

FINAL REPORT

Study Title

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
AEROSOL AND LIQUID DOSING, TWO TIME EXPOSURES AND  
HISTOLOGY  
Using BJ**

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Study Completion Date

October 4, 2005

Performing Laboratory

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

Study Number

05AD41, AD42.350066

Laboratory Project Number

4131

**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: \_\_\_\_\_

\_\_\_\_\_  
Title

Signature: \_\_\_\_\_

**STATEMENT OF COMPLIANCE**

The Bovine Corneal Opacity and Permeability Assay with Aerosol and Liquid Dosing, Two Time Exposures and Histology of the reference substance, BJ, was conducted in compliance with U.S. EPA GLP Standards 40 CFR 160 with the following exceptions:

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

The processing of the corneas and preparation of the slides by Pathology Associates, Inc. was not performed under the GLP guidelines.

Study Director \_\_\_\_\_  
John W. Harbell, Ph.D.

Date \_\_\_\_\_

Submitter: \_\_\_\_\_

Date \_\_\_\_\_

Sponsor's Representative: \_\_\_\_\_

Date \_\_\_\_\_

## QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Aerosol and Liquid Dosing, Two Time Exposures and Histology

Study Number: 05AD41, AD42.350066

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:

<b>Phase Inspected</b>	<b>Audit Date(s)</b>	<b>Reported to Study Director</b>	<b>Reported to Management</b>
Protocol and Initial Paperwork	05-Jul-05	05-Jul-05	05-Jul-05
Final Opacity Measurement	05-Jul-05	06-Jul-05	06-Jul-05
Histology – Negative Control and 05AD41-A 3 minute exposure	08-Sep-05	08-Sep-05	19-Sep-05
Draft Report and Data	19-Sep-05	19-Sep-05	19-Sep-05
Final Report	04-Oct-05	04-Oct-05	04-Oct-05

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

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Amanda K. Ulrey, RQAP-GLP  
Quality Assurance

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Date

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**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
AEROSOL AND LIQUID DOSING, TWO TIME EXPOSURES AND  
HISTOLOGY**

Initiation Date: June 30, 2005

Completion Date: October 4, 2005

Sponsor:

Sponsor's Representative:

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

Archive Location: Institute for In Vitro Sciences, Inc.  
Gaithersburg, MD 20878

Study Director: \_\_\_\_\_  
John W. Harbell, Ph.D. Date

Lead Biologist: Christopher Reyes, B.S.

Laboratory Management: Greg Mun, B.S.

Principle Investigator: David A. Hodge HT (ASCP)  
(Pathology Associates)

Histology Evaluation Performed by: John W. Harbell, Ph.D.

**TEST/REFERENCE SUBSTANCE RECEIPT**

<b>IIVS Test Substance Number</b>	<b>Sponsor's Designation</b>	<b>Physical Description</b>	<b>Receipt Date</b>	<b>Storage Conditions *</b>
05AD42	BJ	clear colorless non-viscous liquid	6/2/05	room temperature

\* - Protected from exposure to light

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
AEROSOL AND LIQUID DOSING, TWO TIME EXPOSURES AND  
HISTOLOGY**

## SUMMARY

The ocular irritancy of BJ was evaluated in the Bovine Corneal Opacity and Permeability (BCOP) Assay. Positive and negative control materials were tested concurrently in the study. The reference substance, BJ, and the positive and negative control materials were exposed to the corneas using the direct instillation procedure using 750 $\mu$ L per cornea. Each treatment condition was applied to three corneas. The corneas were exposed to the test and reference substances for periods of 3 and 10 minutes followed by a post-exposure incubation of 2 hours. After determination of the final opacity measurements and assessment of the penetration of fluorescein, each cornea was fixed for histological evaluation.

**Summary Table**  
**BCOP Results of the Test and Reference Substances**

Assay Date	IIVS Test/Reference Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD <sub>490</sub> Value	<i>In Vitro</i> Score	pH
7/5/05	05AD42-I	BJ	Neat	3 minutes	4.3	0.258	8.2	12.5
				10 minutes	29.0	1.704	54.6	

A- Aerosol method of exposure.

I- Instillation method of exposure.

The response of the positive control, neat ethanol, was within the normal range and therefore the assay was considered valid according the acceptance criteria of the protocol.

## INTRODUCTION

The purpose of this study was to evaluate the potential ocular irritancy of the test substance, BJ, in the Bovine Corneal Opacity and Permeability (BCOP) Assay. The assay procedures generally followed those described by Sina *et al.* (1995)<sup>1</sup>. The reference substance, BJ, and the positive and negative control materials were exposed to the corneas using the direct instillation procedure using 750 $\mu$ L per cornea. This study was performed at the Institute for In Vitro Sciences, Inc. (IIVS), 21 Firstfield Road, Suite 220, Gaithersburg, MD, 20878. The protocol was signed by the Study Director on June 30, 2005, and the assay was begun on July 5, 2005. At the conclusion of the opacity and permeability measurements, the corneas were fixed and sent out for histological preparation. The slides of the test substance and control treated corneas were evaluated at IIVS. The in-life phase of the study concluded, following examination of histological slides, on September 9, 2005.

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<sup>1</sup> Sina, 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.

## MATERIALS AND METHODS

### Test and Reference Substance Preparation

As instructed by the sponsor, the test and reference substances were administered to the test system without dilution.

### Test and Reference Substance pH Determination

The pH values of the test and reference substances were determined using pH paper (EMD Chemicals Inc./ Em Science). Initially, the test and reference substances were added to 0-14 pH paper with 1.0 pH unit increment to approximate a narrow pH range. Next, the test and reference substances were 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.

### Assay 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).

### 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.

### 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 reference substance, BJ, was tested neat. The reference substance, BJ, and the assay controls were tested using only the instillation method of exposure. For instillation exposure, an aliquot of 750  $\mu\text{L}$  of the test substance, reference 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 the reference substance at  $32 \pm 1^\circ\text{C}$  for 3 minutes. A second set of three corneas was incubated in the presence of the reference substance 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. A set of three corneas was incubated in the presence of the positive control at  $32 \pm 1^\circ\text{C}$  for 10 minutes. After the 3 and 10-minute exposure times, the assay control, test substance, or reference substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control material, or test or reference 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  $\mu\text{L}$  from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm ( $\text{OD}_{490}$ ) was determined using a Molecular Devices Vmax kinetic microplate reader. If the  $\text{OD}_{490}$  value of a control material or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the  $\text{OD}_{490}$  value within the linear range of the platereader). A 360  $\mu\text{L}$  sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

#### 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 prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

## Histological Evaluation

Histology was not performed for the reference material.

## 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 OD<sub>490</sub> for the blank wells was calculated. The mean blank OD<sub>490</sub> was then subtracted from the raw OD<sub>490</sub> of each well (corrected OD<sub>490</sub>). Any dilutions that were made to bring the OD<sub>490</sub> readings into the linear range of the platereader (OD<sub>490</sub> should be less than 1.500), had each diluted OD<sub>490</sub> reading multiplied by the dilution factor. The final corrected OD<sub>490</sub> of the test and reference substances and the positive control was then calculated by subtracting the average corrected OD<sub>490</sub> of the negative control corneas from the corrected OD<sub>490</sub> 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 OD<sub>490</sub> value of each treatment group was calculated by averaging the final corrected OD<sub>490</sub> 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 the reference 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 65.1), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

**Table 1**  
**BCOP Results of the Test and Reference Substances**

Assay Date	IIVS Test/Reference Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD <sub>490</sub> Value	<i>In Vitro</i> Score	pH
7/5/05	05AD42-I	BJ	Neat	3 minutes	4.3	0.258	8.2	12.5
				10 minutes	29.0	1.704	54.6	

A- Aerosol method of exposure.

I- Instillation method of exposure.

**Table 2**  
**BCOP Results of the Positive Control**

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD <sub>490</sub> Value	<i>In Vitro</i> Score
7/5/05	Ethanol	10 minutes	32.0	1.285	51.3

### 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 articles 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 rabbit<sup>2</sup>.

Special effort has been made to detect changes in the stromal elements of the corneas. Jester<sup>3</sup>, Maurer<sup>4,5</sup> 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 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 article into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix 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 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 vacuolization 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 vacuolization will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

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<sup>2</sup> 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.

<sup>3</sup> 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.

<sup>4</sup> 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.

<sup>5</sup> 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.

*In vivo*, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through “scar” collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test article-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the beginning of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic eosinophilia. Harbell and Curren<sup>6</sup> 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 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

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<sup>6</sup> 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.

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 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 5. 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 B7716-B7718). 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).

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. In the zone directly under Bowman's Layer, there was a moderate number of keratocytes with more active nuclei (larger) and slight eosinophilic cytoplasmic staining. 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 B7719-B7721), 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/marked collagen matrix vacuolization extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 8) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization (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**

<b>IIVS Number</b>	<b>Sponsor's Designation</b>	<b>Observations</b>	<b>Figure #</b>
05AD42 Instillation Slides B7746- B7748	BJ, neat- installation exposure, 3- minute exposure, 120- minute post- exposure, 07/05/05	Epithelium: Much of the squamous cell layer was lost (through coagulation and lost of the cellular contents). In a minority of fields, the full squamous layer was lost. The wing and basal layers were intact (similar to the negative control corneas) except for a slight increase in cytoplasmic vacuolization (Figures 28 and 29). Stroma: The test article-treated corneas were thicker than the negative control-treated corneas (Figure 30). Moderate collagen matrix vacuolization extended to mid depth. Below mid depth, the collagen matrix was normal. In the zone directly below Bowman's Layer, there was a slight to moderate increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia (Figure 31). Endothelium: Similar to the endothelium of the negative control-treated corneas.	11-14
05AD42 Instillation Slides B7749- B7751	BJ, neat- installation exposure, 10- minute exposure, 120-	Epithelium: The full squamous cell and wing cell layers were coagulated and had lost most of their nuclear and cytoplasmic contents. In many fields, the basal cells showed marked nuclear and cytoplasmic vacuolization and decreased staining	15-18

	minute post-exposure, 07/05/05	<p>(blanching) suggestive of the loss of stainable components within the cells. The remaining basal cells showed marked abnormal chromatin condensation and cytoplasmic eosinophilia (Figures 32 and 33). Most of the epithelium was probably not viable at the time of fixation. Bowman's Layer was intact in all sections. Stroma: The test article-treated corneas were appreciably thicker than the negative control-treated corneas (Figure 34). Marked collagen matrix vacuolization extended to mid depth and moderate vacuolization extended well past mid depth. Where the endothelium was damaged, increased vacuolization was observed directly above Descemet's Layer. There was a marked increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia in the upper 2/3s of the stroma (Figure 35). This kind of keratocyte change is observed in corneas where the epithelium has been removed mechanically (Harbell and Curren, 2005). Thus, the change may be the result of the stromal swelling rather than direct test article action on the cells.</p> <p>Endothelium: The endothelium was compromised in at least half of the fields examined. The stroma above these fields showed increased vacuolization indicating that the loss of functional endothelium occurred before fixation.</p>	
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Histological evaluation summary: The reference substance BJ was exposed to the corneas by the installation route. The corneas treated for 3 minutes showed some coagulation in the surface epithelium and some increased cytoplasmic vacuolization in the deeper epithelium. The upper stroma showed moderate vacuolization and a moderate increase in keratocytes with enlarged nuclei and cytoplasmic eosinophilia. These stromal changes may reflect the reduction of epithelial barrier integrity that allowed water to enter the stroma rather than direct action of the reference substance on the stroma. The 10-minute exposure produced appreciable epithelial damage and resulting loss of epithelial barrier integrity. Stromal changes included marked collagen matrix vacuolization and a marked increase in the frequency of keratocytes with enlarged nuclei and cytoplasmic eosinophilia. Again, these keratocyte changes may reflect the impact of stromal swelling rather than direct action of the reference substance on the cells themselves<sup>7</sup>. These corneas also showed some loss of endothelial cells. These data suggest that the reference substance has more eye irritation potential than does the test substance when compared using the instillation route of exposure.

<sup>7</sup> 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.

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  $\mu\text{m}$ . 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)

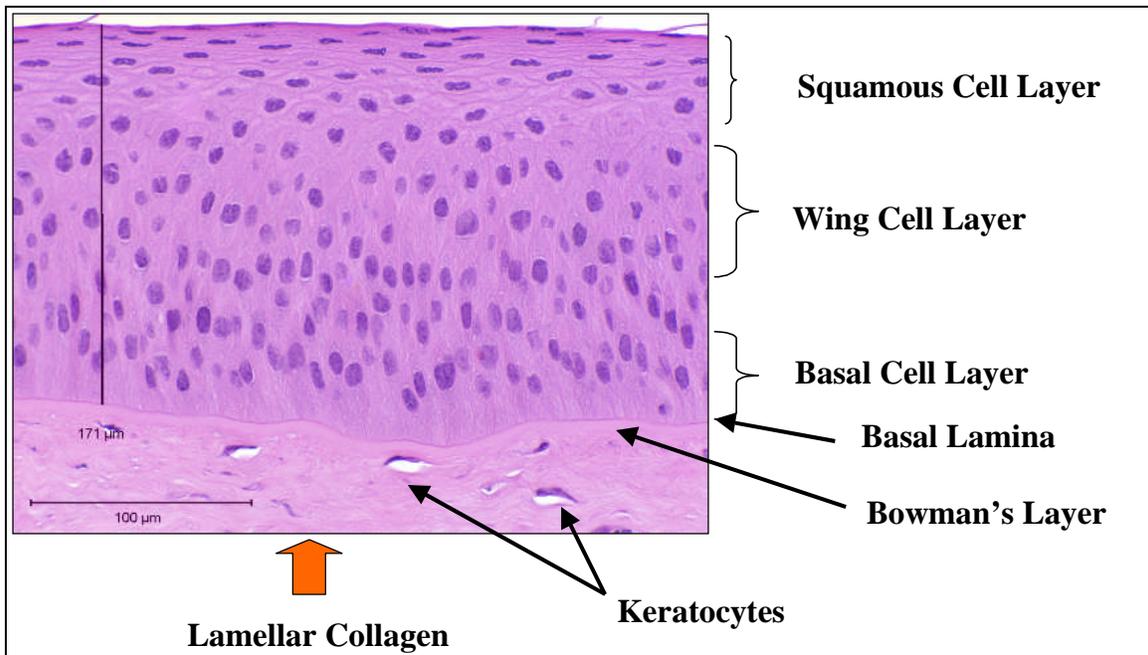


Figure 2. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

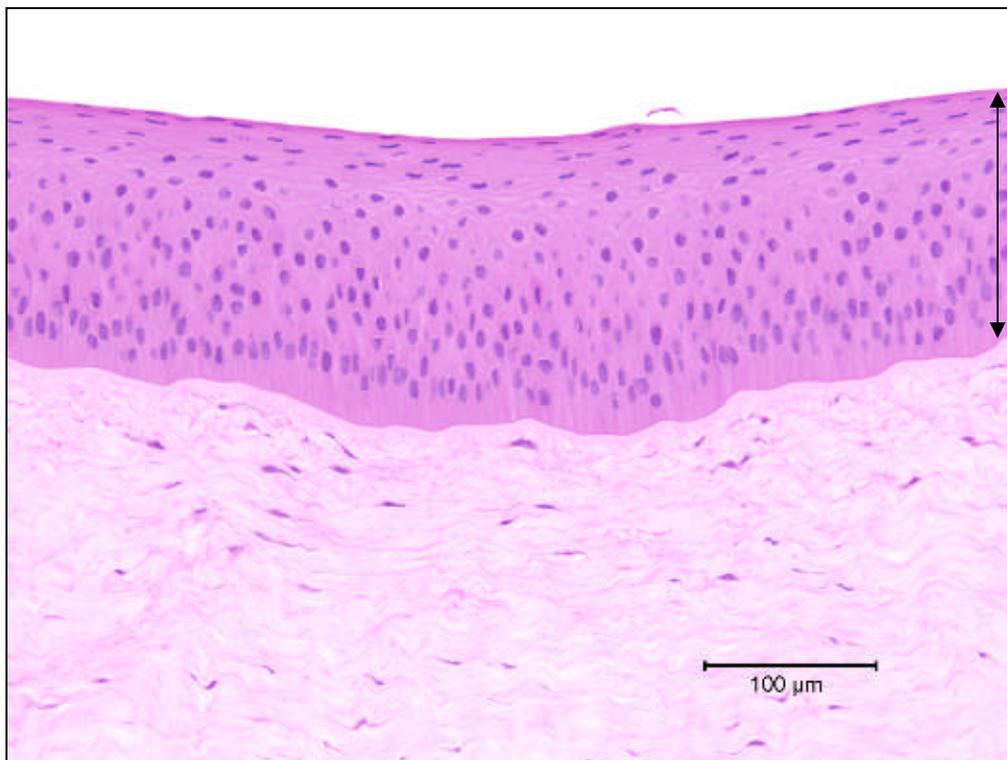


Figure 3. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (detail) (magnification 475x)

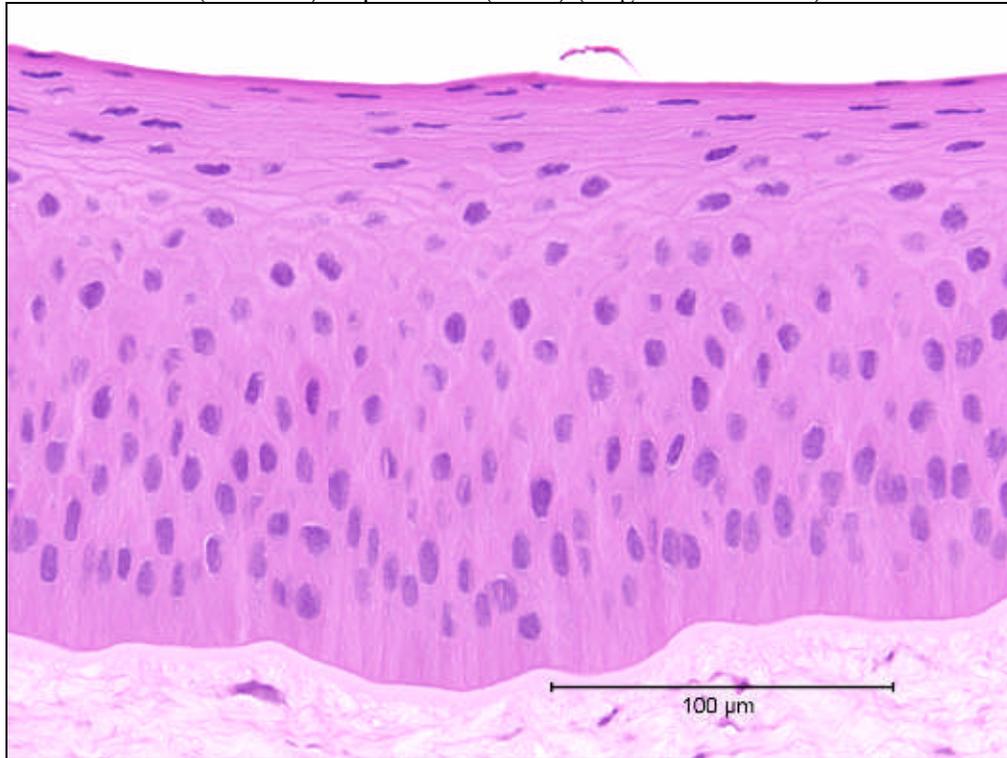


Figure 4. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer (magnification 475x)

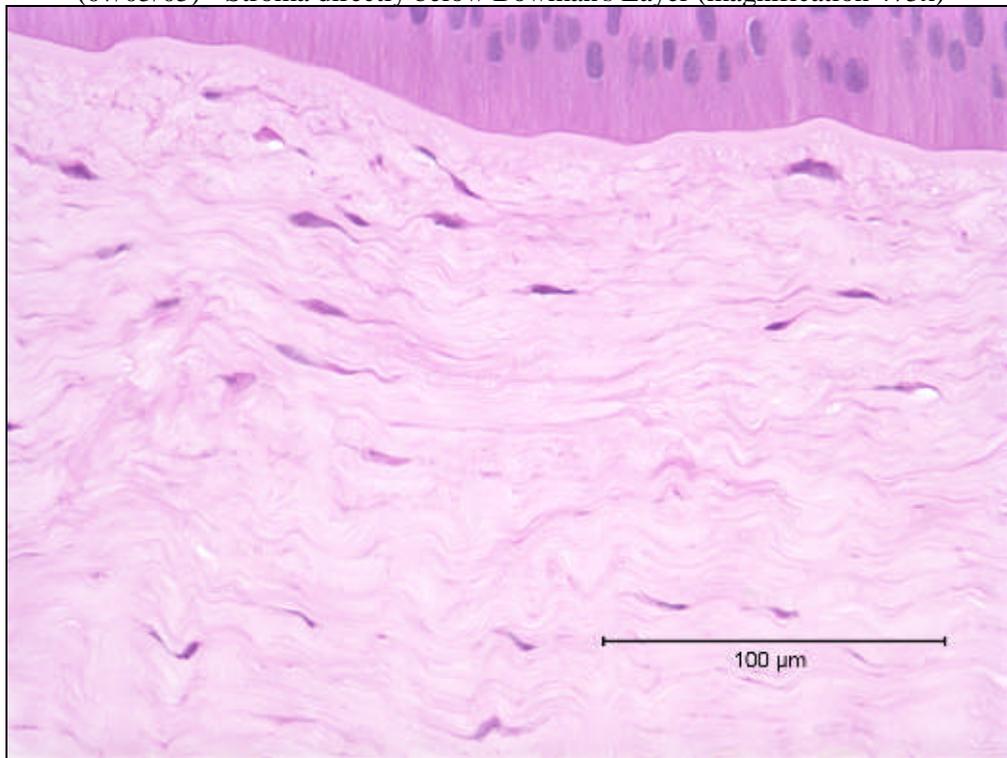


Figure 5. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

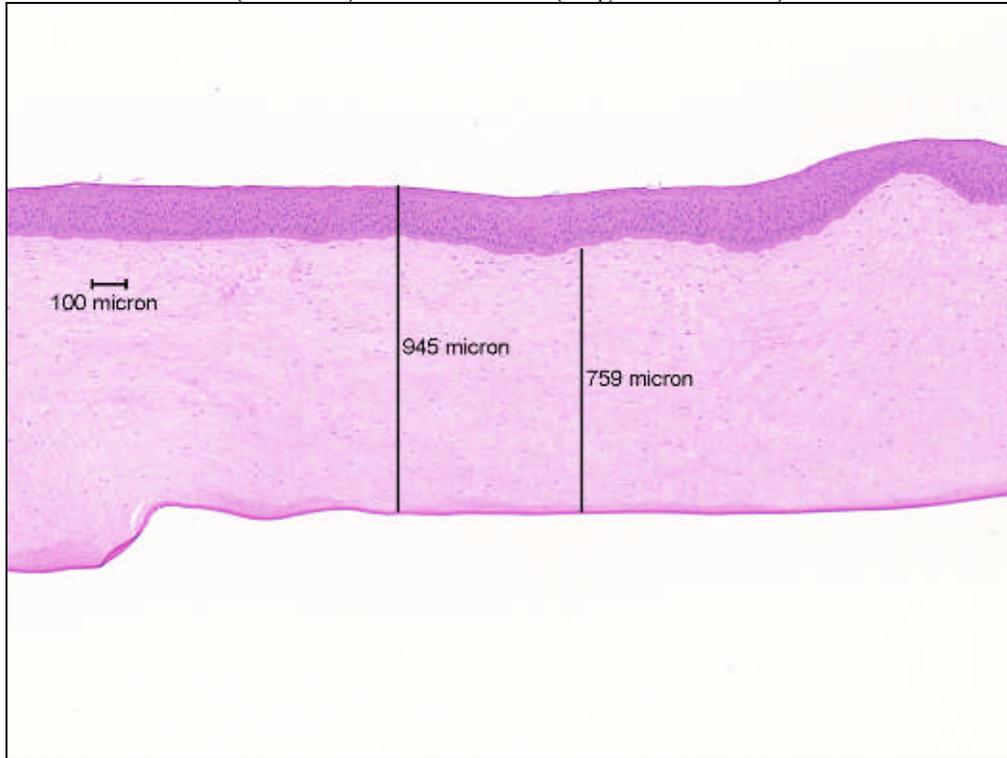


Figure 6. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Epithelium (probably not viable at the time of fixation) (magnification 237x)

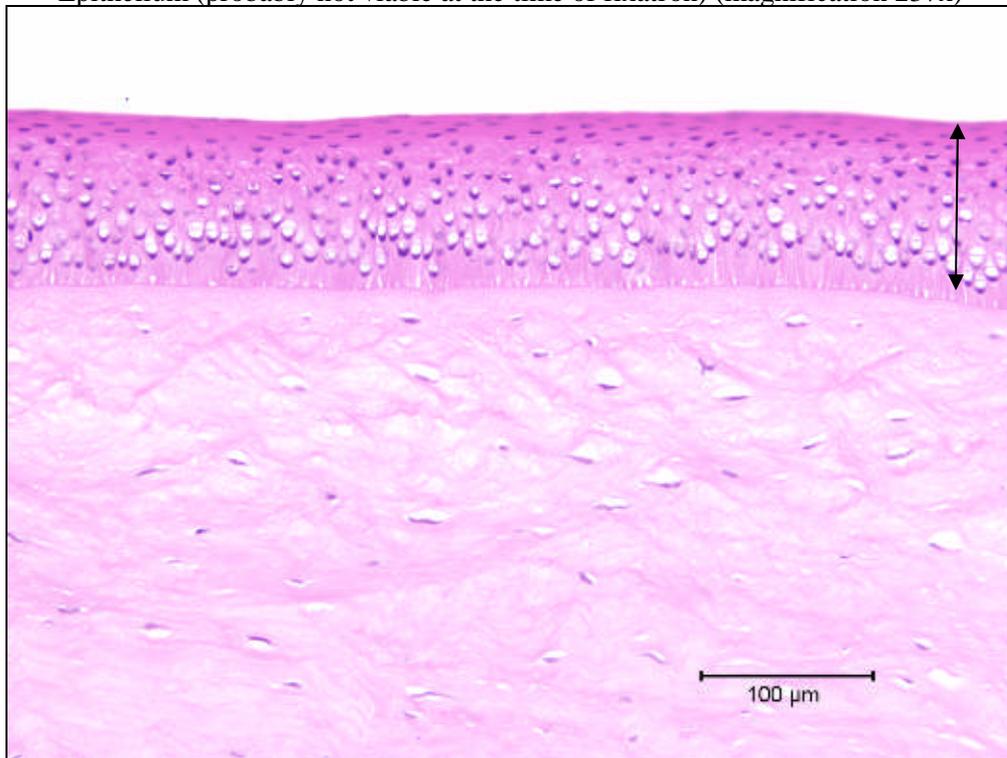


Figure 7. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Full thickness (magnification 48x)

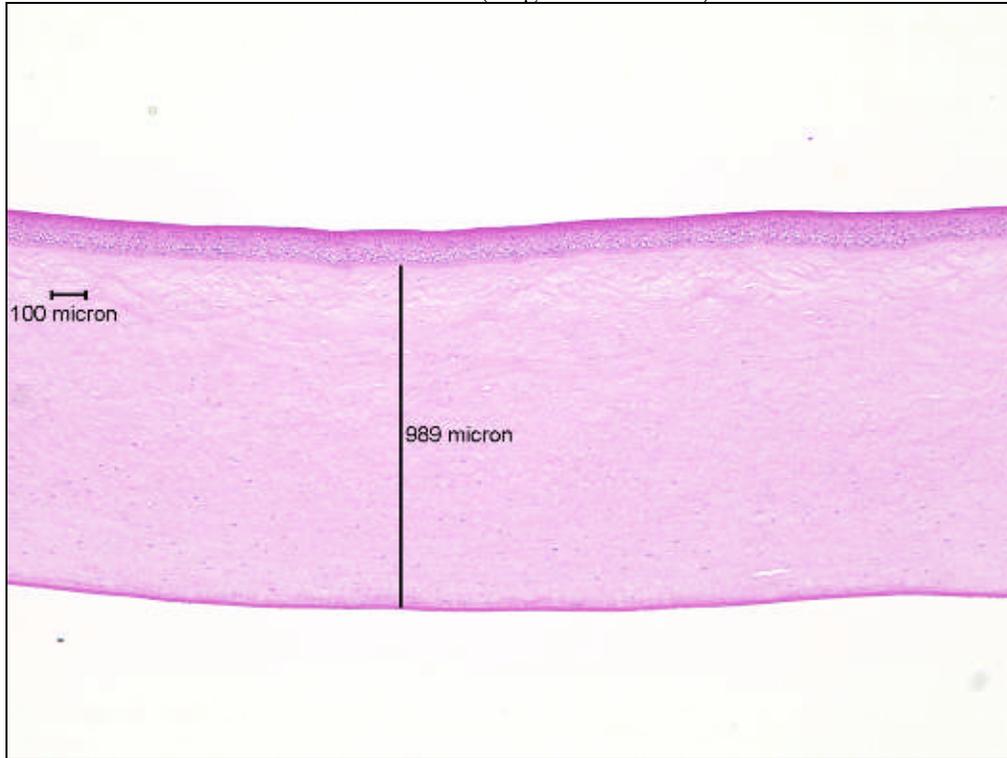


Figure 8. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Upper stroma showing hyperchromatic staining in the zone directly below Bowman's Layer and the decrease in the density of viable keratocytes (magnification 237x)

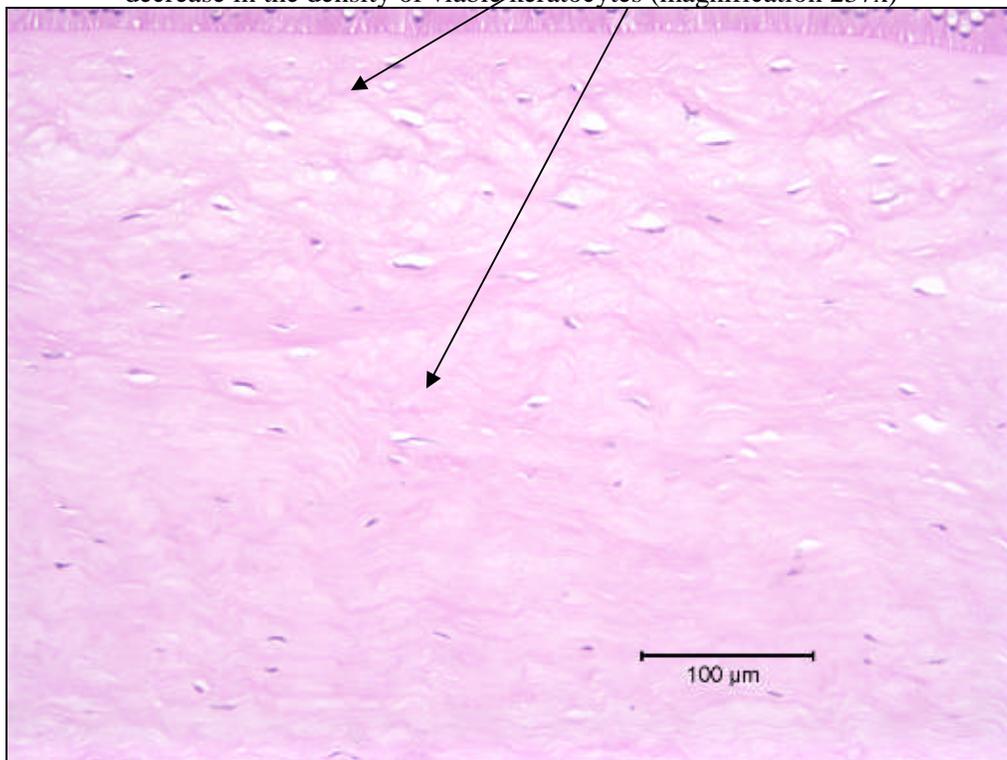


Figure 9. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and an increased frequency of keratocytes with abnormal chromatin condensation (magnification 475x)

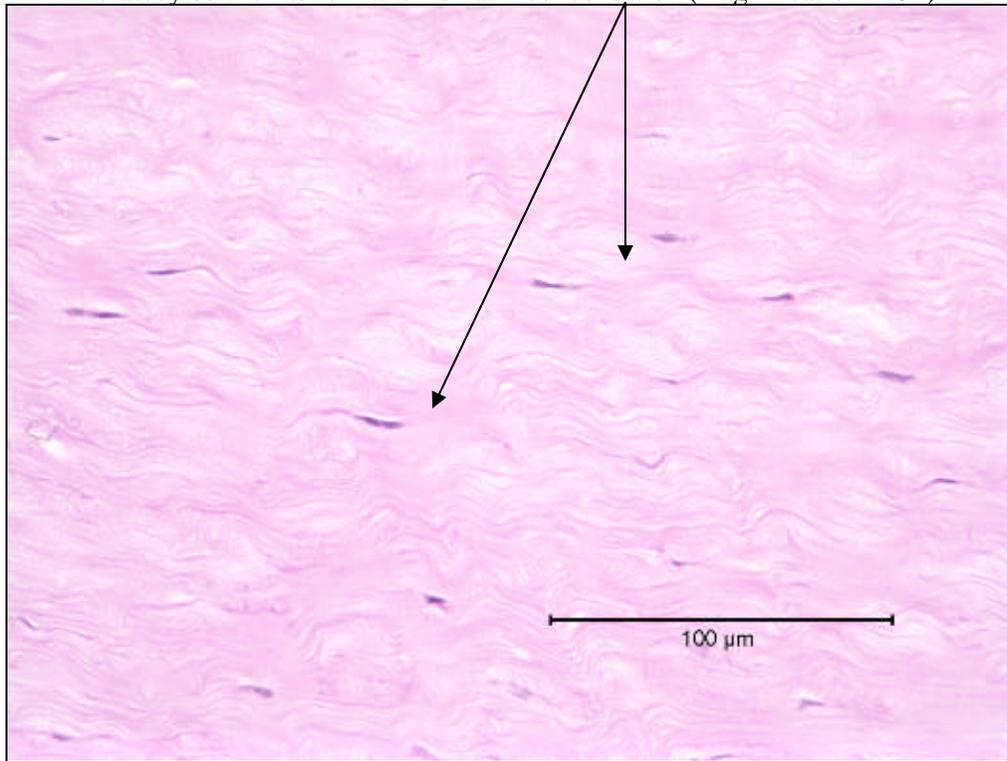


Figure 10. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (07/05/05) - Stroma near mid depth showing keratocyte nuclear enlargement and cytoplasmic eosinophilia (magnification 475x)

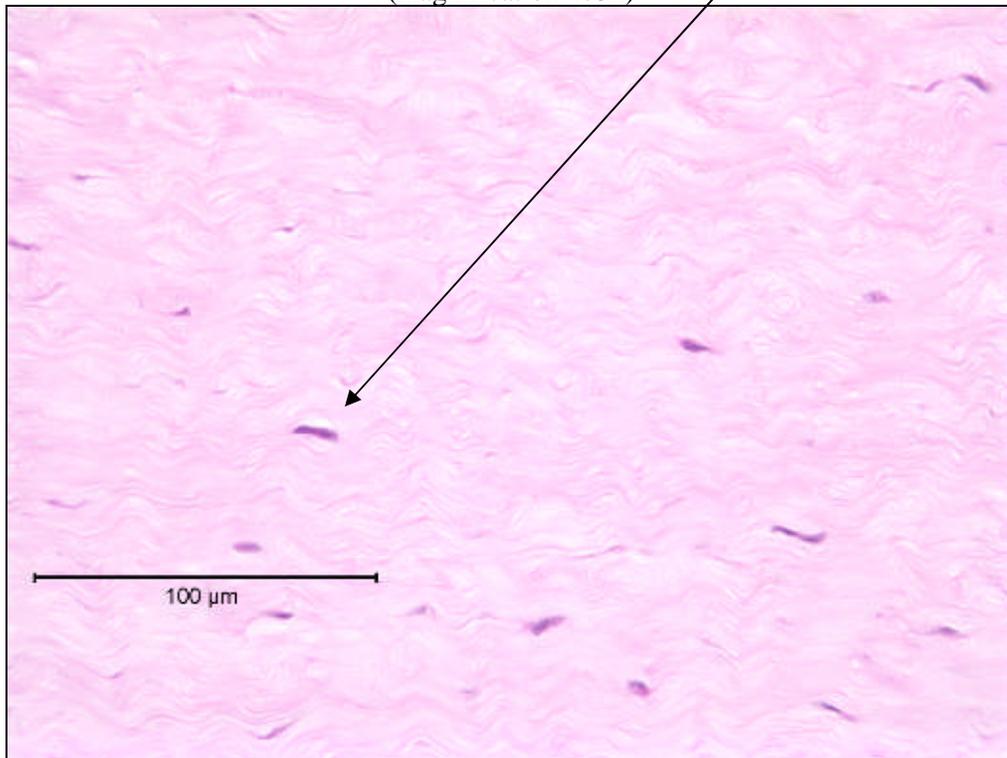


Figure 11. BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

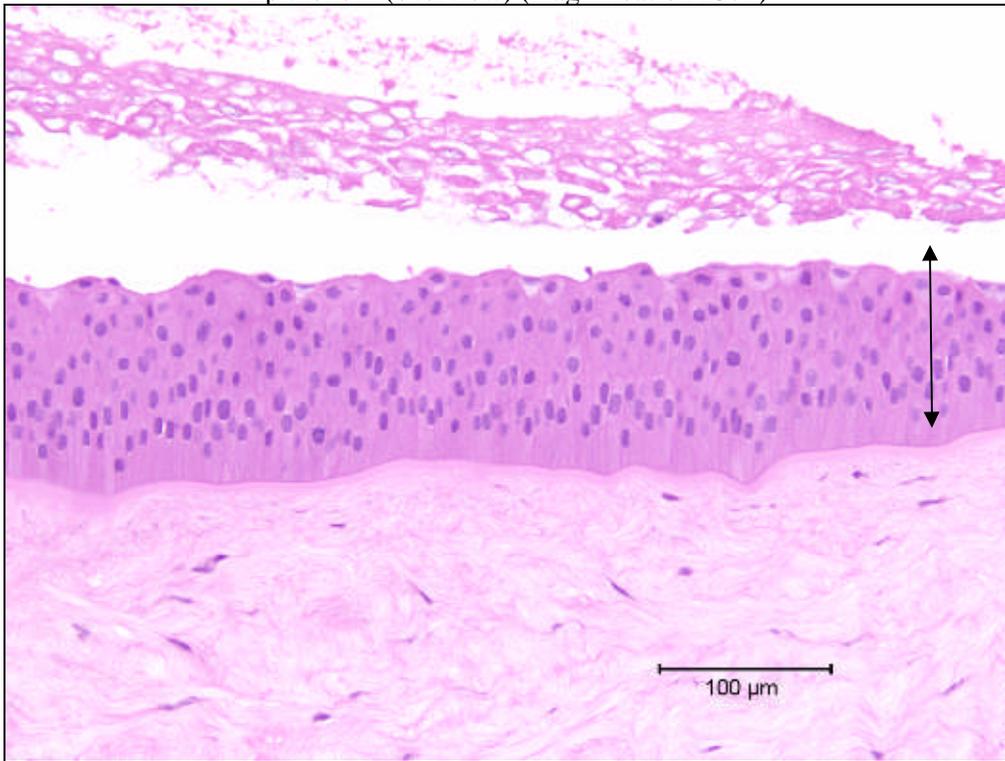


Figure 12. BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium showing the loss of the squamous cell layer and an increase in the number of wing and basal cells with cytoplasmic vacuolization (magnification 475x)

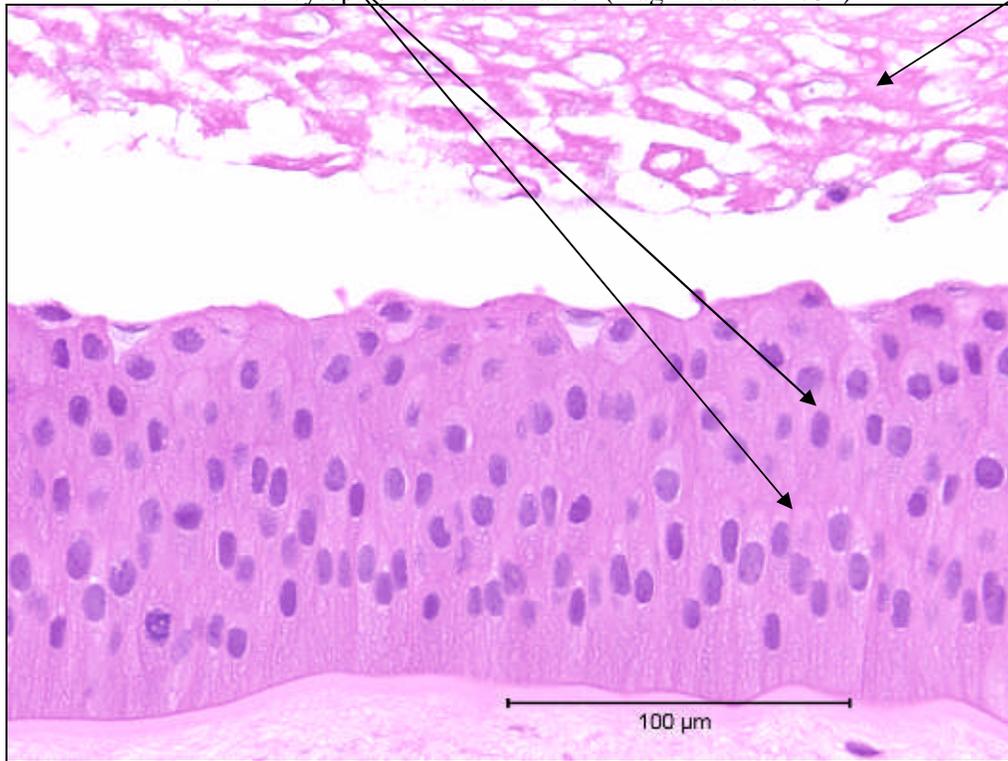


Figure 13. BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Full thickness (magnification 48x)

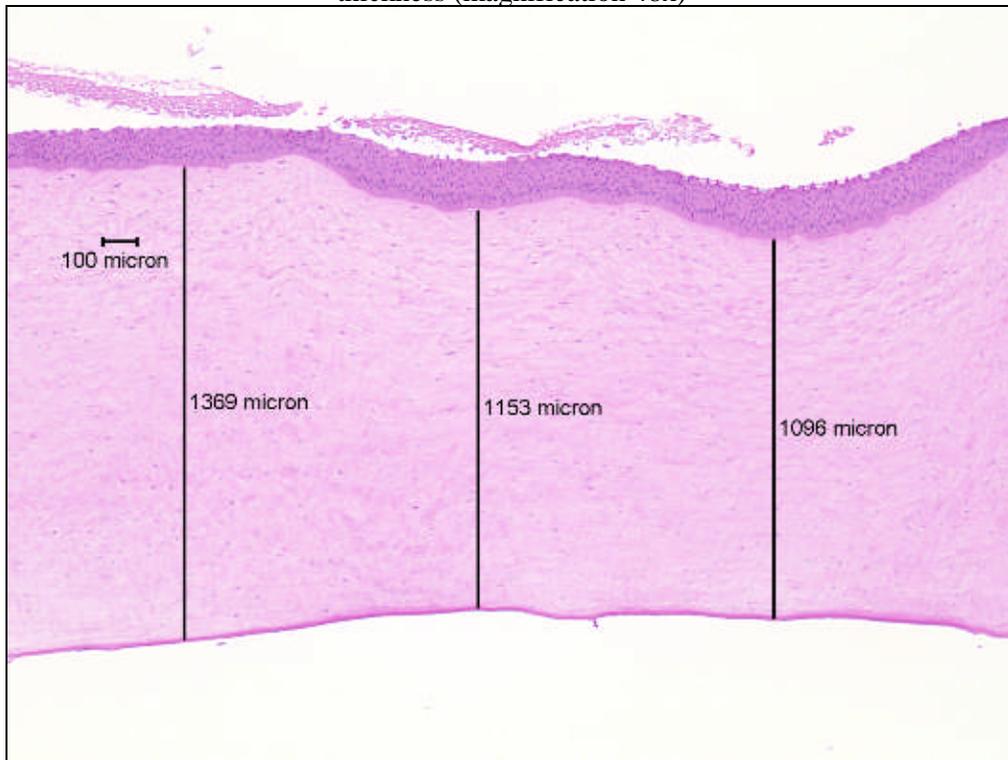


Figure 14.BJ, neat-installation exposure, 3-minute exposure, 120- minute post-exposure (07/05/05) - Stroma directly below Bowman's Layer showing moderate collagen matrix vacuolization and an increased number of keratocytes with nuclear enlargement and cytoplasmic eosinophilia (magnification 475x)

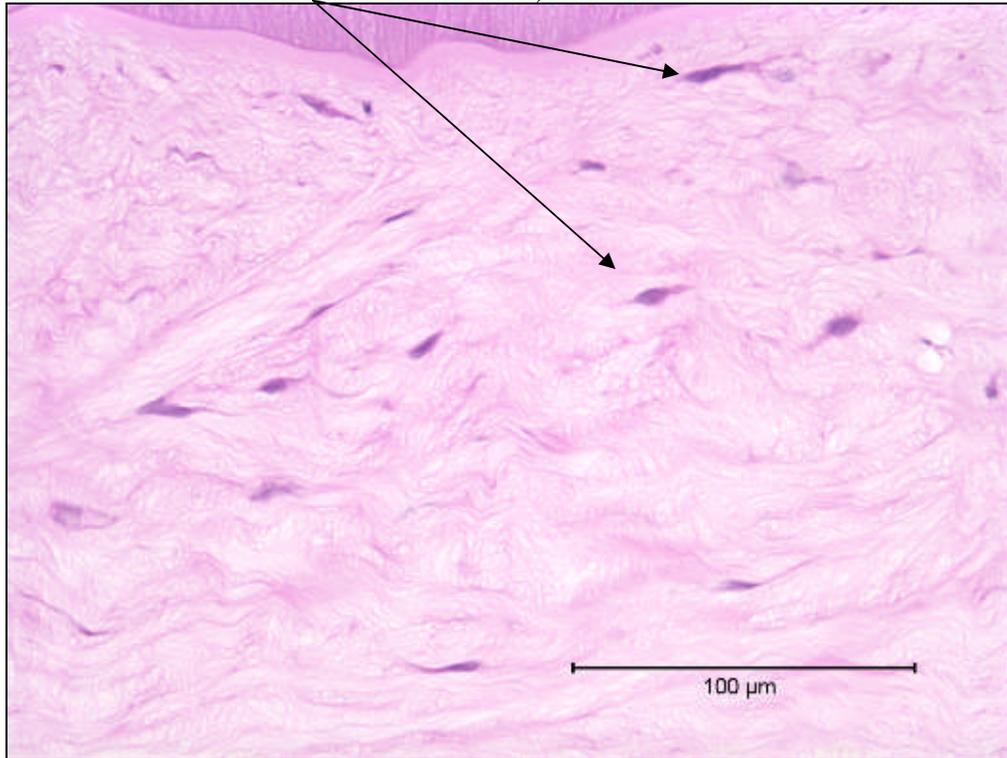


Figure 15. BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium (overview) (magnification 237x)

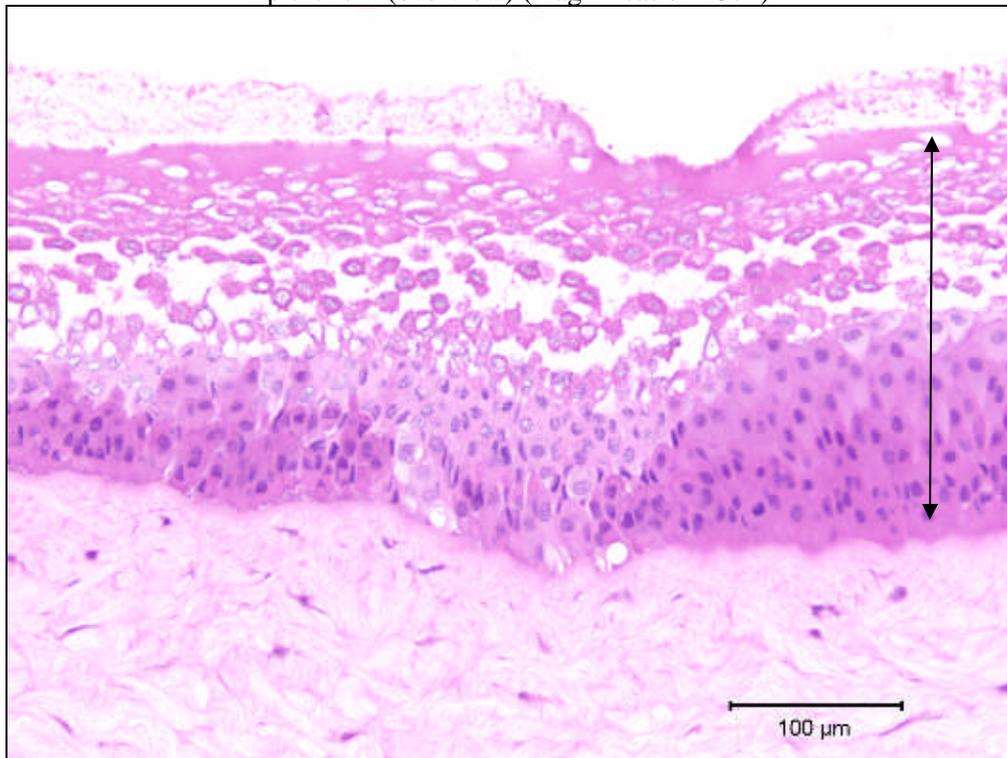


Figure 16.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Epithelium showing coagulation/loss of the squamous and wing cell layers, blanching of parts of the basal layer and abnormal chromatin condensation and cytoplasmic eosinophilia in the remaining basal cells (magnification 475x)

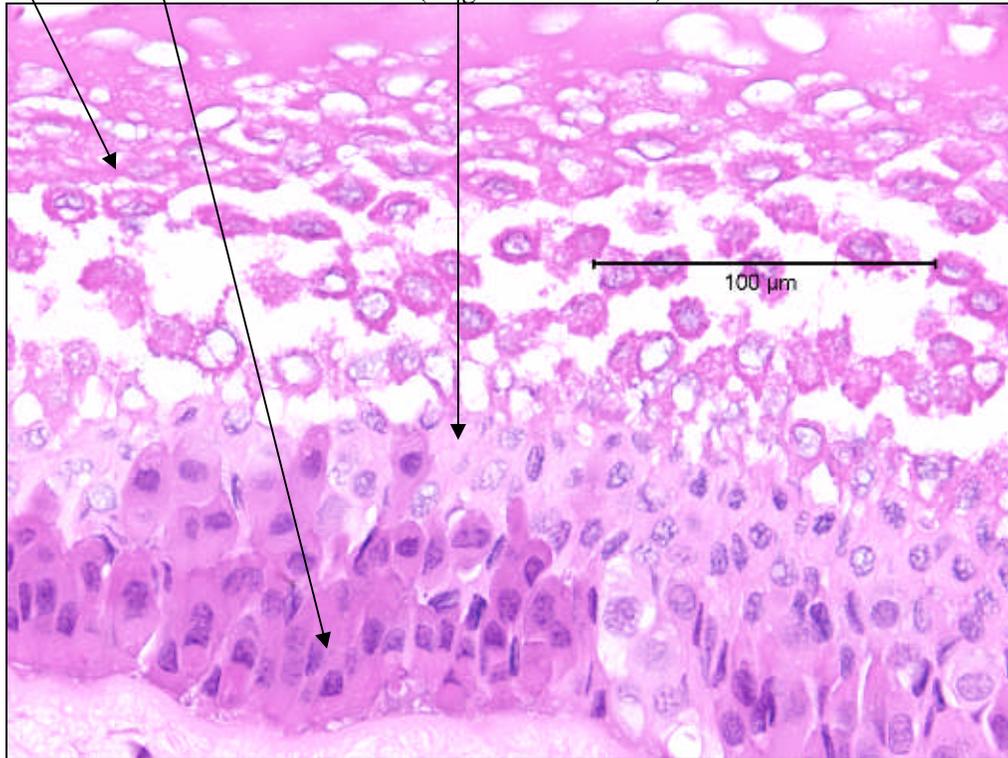


Figure 17.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Full thickness (magnification 48x)

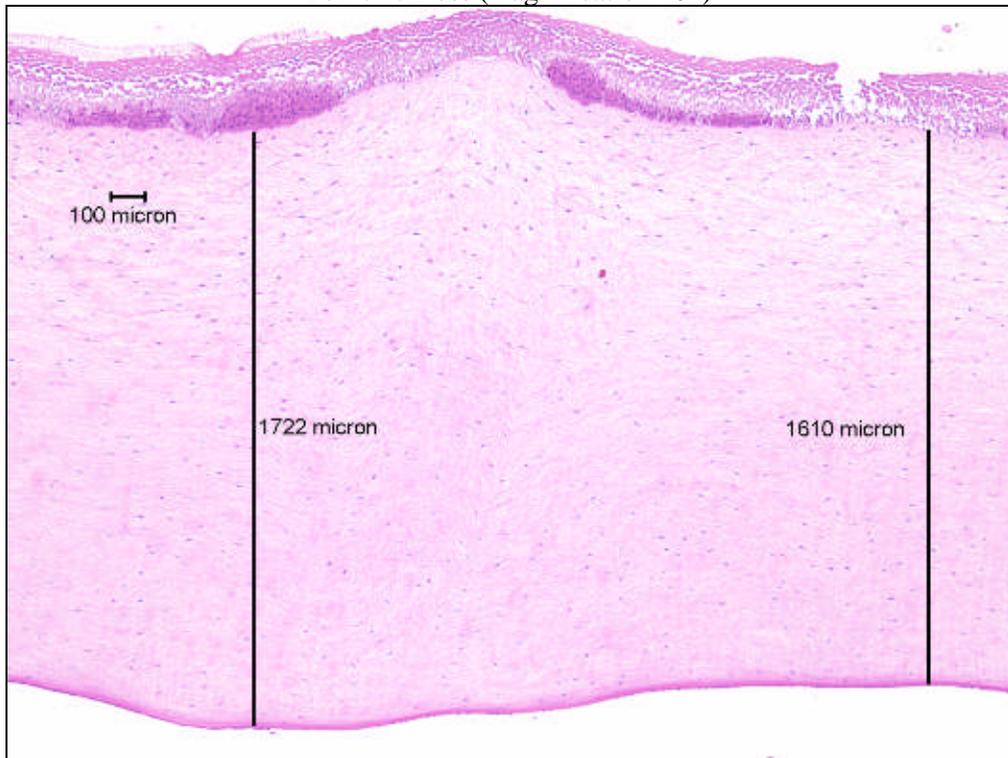
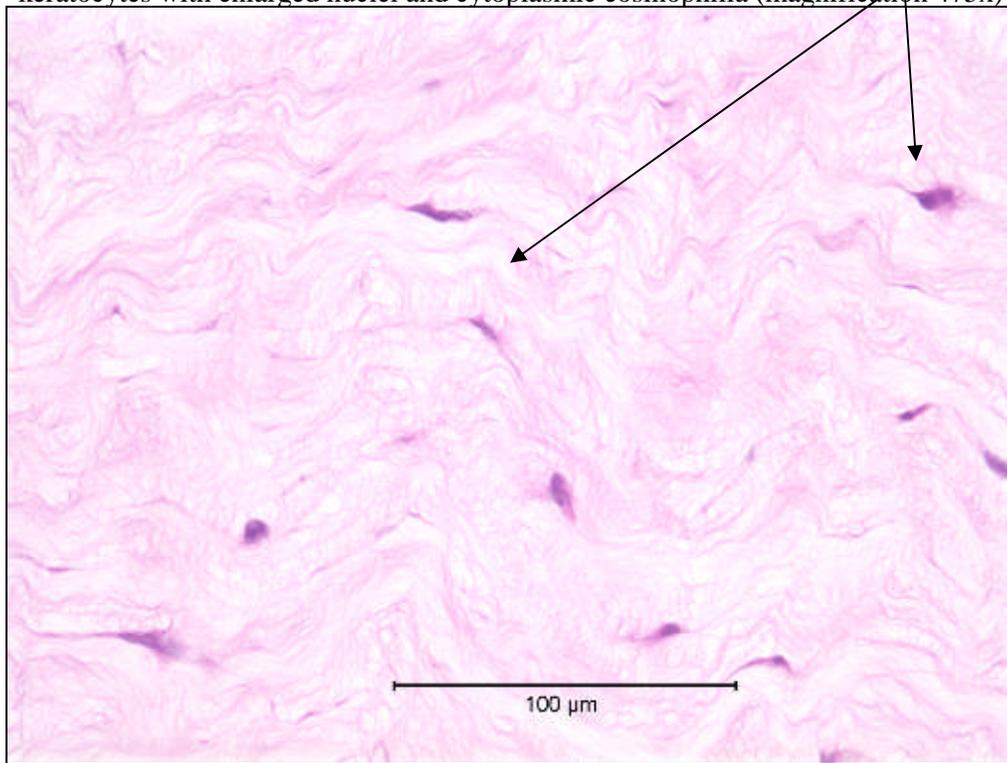


Figure 18.BJ, neat-installation exposure, 10-minute exposure, 120- minute post-exposure (07/05/05) - Stroma at 20% depth showing marked collagen matrix vacuolization and a marked increase in keratocytes with enlarged nuclei and cytoplasmic eosinophilia (magnification 475x)



## **APPENDIX A**

## **APPENDIX B**

**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>
<b>05AD42-1</b>	34	3	11	8	8.3		
<b>Neat</b>	35	3	7	4	4.3		
<b>3 minutes</b>	37	5	5	0	0.3	4.3	4.0
<b>05AD42-1</b>	38	4	35	31	31.3		
<b>Neat</b>	39	4	36	32	32.3		
<b>10 minutes</b>	40	4	27	23	23.3	29.0	4.9
<b>Neg. Control</b>	1	4	3	-1	NA		
<b>Sterile, DI water</b>	2	4	4	0	NA		
<b>10 minutes</b>	3	4	4	0	NA	-0.3	
<b>Pos. Control</b>	4	3	36	33	33.3		
<b>Ethanol</b>	6	4	34	30	30.3		
<b>10 minutes</b>	8	2	34	32	32.3	32.0	1.5
	*9	1					
	*10	4					
	*11	3					
	*12	5					
	*13	2					
	*14	4					
	*15	3					
	*16	3					
	*17	2					
	*18	3					
	*19	4					
	*20	3					
	*29	3					
	*36	6					
	*41	4					
	*42	4					
	*45	3					
	*48	3					

**Initial corneal opacity average:** 3

\* - 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	0.006
2	0.006
3	0.005
-----	
Avg.	0.006

**05AD42-1  
 Neat  
 3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
34	0.366	1	0.360
35	0.264	1	0.258
37	0.162	1	0.156
-----			
Avg. =			0.258
STDEV =			0.102

**Pos. Control  
 Ethanol  
 10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.314	1	1.308
6	1.179	1	1.173
8	1.379	1	1.373
-----			
Avg. =			1.285
STDEV =			0.102

**05AD42-1  
 Neat  
 10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
38	1.381	1	1.375
39	1.412	1	1.406
40	0.467	5	2.329
-----			
Avg. =			1.704
STDEV =			0.542

**IN VITRO SCORE**

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

<b>Test Article</b>	<b>Concentration</b>	<b>Exposure Period</b>	<b>Mean Opacity</b>	<b>Mean OD490</b>	<b>In vitro Score</b>
<b>05AD42-1</b>	Neat	3 minutes	4.3	0.258	8.2
<b>05AD42-1</b>	Neat	10 minutes	29.0	1.704	54.6
<b>Ethanol</b>	Neat	10 minutes	32.0	1.285	51.3