or nuclear pyknosis (magnification 475x)



APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
05AE36	9	3	89	86	85.7		
Neat	10	4	76	72	71.7		
3 minutes	11	5	85	80	79.7	79.0	7.0
05AE36	12	5	94	89	88.7		
Neat	13	4	101	97	96.7		
10 minutes	14	3	78	75	74.7	86.7	11.1
05AE37	15	3	4	1	0.7		
Neat	16	3	4	1	0.7		
3 minutes	17	3	3	0	-0.3	0.3	0.6
05AE37	19	4	9	5	4.7		
Neat	20	3	9	6	5.7		
10 minutes	21	4	9	5	4.7	5.0	0.6
05AE38	22	3	120	117	116.7		
Neat	23	6	110	104	103.7		
3 minutes	24	4	106	102	101.7	107.3	8.1
05AE38	25	3	179	176	175.7		
Neat	26	4	171	167	166.7		
10 minutes	28	5	161	156	155.7	166.0	10.0
05AE39	31	5	4	-1	-1.3		
Neat	32	4	4	0	-0.3		
3 minutes	33	4	4	0	-0.3	-0.7	0.6
05AE39	34	5	4	-1	-1.3		
Neat	35	5	4	-1	-1.3		
10 minutes	36	4	4	0	-0.3	-1.0	0.6
05AE40	38	3	4	1	0.7		
Neat	41	4	4	0	-0.3		
3 minutes	42	4	4	0	-0.3	0.0	0.6
05AE40	43	4	4	0	-0.3		
Neat	44	5	5	0	-0.3		
10 minutes	45	3	4	1	0.7	0.0	0.6
Neg. Control	1	4	4	0	NA		
Sterile, DI water	2	3	4	1	NA		
10 minutes	3	4	4	0	NA	0.3	
Pos. Control	6	3	29	26	25.7		
Ethanol	7	4	28	24	23.7		
10 minutes	8	4	33	29	28.7	26.0	2.5
	*46	4					
	*47	5					
	*48	4					

Initial corneal opacity average: 4

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

PERMEABILITY SCORE

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

Cornea #	OD490
1	0.005
2	0.005
3	0.005
Avg.	0.005

**Pos. Control
Ethanol
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
6	1.363	1	1.358
7	0.339	5	1.690
8	1.205	1	1.200
Avg. =			1.416
STDEV =			0.250

**05AE36
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
9	0.558	5	2.785
10	0.792	5	3.955
11	0.823	5	4.110
Avg. =			3.617
STDEV =			0.724

**05AE36
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
12	0.707	5	3.530
13	0.737	5	3.680
14	0.680	5	3.395
Avg. =			3.535
STDEV =			0.143

**05AE37
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
15	0.211	1	0.206
16	0.266	1	0.261
17	0.181	1	0.176
Avg. =			0.214
STDEV =			0.043

**05AE37
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
19	1.095	1	1.090
20	0.933	1	0.928
21	1.137	1	1.132
Avg. =			1.050
STDEV =			0.108

**05AE38
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
22	1.379	1	1.374
23	0.419	5	2.090
24	0.327	5	1.630
Avg. =			1.698
STDEV =			0.363

**05AE38
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
25	0.586	5	2.925
26	0.450	5	2.245
28	0.602	5	3.005
Avg. =			2.725
STDEV =			0.418

**05AE39
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
31	0.022	1	0.017
32	0.010	1	0.005
33	0.051	1	0.046
Avg. =			0.023
STDEV =			0.021

**05AE39
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
34	0.093	1	0.088
35	0.116	1	0.111
36	0.118	1	0.113
Avg. =			0.104
STDEV =			0.014

**05AE40
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
38	0.137	1	0.132
41	0.115	1	0.110
42	0.113	1	0.108
Avg. =			0.117
STDEV =			0.013

**05AE40
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
43	0.353	1	0.348
44	0.449	1	0.444
45	1.052	1	1.047
Avg. =			0.613
STDEV =			0.379

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
05AE36	Neat	3 minutes	79.0	3.617	133.3
05AE36	Neat	10 minutes	86.7	3.535	139.7
05AE37	Neat	3 minutes	0.3	0.214	3.5
05AE37	Neat	10 minutes	5.0	1.050	20.8
05AE38	Neat	3 minutes	107.3	1.698	132.8
05AE38	Neat	10 minutes	166.0	2.725	206.9
05AE39	Neat	3 minutes	-0.7	0.023	-0.3
05AE39	Neat	10 minutes	-1.0	0.104	0.6
05AE40	Neat	3 minutes	0.0	0.117	1.8
05AE40	Neat	10 minutes	0.0	0.613	9.2
Ethanol	Neat	10 minutes	26.0	1.416	47.2

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
05AE40	11	3	4	1	0.3		
Neat	12	4	4	0	-0.7		
3 minutes	13	5	4	-1	-1.7	-0.7	1.0
05AE40	15	3	4	1	0.3		
Neat	17	4	6	2	1.3		
10 minutes	18	3	5	2	1.3	1.0	0.6
05AE41	20	3	5	2	1.3		
Neat	21	4	6	2	1.3		
3 minutes	22	4	5	1	0.3	1.0	0.6
05AE41	23	5	12	7	6.3		
Neat	24	4	8	4	3.3		
10 minutes	25	4	9	5	4.3	4.7	1.5
05AE42	26	5	5	0	-0.7		
Neat	27	3	4	1	0.3		
3 minutes	28	5	5	0	-0.7	-0.3	0.6
05AE42	29	3	5	2	1.3		
Neat	30	4	6	2	1.3		
10 minutes	31	5	6	1	0.3	1.0	0.6
05AE43	32	3	4	1	0.3		
Neat	33	6	5	-1	-1.7		
3 minutes	34	5	5	0	-0.7	-0.7	1.0
05AE43	35	5	6	1	0.3		
Neat	36	4	5	1	0.3		
10 minutes	46	4	4	0	-0.7	0.0	0.6
Neg. Control	3	2	3	1	NA		
Sterile, DI water	4	4	5	1	NA		
10 minutes	5	3	3	0	NA	0.7	
Pos. Control	6	3	41	38	37.3		
Ethanol	7	4	30	26	25.3		
10 minutes	8	4	30	26	25.3	29.3	6.9
	*9	3					
	*38	7					
	*40	7					
	*41	7					
	*44	7					
	*47	7					
	*48	4					
	*49	4					
	*50	4					

Initial corneal opacity average: 4

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

PERMEABILITY SCORE

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

Cornea #	OD490
3	0.003
4	0.005
5	0.002
Avg.	0.003

**Pos. Control
Ethanol
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
6	1.049	1	1.046
7	1.049	1	1.046
8	1.103	1	1.100
Avg. =			1.064
STDEV =			0.031

**05AE40
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
11	0.213	1	0.210
12	0.251	1	0.248
13	0.222	1	0.219
Avg. =			0.225
STDEV =			0.020

**05AE40
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
15	0.617	1	0.614
17	0.917	1	0.914
18	0.563	1	0.560
Avg. =			0.696
STDEV =			0.191

**05AE41
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
20	0.088	1	0.085
21	0.101	1	0.098
22	0.137	1	0.134
Avg. =			0.105
STDEV =			0.025

**05AE41
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
23	0.100	1	0.097
24	0.306	1	0.303
25	0.205	1	0.202
Avg. =			0.200
STDEV =			0.103

**05AE42
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
26	0.002	1	-0.001
27	0.003	1	0.000
28	0.003	1	0.000
Avg. =			-0.001
STDEV =			0.001

**05AE42
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
29	0.022	1	0.019
30	0.004	1	0.001
31	0.003	1	0.000
Avg. =			0.006
STDEV =			0.011

**05AE43
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
32	0.004	1	0.001
33	0.005	1	0.002
34	0.006	1	0.003
Avg. =			0.002
STDEV =			0.001

**05AE43
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
35	0.022	1	0.019
36	0.008	1	0.005
46	0.025	1	0.022
Avg. =			0.015
STDEV =			0.009

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
05AE40	Neat	3 minutes	-0.7	0.225	2.7
05AE40	Neat	10 minutes	1.0	0.696	11.4
05AE41	Neat	3 minutes	1.0	0.105	2.6
05AE41	Neat	10 minutes	4.7	0.200	7.7
05AE42	Neat	3 minutes	-0.3	-0.001	-0.3
05AE42	Neat	10 minutes	1.0	0.006	1.1
05AE43	Neat	3 minutes	-0.7	0.002	-0.6
05AE43	Neat	10 minutes	0.0	0.015	0.2
Ethanol	Neat	10 minutes	29.3	1.064	45.3