APPLICATION OF HISTOLOGICAL EVALUATION TO ENHANCE THE BOVINE OPACITY AND PERMEABILITY (BCOP) ASSAY

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Abstract

The BCOP assay was optimized to its present form by Drs. Pierre Gautheron and Joe Sina to address ocular irritation potential of pharmaceutical intermediates and is now widely applied across industries and chemical/formulation classes. Membrane lysis, protein coagulation, and saponification are common modes of action that lead to ocular irritation. In our experience, the opacity and permeability endpoints (generally combined into an "in vitro score") have been able to identify the epithelial and stromal changes associated with these types of damage. However, chemicals that react with nucleic acids, mitochondrial proteins, or other cellular targets, that do not lead to immediate loss of cellular integrity or protein precipitation, have proven more difficult to identify without the addition of histological evaluation of the treated corneas. In this study, a series of reference chemicals (i.e., surfactants, solvents, acids, alkalis and oxidizers) were exposed to the corneas under standard assay conditions. The opacity and permeability to fluorescein were measured and the corneas fixed for histological evaluation. The test substances were chosen to induce corneal lesions consistent with the various modes of action. Histological evaluation was performed on the three layers of the cornea to provide a direct measure of the depth of injury. This evaluation was used to compare depth of injury with the opacity and permeability scores and to identify lesions that were not fully expressed in opacity or permeability changes. For materials inducing membrane lysis, coagulation, and saponification, the opacity and permeability scores paralleled the depth of injury and histology provided data on specific changes in the tissue. Furthermore, the action of reactive materials (i.e., peroxide) was detected with histology and its selective action on the stromal keratocytes demonstrated. Histological evaluation of the treated corneas can be useful in directly evaluating lesions and enhancing the interpretation of the opacity and permeability endpoints.

INTRODUCTION

Drs. Jim Maurer (1996, 2001 and 2002) and James Jester (1998) have collaborated on a series of seminal studies that relate the initial depth of corneal injury in the rabbit (and some cases the rat) eye with the eventual degree and duration of the injury across a range of chemical classes and resulting modes of action of the corneal tissue. The BCOP assay allows precise control over the concentration and duration of test material exposure and assessment of the degree and location of any resulting lesions. The classic assay endpoints of opacity and penetration of fluorescein (permeability) provide a quantitative assessment of test substance-induced damage to the cornea. Membrane lysis, protein coagulation, and saponification are common modes of action that lead to ocular irritation. In our experience, the opacity and permeability endpoints (generally combined into an "in vitro score") have been able to identify the epithelial and stromal changes associated with this type of damage. However, chemicals that react with nucleic acids, mitochondrial proteins, or other cellular targets, that do not lead to immediate loss of cellular integrity or protein precipitation, have proven more difficult to identify without the addition of histological evaluation of the treated corneas (Curren et al, 1999) (Swanson et al., 2003).

Histological evaluation is performed on the three major layers of the cornea and provides a direct measure of the depth of injury (Harbell et al., 1999). This evaluation may be used to better understand the types of lesions produced, rule out occult lesions (either by potentially reactive materials or very mild formulations) or compare depth of injury between unknown and reference test materials. In this study, we have used a series of reference chemicals/formulations to illustrate the types of lesions that can be induced by membrane lysis, protein denaturation, saponification, and reactive/oxidative damage.

MATERIALS AND METHODS

Test and Control Substances

Test substance: Sodium lauryl sulfate (SLS), benzalkonium chloride (BAC), trichloroacetic acid (TAC), and sodium hydroxide (NaOH) were diluted to their final concentration in sterile, deionized water. The solvent formulation and peroxide-containing cleaning formulation were tested neat.

Controls: The negative control was sterile, deionized water (exposed in parallel with the test substances) and the positive control was neat ethanol (10-minute exposure).

Preparation of the Corneas

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals and transferred to the laboratory in cold Hanks' Balanced Salt Solution (HBSS). The corneas were inspected for damage and any with defects were discarded. Corneas were mounted in corneal holders and the two compartments of the holder were filled with minimum essential medium Eagle (MEM) with 1% Fetal Bovine Serum (FBS) (complete MEM), starting with the posterior chamber and the holders incubated at 32±1oC for a minimum of one hour. Corneal Opacity The opacity was determined for each cornea using the OP-KIT Opacitometer (Spectro Design; Riom, France). The opacity of each test cornea was determined against an air blank. The medium was then removed from the anterior part of the holder and replaced with the test material, negative control (water), or positive control (ethanol).

Assay Procedure

Test Substances (750μL) were introduced into the anterior compartment of the holder while slightly rotating the holder to ensure uniform covering over the surface of the cornea. The holders were then incubated at 32± 1oC for the indicated times. The dosing material was then removed, and the epithelial side of the cornea washed at least three times with complete MEM to remove any remaining test material. The anterior compartment was then refilled with MEM. The corneas were then returned to the incubator for the indicated times after which a measure of relative opacity was obtained.

Corneal Permeability

After the opacity reading, the medium was removed from both chambers of the holder and the posterior compartment refilled with fresh complete MEM. One ml of a 4mg/ml fluorescein solution was added to the anterior compartment. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32±1oC. The medium was then removed from the posterior chamber, an aliquot transferred to a 96-well plate and the optical density (OD) at 490nm determined using a plate reader. As necessary, dilutions were performed to keep the readings in the linier range of the instrument.

Presentation of Data

A net change in opacity for each treated cornea was obtained by subtracting the initial opacity reading from the final opacity reading. The mean change in opacity for each treatment group was calculated and corrected for the change in the negative control group. The corrected mean OD490 value of each treatment group was to be calculated by averaging the OD490 values of the treated corneas for that treatment group and subtracting the mean OD490 for the negative control.

The following formula was used to determine the in vitro score:
In Vitro Score = Corrected Mean Opacity Value + 15 x Corrected Mean OD490 Value

Histology

Each cornea was fixed whole in 10% buffered formalin for at least 24 hours. 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. Corneal sections were scored for lesions in the epithelium, stroma, (swelling and keratocyte damage), and endothelium.

RESULTS

A series of reference substances were evaluated to illustrate membrane lysis, protein denaturation, saponification, and alkylation/oxidative damage. Table 1 provides the average opacity, permeability (OD490) and in vitro score values for the respective treatment groups. The concurrent negative and positive control-treated corneas are evaluated in parallel with the test substance-treated corneas (Figures 1a and 1b show examples of negative control corneas).

Membrane lysis was induced by mechanically removing the epithelium (to evaluate changes to the stroma without chemical action) and by exposure to several concentrations of SLS (1.5% Figure 2a and 15% Figure 2b). Note that the loss of epithelium (and resulting stromal swelling) does not induce any appreciable opacity directly (Table 1).

Protein denaturation (coagulation/precipitation) was induced by 3% TCA. Increasing exposure periods illustrate the relatively slow infiltration of the acid through the epithelium (Figures 3a and b). BAC was used to illustrate the action of a precipitating cationic surfactant (Figures 4a and d). Note that the epithelial cells remained attached to the cornea but the barrier properties of the epithelium were lost.

Saponification was induced by exposure to 4% (1N) NaOH (one-minute exposure) and 1% (0.25 N) NaOH (10-minute exposure). Note the blanching of the nuclei in the epithelium, denaturation of the epithelial proteins, swelling in the stroma and full thickness destruction of the keratocytes (Figures 5a to d).

Parafluoranaline (Figures 6a and b) and a model cleaner containing 1% H2O2 (Figures 7a and b) demonstrate the kind of delayed keratocyte changes that are often observed with "reactive chemistries". Corneas are treated in normal fashion but maintained in culture for up to 20 hours to allow the changes in the keratocytes to be manifested.. Parafluoranaline is a severe eye irritant in vivo but the BCOP In Vitro Score under predicted the degree and duration of the in vivo response. The histological changes in the stroma clearly show the severe irritation potential.

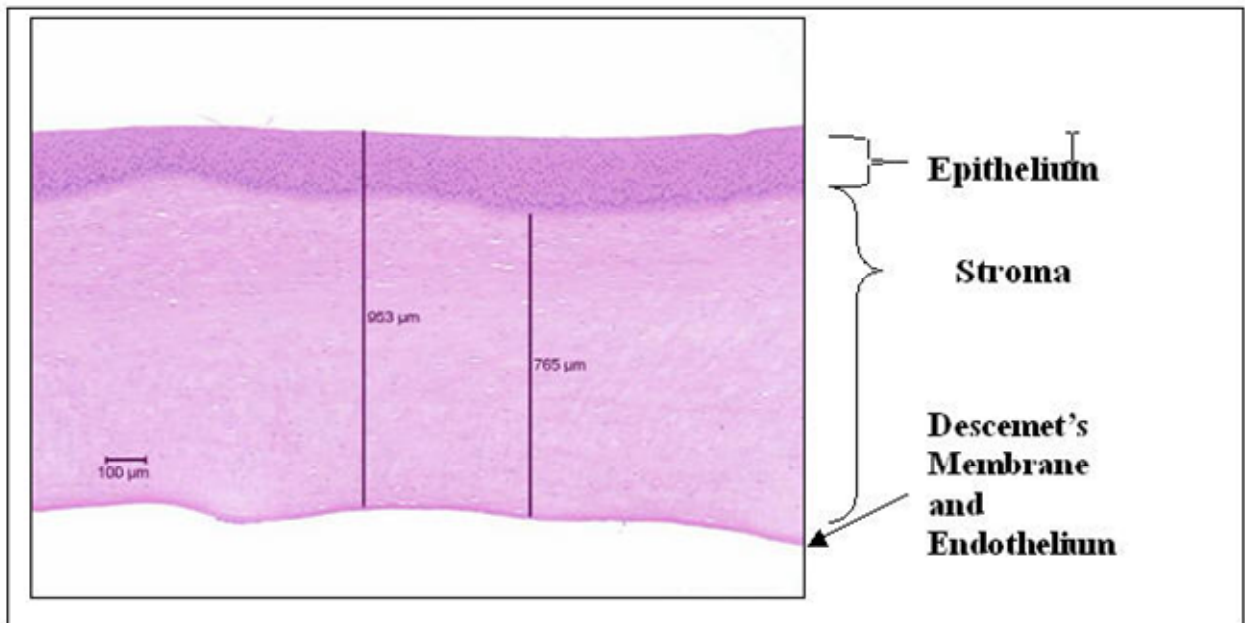**Table 1.** Opacity, Permeability, and In Vitro Scores from the Various Treatment Groups

Treatment	Exposure	Post-Exposure	Opacity	OD_{490}	In Vitro Score	Figure No.
Mechanical de-epithelialization	NA	2 hours	0	2.975 to 4.090		
1.5% SLS	10 min	2 hours	1.7	0.302	6.5	2a
15% SLS	10 min	2 hours	4.7	0.967	19.2	2b
3% TCA	1 min	2 hours	5.7	0.012	5.8	3a
3% TCA	10 min	2 hours	110.5	0.014	110.7	3b
3% TCA	30 min	2 hours	218.0	0.000	218.0	3c, d
0.1% BAC	10 min	2 hours	9.2	0.006	9.3	
0.25% BAC	10 min	2 hours	24.7	0.801	31.2	4a
0.5% BAC	10 min	2 hours	29.7	1.398	51.8	
1% BAC	10 min	2 hours	49.7	1.456	73.4	4b
4% NaOH	1 min	2 hours	101.0	3.364	151.5	5a, b
1% NaOH	10 min	2 hours	98.7	4.193	161.6	5c, d
Parafluoranaline	10 min	2 hours	18.0	0.862	30.9	6a
Parafluoranaline	10 min	20 hours	21.5	0.467	28.5	6b
1% H_2O_2 (cleaner)	10 min	4 hours	9.7	0.349	14.9	7a
1% H_2O_2 (cleaner)	10 min	20 hours	24.3	0.763	35.8	7b
Ethanol (historical range)	10 min	2 hours	31.4±4.8	1.404±0.24	52.5±6.2	

Figure 1. Negative control-treated corneas
**a) Epithelium and upper stroma**

Squamous Cell Layer

Wing Cell Layer

Basal Cell Layer

Basal Lamina

Bowman's Layer

Keratocytes

Lamellar Collagen

171 µm

100 µm
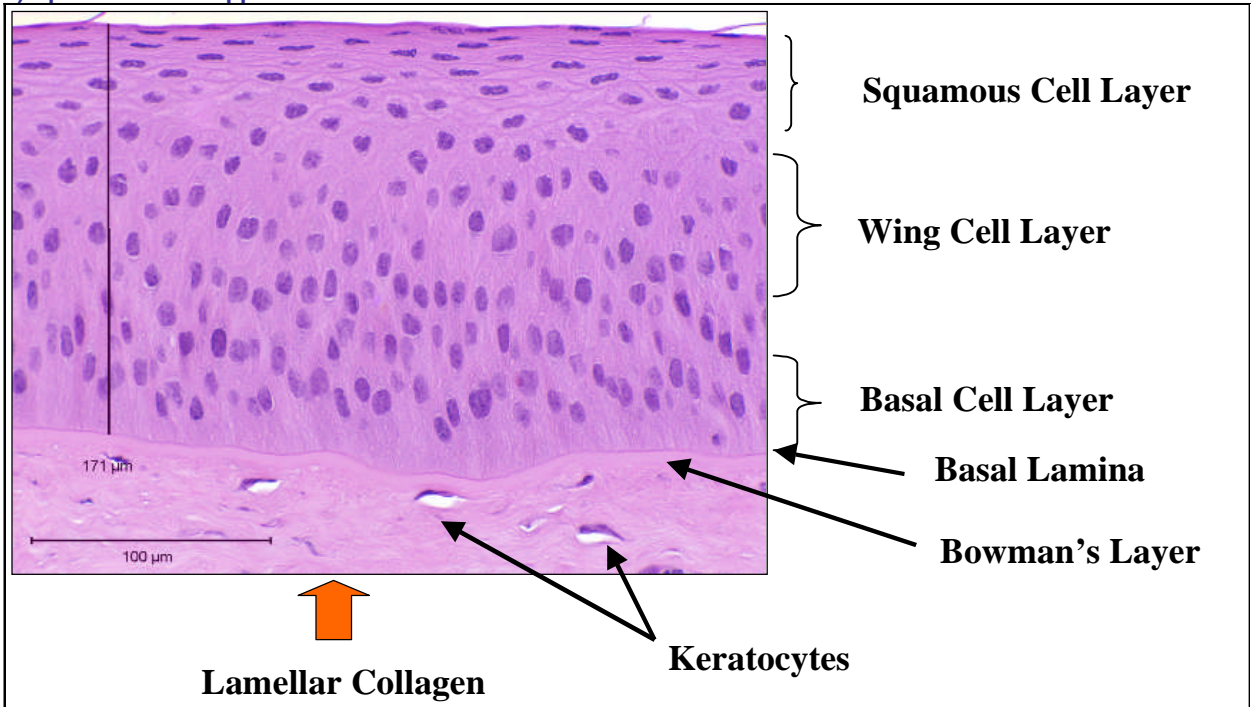
b) Full thickness Control corneas generally range in thickness between 0.9 and 1.0 mm.

Epithelium

Stroma

Descemet's Membrane and Endothelium

953 µm

765 µm

100 µm

Figure 2. Corneas treated with SLS for 10 minutes at the indicated concentration.
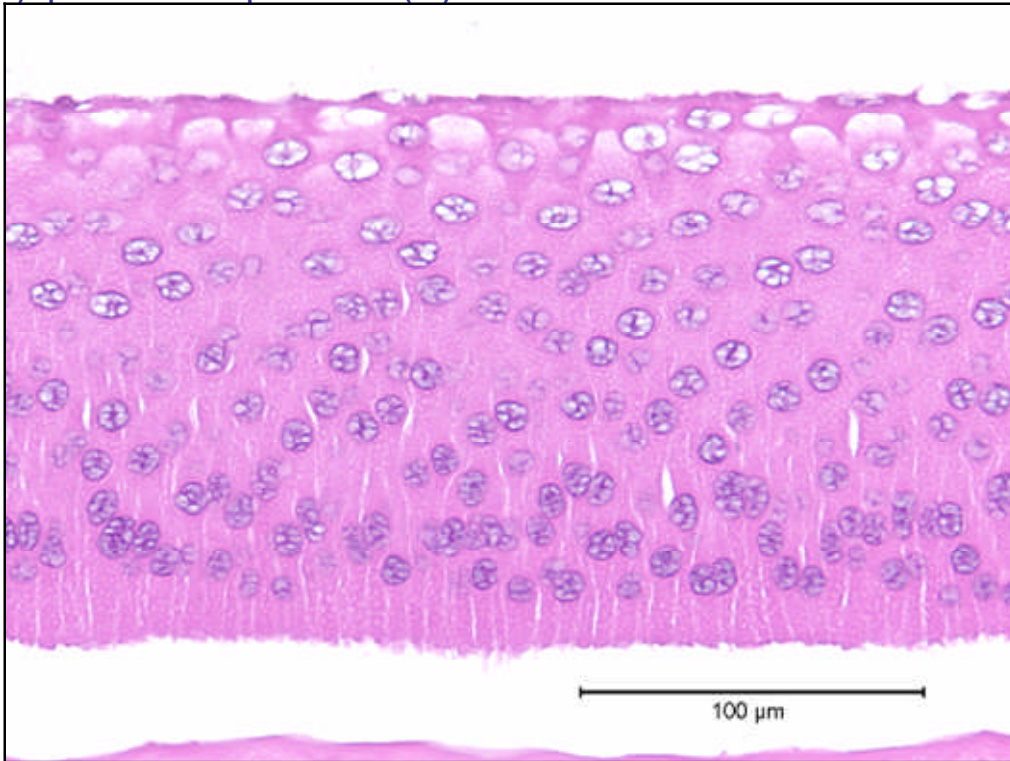a) Epithelium after exposure to 1.5% SLS
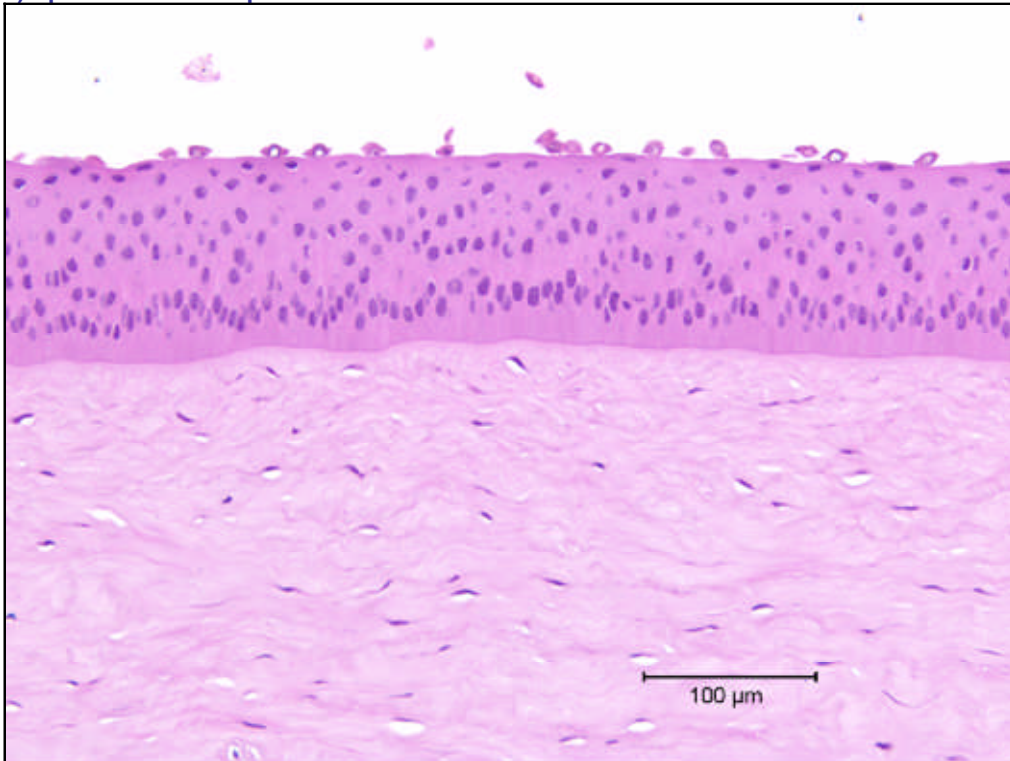
b) Epithelium after exposure to 15% SLS
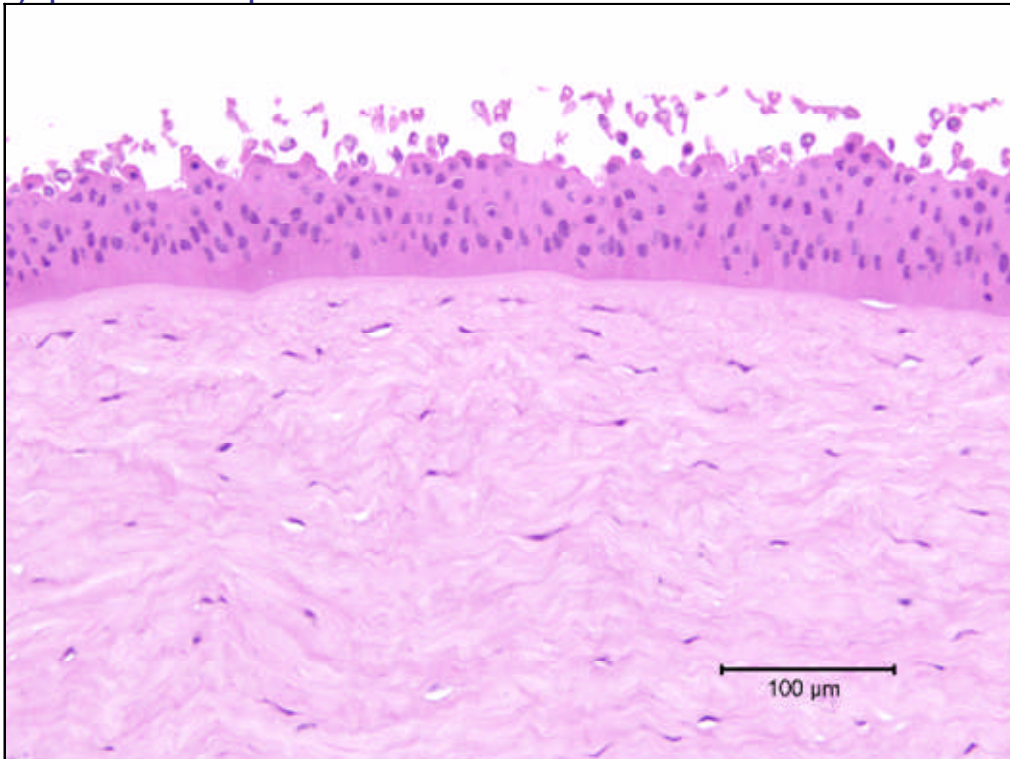
Figure 3. Corneas treated with 3% TCA for the indicated periods of exposure.
a) Epithelium after a 1-minute exposure

100 μm
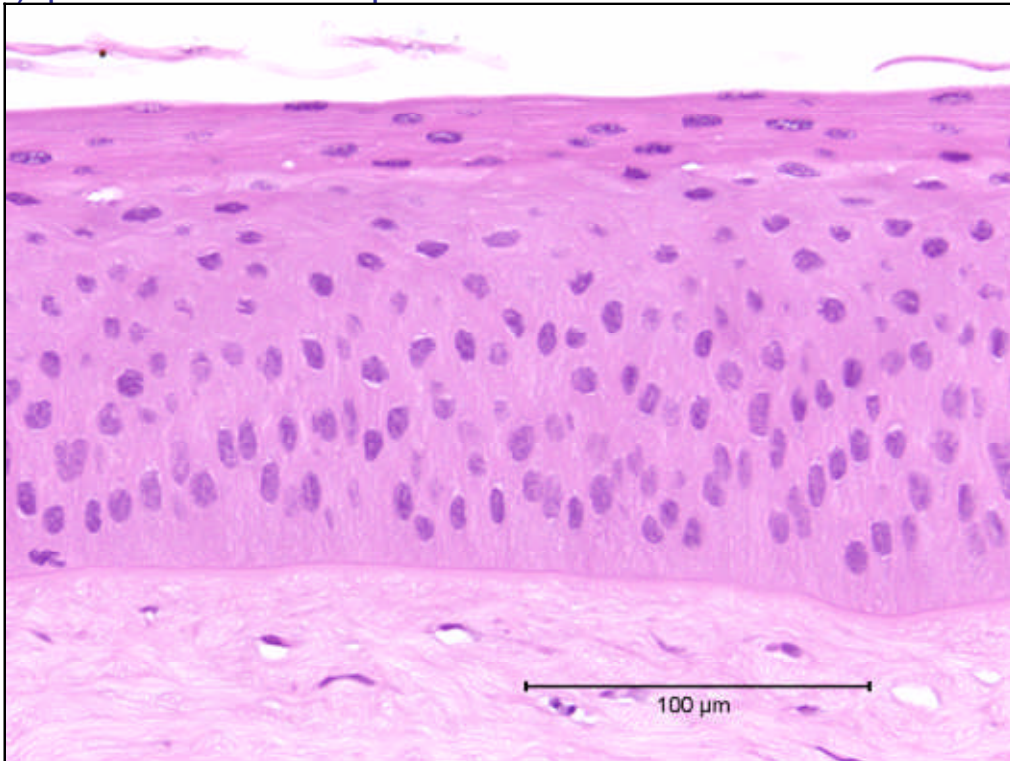
b) Epithelium after a 10-minute exposure

100 μm
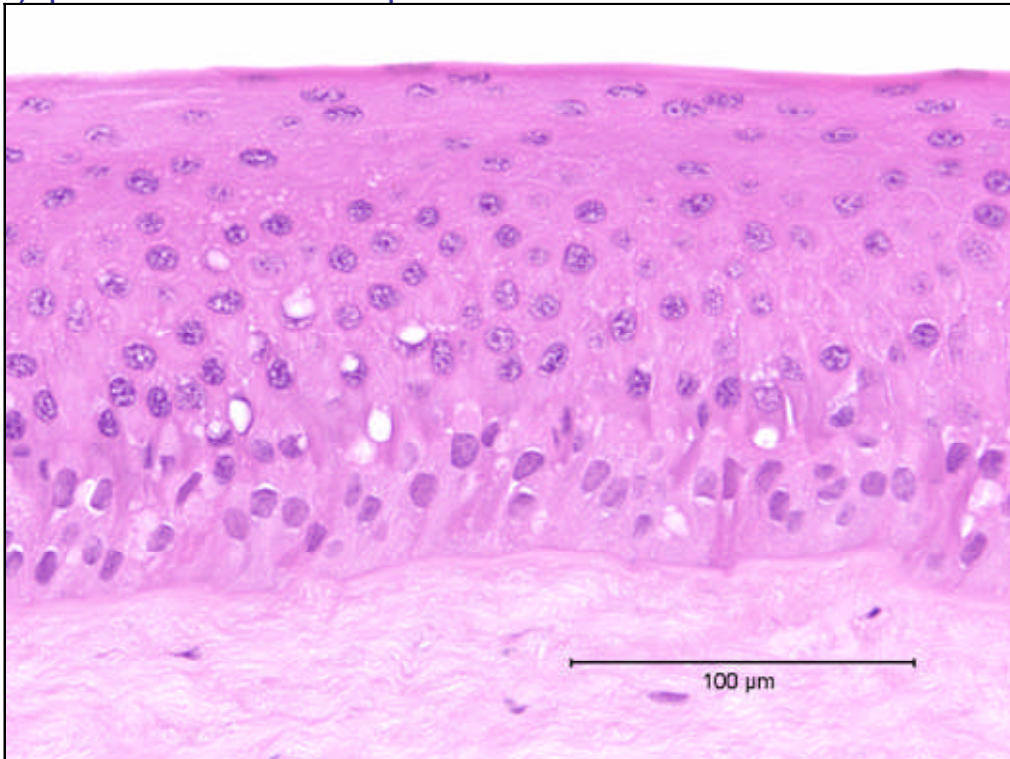
c) Epithelium after a 30-minute exposure

100 µm
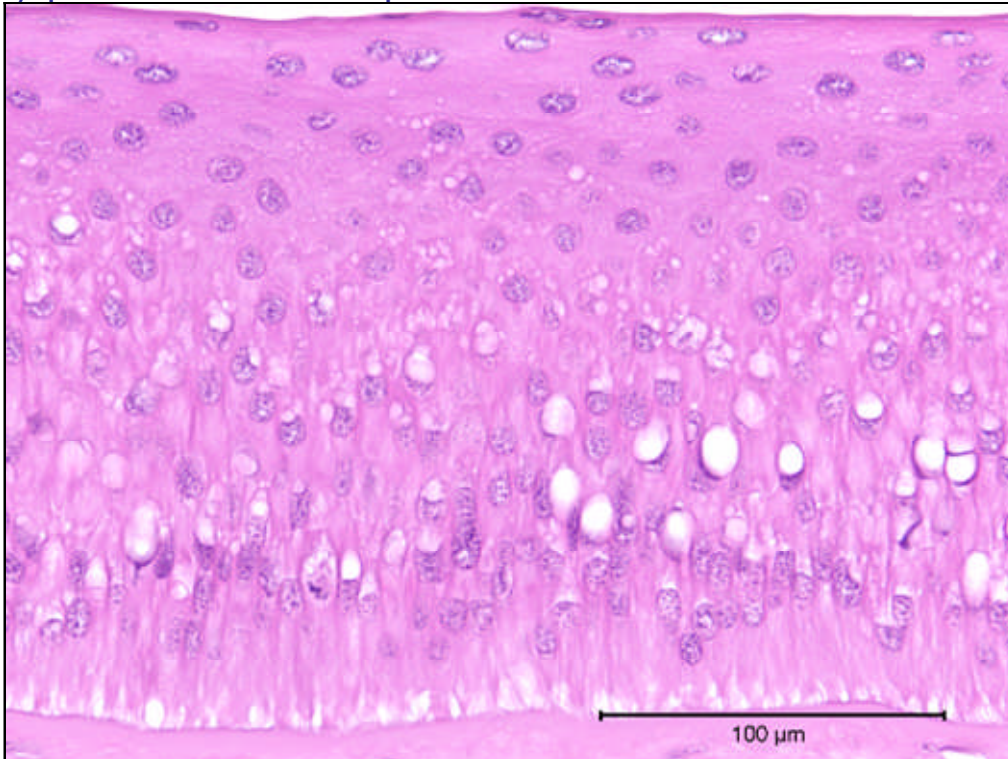
d) Cross-section of a cornea treated for 30 minutes as above. However, the coagulated epithelium was removed after exposure and the cornea carried through the opacity and permeability steps (Opacity=17, OD490=4.589, In Vitro Score=85.8). This cornea shows that most of the opacity was in the coagulated epithelium but that some stromal denaturation was also present (note the hyperchromic staining in the upper stroma).

100 micron

1686 micron
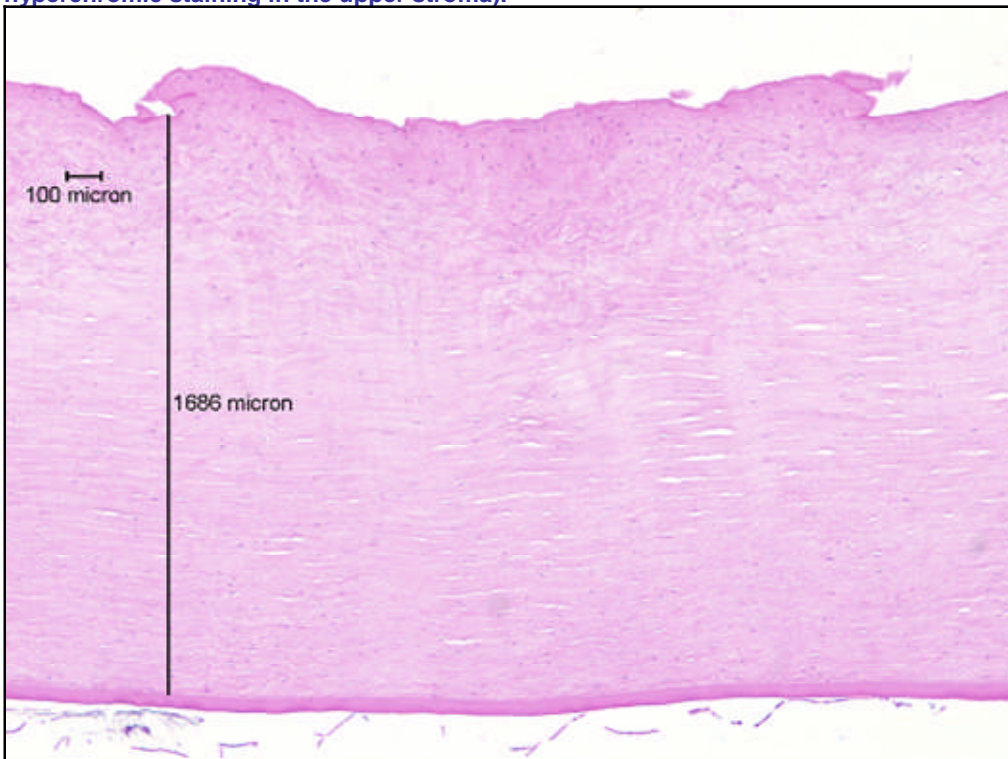
Figure 4. Corneas treated with BAC for 10 minutes at the indicated concentration.
a) Epithelium after exposure to 0.25% BAC
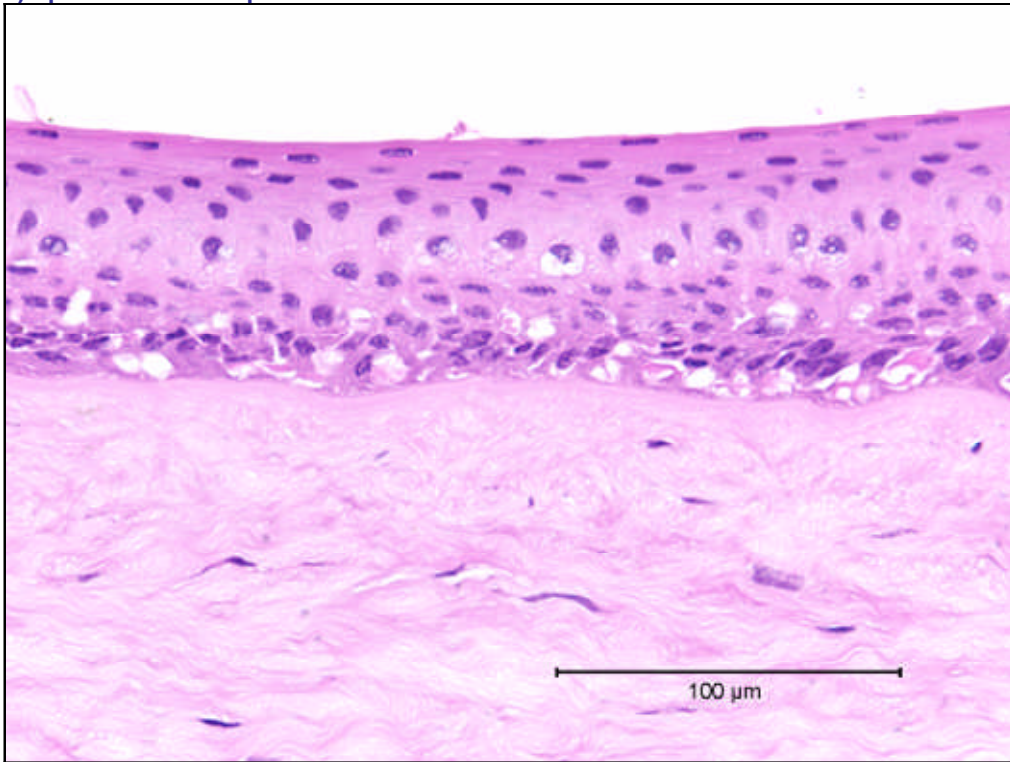
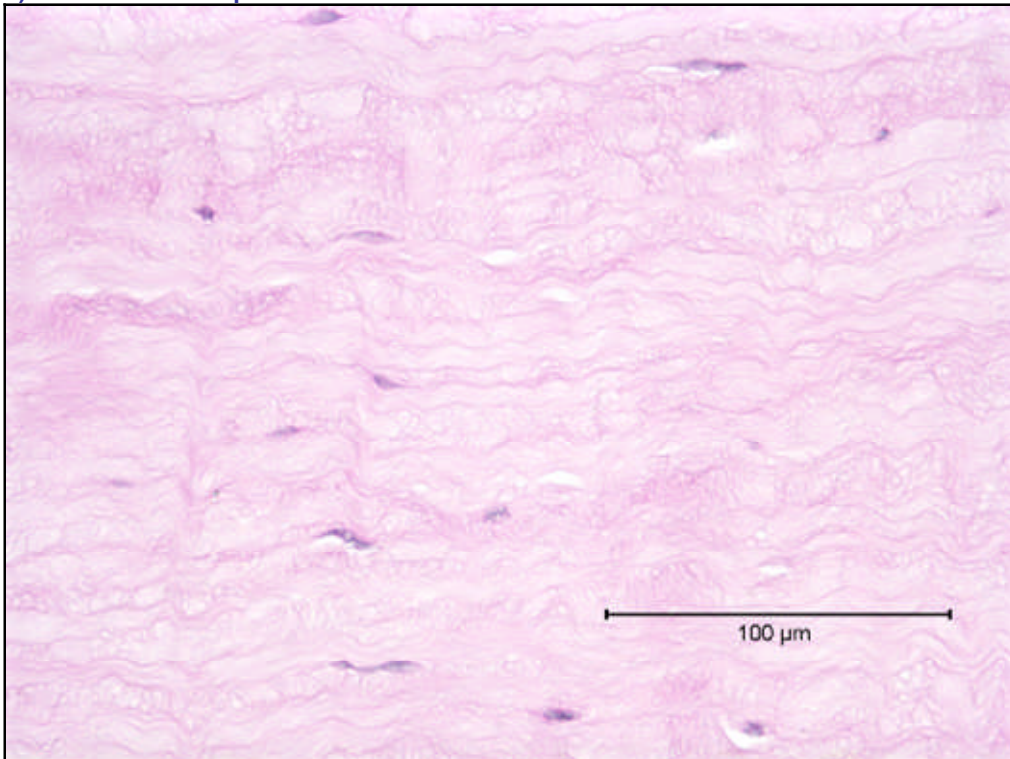**b) Epithelium after exposure to 1% BAC**
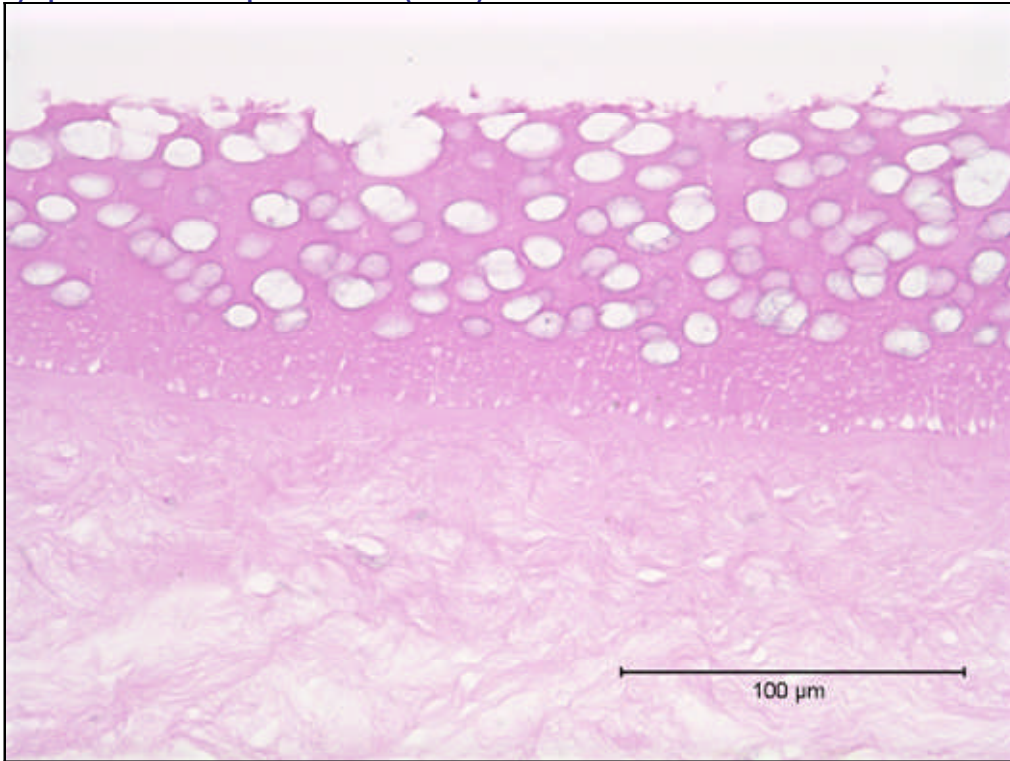
Figure 5. Corneas treated with NaOH

a) Epithelium after exposure to 4% (1N) NaOH for one minute

b) Stroma at 20% depth

c) Epithelium after exposure to 1% (0.25N) NaOH for 10 minutes

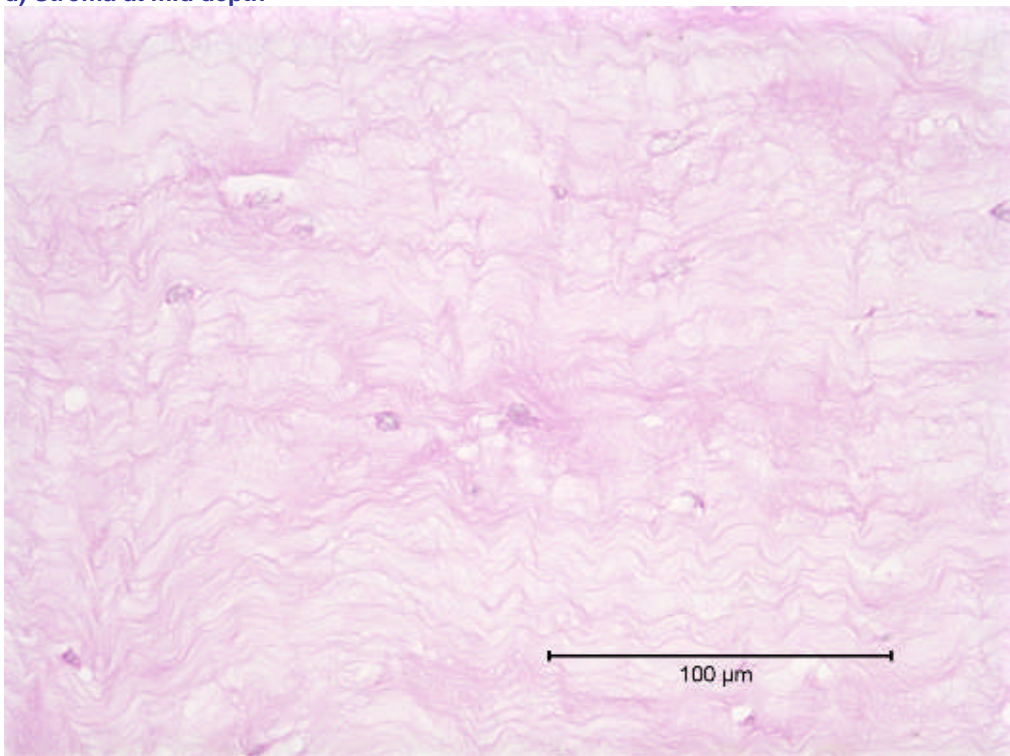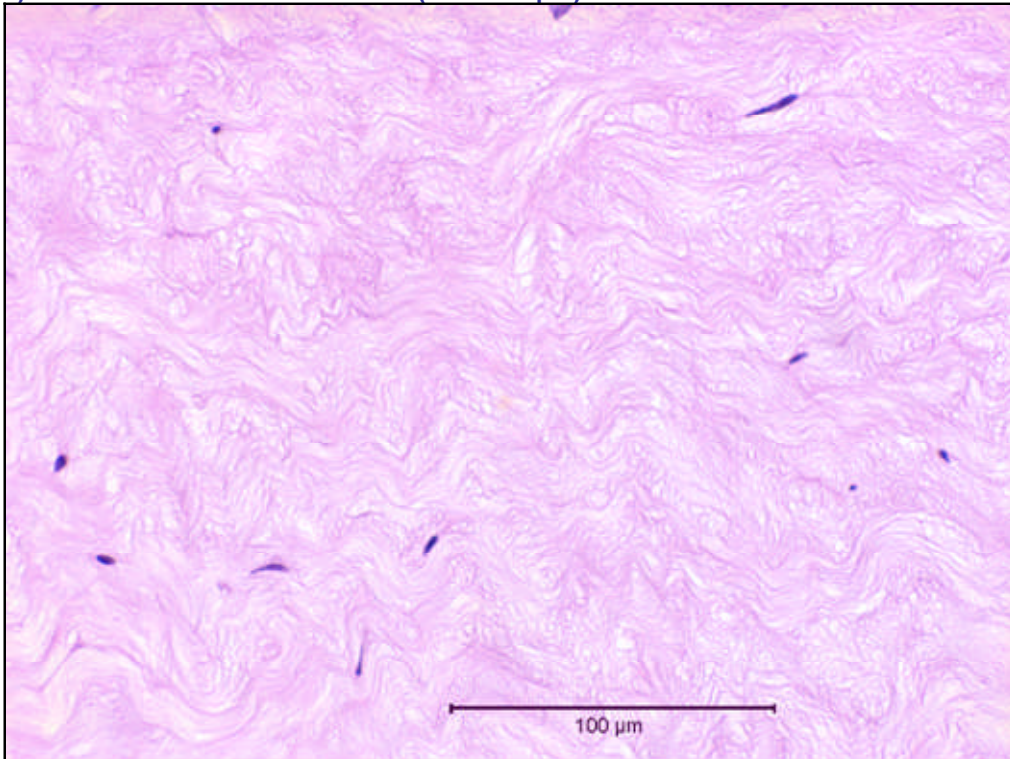100 μm

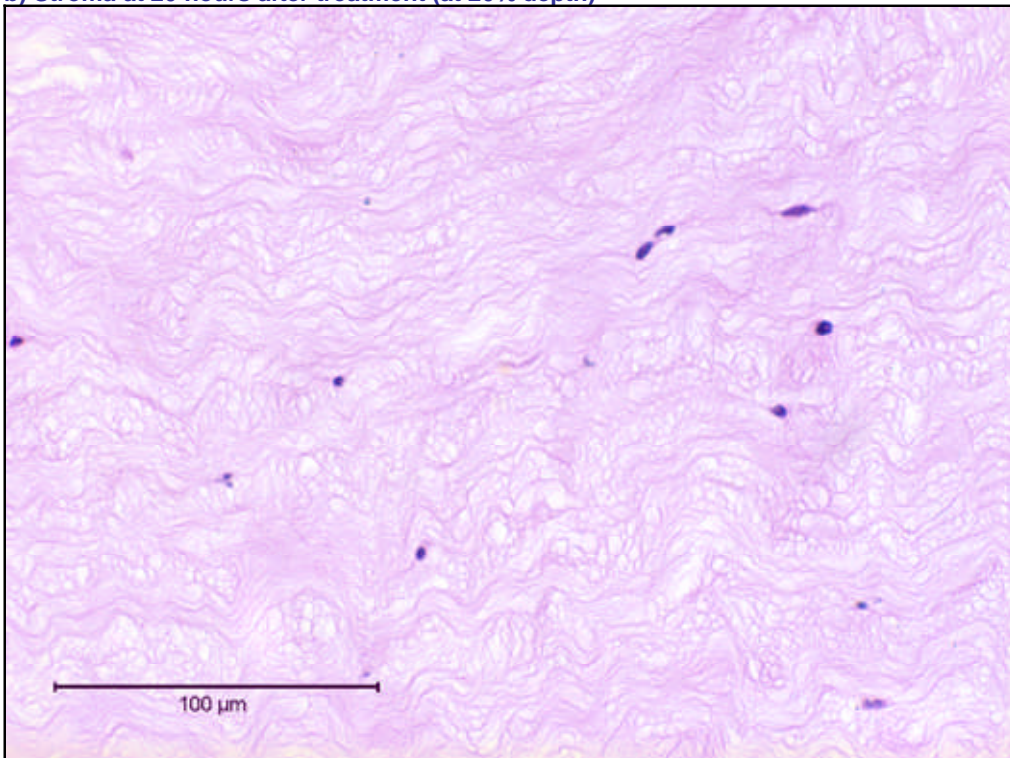**d) Stroma at mid depth**

100 μm

Figure 7. Corneas treated with a model peroxide cleaner (from Swanson et al., 2003).

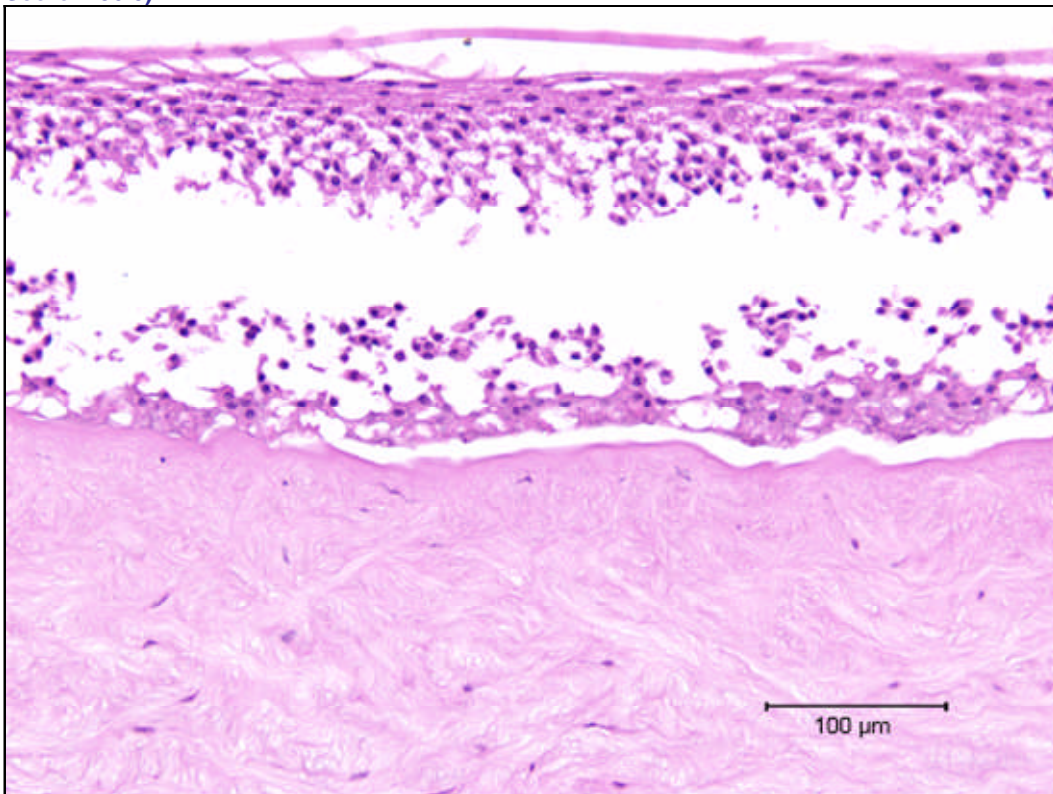**a) Stroma at 4 hours after treatment (at 10% depth)**

**b) Stroma at 20 hours after treatment (at 20% depth)**

Figure 8. Corneas treated with an organic solvent-containing formulation for 3 minutes showing the impact of the loss of the precipitated epithelium
a) Cornea with precipitated epithelial components in place (opacity = 37.3, OD490 = 1.245, In Vitro Score = 56.0)

b) Cornea where the precipitated epithelium was lost during incubation (opacity = 12.3, OD490 = 2.115, In Vitro Score = 44.0)

100 µm
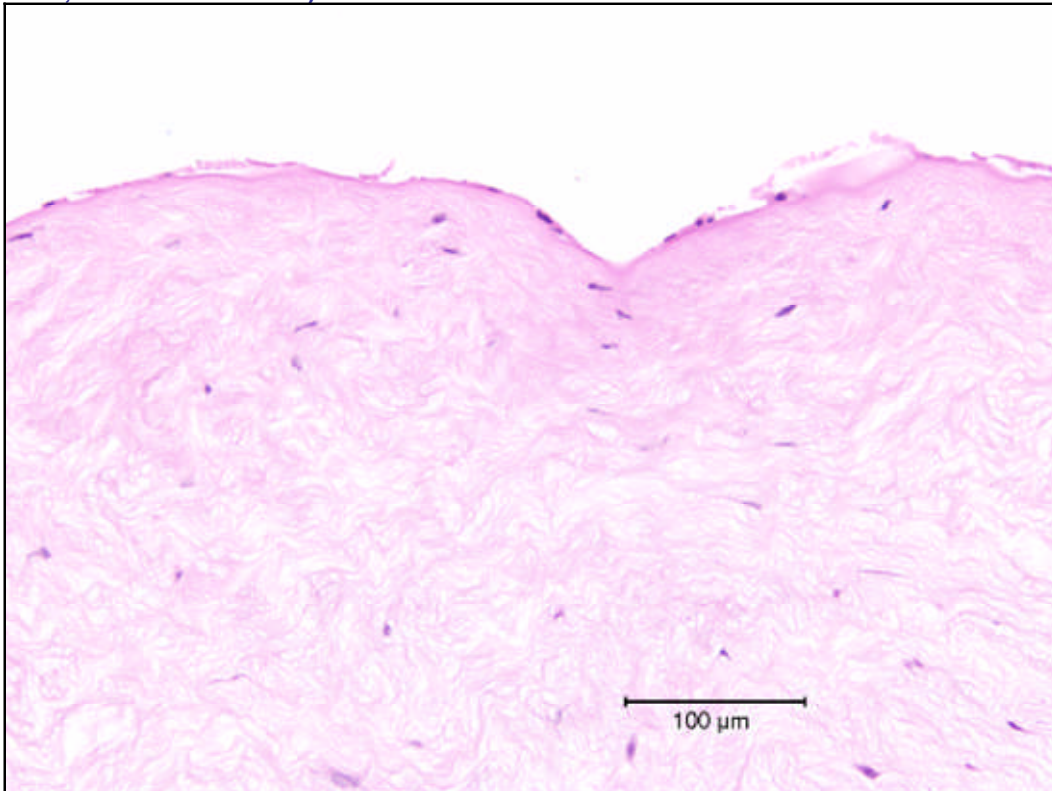
CONCLUSIONS

1) While the opacity and permeability endpoints provide good measures of tissue injury, histology is added to the study to:
   a) Directly determine depth and character of lesions developed (often in comparison to a benchmark material)(Cater et al, 2003 and Cuellar et al, 2003).
   b) Confirm very mild/nonirritating responses.
   c) Enhance submissions to regulatory agencies.

2) Certain chemicals (chemical classes) produce lesion in the cornea without inducing corresponding increases in opacity or permeability scores. Formulations containing such active ingredients require histology for proper assessment.

3) The ICCVAM EXPERT PANEL REPORT (March 2005), Chapter III, has recommended that histology be added to the BCOP assay on chemical/formulation classes when the predictive capacity of the opacity and permeability endpoints is not well documented.

4) To adequately predict completely unknown chemicals, it suggested that both neat and 20% test article (if a solid) be tested using an unbuffered dosing solution. The protocol should also include a short (2 to 4-hour) and long (20-hour) post-exposure incubation, and histopathological evaluation.

5) Corneas from the concurrent controls must be evaluated in parallel with the treated corneas. In particular, the negative control-treated corneas provide a baseline for assessing the quality of tissue processing, slide preparation, and staining, as well as pre-Existing lesions.

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