Institute for In Vitro Sciences, Inc.

Advancing **Science &** Animal **Welfare** Together

November 18, 2004

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Dear Dr. Stokes:

This public comment is delivered in response to Federal Register Notice Volume 69, Number 212, Pages 64081-64082. It addresses the Background Review Document (BRD), "Current Status of In Vitro Test Methods for Identifying Ocular Corrosives and Severe Irritants: The Bovine Opacity and Permeability (BCOP) Test Method", November 1, 2004.

We believe that the BCOP assay provides much useful information about the potential for, and the mechanisms of, chemically-induced eye irritation. Such information can be extremely valuable to both industry and the regulatory community. We are pleased that the US EPA has nominated the BCOP (and other) assay(s) for review by ICCVAM, and we appreciate the time that NICEATM has spent developing this BRD.

Our purpose in writing this letter is to emphasize the value of histology (assessing depth and degree of injury) as an additional endpoint in the BCOP test method, especially when evaluating unknown or novel materials. Considerable information on this additional endpoint was provided directly to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) during the development of this BRD. Those documents included the full Standard Operating Procedure (SOP) from the Institute for In Vitro Sciences, Inc., results of in-house studies (e.g., evaluation of under-predicted chemicals from the European Commission/British Home Office study) and numerous studies conducted for clients and presented at national meetings. However, the BRD reflects very little of this information. In our minds, the histological assessment of depth and degree of damage is an essential endpoint in the prediction of irritation potential of certain classes of chemicals (e.g., peroxides and other reactive chemicals) and for chemicals where the mode of action cannot be easily predicted. This public comment will outline the mechanistic basis, standard tissue and slide preparation procedures, descriptions of how control and treated corneas are scored, and examples demonstrating the use of histology in understanding irritation potential, including those chemicals where irritation potential might be underestimated by opacity and permeability measurements alone.

Sincerely yours,

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Depth of Injury as a Predictor of Degree and Duration of Ocular Injury

In their 1998 publication "Ophthalmologic Perspectives on Eye Irritation Testing", Nussenblatt[1] and colleagues stressed the paramount importance of predicting eye irritation in humans. They stated, "We note that two major themes should permeate all future work to further development of alternative tests. First, we unanimously agreed that the Draize rabbit eye method as currently used should not be considered the primary standard for the evaluation of new methods. Despite its long use, there has been a lack of rigorous scientific correlation of Draize test data with the human experience. We believe that the ability of a test to predict eye irritation potential in humans should be paramount.". These experts also felt that animal-based research was currently necessary but that it should be focused so as to provide data that could be used to address human exposures and responses. In a follow on presentation at the 1998 ARVO meeting, these experts proposed an initial human-based classification scheme for accidental ocular irritation based on depth of injury[2].

Drs. Jim Maurer[3,4] and James Jester[5] have collaborated on a series of seminal studies. This work has related the initial depth of injury in the rabbit (and some cases the rat) eye, using the low volume dosing method, with the degree and duration of the injury. These studies included a wide range of chemical classes and resulting modes of action: anionic, nonionic and cationic surfactants, acids, an alcohol, an aldehyde, alkalis, an aromatic amine, bleaches and a ketone. Their key findings were summarized in their 2002 review paper[6]. In their conclusions section, they state:

"Based on our studies characterizing the extent of initial injury and repair associated with different materials causing different levels of ocular irritation *in vivo*, we conclude that extent of initial injury is an appropriate mechanistically based criterion for developing and validating *ex vivo* or *in vitro* alternatives. Further, we make the following recommendations for developing and validating alternative assays to replace the use of animals in eye irritation testing:

- Replacement assays used to assess extent of injury should be three-dimensional, as injury is a three-dimensional process.
- Extent of injury may be assessed by determining extent of cytotoxicity within the cornea.
- Replacement assays should include a means to assess potential injury to the epithelium, stroma, and endothelium.
- Replacement tests should be able to differentiate injury that is diffuse from injury that is focally extensive.

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[1] Nussenblatt, R.B., Bron, A., Chambers, W., McCulley, J.P., Pericoi, M., Ubels, J.L., and Edelhauser, H.F. (1998) Ophthalmologic perspectives on eye irritation testing. **J. Toxicol – Cut. & Ocular Toxicol**. 17(2&3):103-109.
[2] Maurer, J.K., McCulley, J.P., Edelhauser, H.F., and Nussenblatt, R.B. (1998) A proposed new classification scheme for chemical injury to the human eye. The Association for Research in Vision and Ophthalmology Annual Meeting.
[3] 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.
[4] Maurer, J.K., Molai, A., Parker, R.D., Li, L., Carr, G.J., Petroll, M.W., Cavanagh, D.H., and Jester, J.V. (2001) Pathology of ocular irritation with bleaching agents in the rabbit low-volume eye test. **Toxicological Pathology** 29(3):308-319.
[5] 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.
[6] 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.

- Replacement tests should assess for initial injury at different time points to predict accurately the ocular irritation potential.

Our studies of surfactants and nonsurfactants support the hypothesis that by defining the extent of initial injury associated with a putative ocular irritant it is possible to predict the subsequent responses and final outcome. Therefore, we believe that the best approach for the development of mechanistically based alternative ocular irritation tests would be the microscopic or biochemical measurement of initial injury using either an *ex vivo* or *in vitro* corneal equivalent system composed of a defined corneal epithelial, stromal keratocyte, and corneal endothelial cell layers."

In its current form, the BCOP assay was developed by Drs. Pierre Gautheron[7] and Joe Sina[8] to address ocular irritation potential of pharmaceutical intermediates. The method is now widely applied across industries and chemical/formulation classes. For many, if not most, of these chemical/formulation classes, the mode of action(s) of the test material is generally known. Membrane lysis, protein coagulation, saponification are common modes of action that lead to ocular irritation. In our experience, the opacity and permeability (measured by the passage of fluorescein through the cornea; OD_{490}) endpoints (generally combined into an "in vitro score") have been able to identify the epithelial and stromal changes associated with this type of damage. Endothelial cell damage has been harder to assess through permeability changes unless the endothelium is largely destroyed. In such cases, the passage of fluorescein is greatly enhanced (data unpublished). However, the prediction of ocular irritation from chemicals that react with nucleic acids, mitochondrial proteins, or other cellular targets, that do not lead to immediate loss of cellular integrity (particularly in the epithelium), has proven more difficult using only the opacity and permeability endpoints. This difficulty was evident in the results of the EC/HO study where certain strong irritants were under-predicted by the in vitro score. It was this study that prompted us to begin the active examination of histological changes (as described by Jester and Maurer in the preceding paragraphs) in conjunction with the primary opacity and permeability measurements[9].

With the addition of the histological assessment of depth of injury, the BCOP would seem to address most of the requirements proposed by Maurer et al. (above).

- The BCOP assay uses a three-dimensional tissue that contains the three layers of interest in the cornea.
- The corneal tissue is very much alive using the standard organ culture procedures of the protocol and can be maintained, with repeated medium changes, for 24 hours (see #9).
- Cytotoxicity and structural damage to the three layers of the cornea are measured with several, complementary endpoints.
- One can determine initial injury after different periods of exposure and can follow the progression of damage over the first 20+ hours after exposure.

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[7] Gautheron, P.D., Dukic, M., Alix, D., and Sina, J.F. (1992) Bovine Corneal Opacity and Permeability Test: An *in Vitro* Assay of Ocular Irritancy. **Fundamental and Applied Toxicology** 18:442-449.

[8] 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.

[9] Curren, R., Evans, M., Raabe, H., Ruppalt, R., Harbell, J. (2000) Correlation of histopathology, opacity, and permeability of bovine corneas exposed in vitro to known ocular irritants. **Veterinary Pathology** 37(5):557.

One small shortcoming is that because the entire surface of the cornea is exposed, the standard protocol does not directly differentiate between focal and diffuse injury.

When conducting BCOP studies at the Institute for In Vitro Studies, Inc. (IIVS), histology may be included in the study protocol for several reasons. Some clients wish to understand the tissue changes associated with exposure to the products under study. In other cases, the depth of injury associated with the new formulation will be compared to the depth of injury induced by a reference material(s). (Clients are strongly encouraged to include concurrent benchmark formulations to facilitate interpretation of the study; positive and negative control corneas are always used)[10].This approach is common in final product testing or when the study is intended for submission to a regulatory agency (e.g., USEPA). The histology allows the agency reviewer to more completely use the data from the reference article(s) to determine the labeling requirements for the new formulation. When formulations contain reactive chemicals (e.g., peroxides, bleaches, etc), histology is definitely recommended. Maurer et al[11] have shown the oxidizing agents induce a delayed toxicity in vivo and act more profoundly on the keratocytes. Our experience has shown that peroxide-containing formulations require histological assessment to elucidate the full depth of injury[12]. New chemistries, where the mode of action cannot be predicted or "reactive chemistry" is expected, require special handling (discussed below), including histology[13].

Overview of the Histology Procedures Used at IIVS

This overview and subsequent discussion is offered as an example of how histology is incorporated into the BCOP assay at IIVS. No effort has been made to assess approaches at other laboratories, due to the limited amount of time available to respond to this BRD

Corneal Accession Numbers

Corneas are assigned numbers sequentially with A#### (B, C, D etc. are used in sequence as needed). The accession #, test article or control designation, exposure time, harvest date, cornea # (from that day's assay) and any needed comments are recorded on the histology record. A copy of these data is placed in the study notebook and histology logbook.

Fixation of the Corneas

The corneas are fixed after the completion of the fluorescein penetration step of the assay. In-house evaluation has shown that the fluorescein exposure does not impact tissue morphology. In addition, fixing the corneas at this step allows the histological evaluation to be performed on the same corneas from which the opacity and permeability values were taken. Once the posterior chamber of the corneal holder has been sampled, the remaining fluids are removed and the chamber dismantled. Each cornea is placed into a tissue cassette that has been pre-labeled with its accession number (as above). The cassette is fitted with a "histology sponge" to help protect the endothelial surface. The cornea will be placed onto the sponge with the epithelium facing up. Care is taken to prevent the cornea from drying during the transfer process. The cassette is closed and immersed in 10% neutral

[10] Cuellar, N., Merrill, J.C., Clear, M.L., Mun, G.C., and Harbell, J.W. (2002) The application of benchmarks for the evaluation of the potential ocular irritancy of aerosol fragrances. **The Toxicologist** 66:243-244.
[11] Ibid #4.
[12] Swanson, J.E., White, B.T., Gran, B.P., Merrill, J.C., and Harbell, J.W. (2003) Evaluating oxidizing/reactive cleaning products in the bovine corneal opacity and permeability (BCOP) assay. **The Toxicologist** 72:220-221.
[13] Ibid #9.

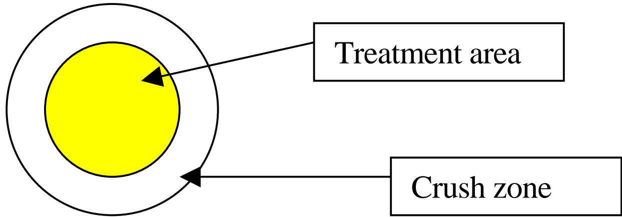
buffered formalin. It is important to be sure that the tissue is fully submerged in the formalin. Approximately 20 cassettes are fixed in a volume of 300 mL. Corneas should be fixed at room temperature for at least 24 hours before processing.

Preparation of the Slides

IIVS does not possess its own tissue preparation capacity. Corneas are trimmed, embedded, sectioned and stained by an approved contractor. Generally, this work is performed by Pathology Associates – A Charles River Company, Frederick, MD and the details of each step covered by their SOPs.

The following general guidance is provided to the histology laboratory:

The actual size of the cornea and the treatment area are shown below:



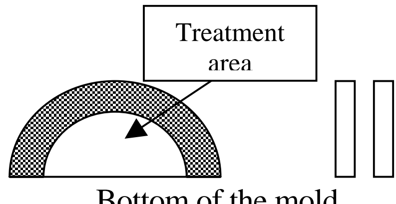
The center shows the area treated and the area of interest for histological examination.

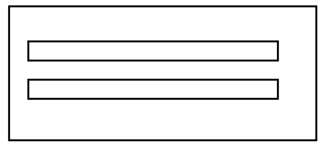
The fixed corneas are transferred to your lab. Placement paperwork (and study protocol for GLP studies) will be sent with the corneas. Upon receipt by the lab, the samples will be received into their tissue accession system.

The corneas may be trimmed to remove some of the outer crush zone if necessary however the cornea will be infiltrated whole. Before the corneas are placed on the tissue infiltration machine, a second sponge will be placed on the anterior surface of the cornea. This second sponge is intended to reduce the chances of tissue warping during infiltration. Corneas are always mounted in the cassette at IIVS with the anterior surface facing the upper lid. Infiltration will proceed according to the current SOP. Elevated temperatures should not be used with the infiltrating solvents.

Once infiltrated with paraffin, the cornea will be bisected so that the two halves of the cornea can be embedded in the same block. The cornea will have some wrinkles and so it is often helpful to cut across the wrinkles (if they fall in a particular orientation) so that a good cross section can be obtained when the tissue is sectioned.

The microtome cuts must produce as close to a true cross sections (anterior to posterior) of the cornea as possible. The true cross section allows us to accurately measure increases in the thickness of the corneas (swelling as a result of test article exposure) relative to the thickness of the negative control-treated corneas. Large, deep molds must be used and great care in orienting the tissue is required. The two halves of the cornea are placed with the cut side down in the mold and aligned vertically and with their long axis in parallel with the long axis of the mold.





Bottom of the mold   Viewed from the top of the mold

If the tissue is too long to fit into the mold, the outer edges (shown as the dark outer area above) may be trimmed. Ideally, one would like to see some of the dark "crushed" tissue in the section so as to be assured that one is evaluating the whole cross section of the cornea. Having the two tissue strips oriented in parallel along the long axis of the slide (once they are cut) makes scoring much easier.

Sectioning the tissue requires that the area damaged by the bisecting cut be trimmed away (with the microtome) so that the artifacts introduced by the cutting are not mistaken for changes associated with the test material exposure. The sections must include the full cross section of the cornea (epithelium, stroma, and endothelium). The corneal stroma is quite delicate and prone to artifacts from over-stretching of the sections on the water bath. In addition, over-stretching will induce breaks between the epithelium and stroma that might be mistaken for test article-induced damage.

The corneas will be photographed as part of the evaluation and the micrographs included in the final report to the client. Therefore, the flatness of the sections (on the slide) and the reproducibility of the staining from one study to the next (and especially within a study) become more important that if we were simply reading the slides. Our eyes are good at making adjustments for depth and color but the camera is most unforgiving!

The slides are being evaluated for rather subtle changes compared to what one might expect to see from an eye treated in the whole animal. This is because we are looking for the early events (tissue/cellular changes) that would be associated with the initiation of subsequent inflammatory reaction. Since we have no circulator system in the isolated tissue, we will not have leukocyte infiltration to help us. The tissue sections must be of extremely good quality to evaluate stromal and endothelial elements properly because the early changes can be very subtle."

Slides are normally stained with hematoxylin and eosin (H&E) although other stains may be requested. Positive and negative control corneas from each trial are processed with test article treated corneas. The histology of the negative control corneas is used to evaluate the quality/acceptability of the remaining slides.

<u>Recording Observations</u>

Observations of treated corneas are generally recorded electronically. The data include: the IIVS test article number, slide (cornea accession) numbers, sponsor's designation, test article concentration (relative to the test article as supplied), exposure time, post-exposure expression time, date of test article application, observations on each corneal tissue layer, and the related figure numbers (where appropriate). Once the observations are finalized, the table is included in the final report. The finalized observations are signed and dated by the responsible scientist. The original copy is placed into the study notebook.

Preparation of the Photomicrographs

Photomicrographs of the lesions are made to illustrate the degree of damage at the indicated depth observed in the treatment group. They are not intended to document the overall damage, as only a very small portion of the tissue can be photographed. Images are 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 is sometimes corrected to better represent the colors that would be seen through the microscope. Each photomicrograph is documented in a study-associated digital image log. A backup copy of the photomicrograph image files is made. Once finalized, the image log for the study is printed, signed and dated by the scientist responsible. The finalized copy is placed into the study notebook. The photomicrographs are "pasted" electronically into the final report.

Evaluating the Cornea Histology

Evaluation of the Corneal Sections (Overview)

Initial quality control evaluation of the sections: The three tissue layers of the cornea are evaluated for lesions. It is important to distinguish between test article-induced changes and artifacts of handling or processing. To this end, the negative control slides are used to detect artifacts at the batch level. They are also used to assess "normal" staining (degree of hematoxylin or eosin in each layer/cell type), tissue architecture and thickness.

In the ideal, the corneal sections are prepared as true cross sections rather than tangential sections. However, some fraction of the sections (or portions of a section) will not be true cross sections and so the overall thickness and tissue architecture will be distorted from the ideal. There may also be some fields in a section that are tangential even when most of the section is a good cross section. In control or minimally damaged corneas, stromal thickness provides a good indication of how true the cross section is. The thickness of Descemet's Membrane provides a good measure of a true cross section. The Descemet's Membrane in a good section of a control cornea can be used to compare with treated corneas. This measure can be especially helpful in sections of treated corneas where collagen matrix vacuolization and stromal swelling are evident. It should be remembered, however, that Descemet's Membrane increases in thickness with age of the donor and so corneas from older cattle will have thicker membranes. In a cross section, the endothelium is quite thin. The endothelium will be thicker in tangential sections. Where the tissue has been poorly prepared (e.g., overly trimmed or poorly embedded), the epithelium, stroma and Descemet's Membrane may appear very thick.

It is essential that all layers of the cornea be included in the section. Poorly trimmed blocks may produce sections where the full depth of the cornea is not present (e.g., the lower stroma and endothelium missing). It may be necessary to request recuts of such slides.

Slides are usually stained with an automated slide stainer. The intensity of staining depends on several factors and may vary slightly across studies. Occasionally, a malfunction of the stainer will produce variations within the study. Decreased hematoxylin or eosin staining can markedly compromise interpretation and photography. Such slides should be returned for restaining or

recutting. Highly swollen stromal collagen will appear to be poorly stained but, in fact, there is a great deal of empty space between the fibers that is not stained at all.

Evaluating the Negative Control-Treated Corneas

Negative control corneas are treated with sterile, deionized water or saline in parallel with the positive control and test article-treated corneas.

Epithelium: The negative control-treated epithelium is composed of three layers. The basal cell layer is a well-formed, columnar-cell region directly attached to the Bowman's Layer. The basal cells are always tightly attached to each other. Several layers of wing cells cover the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Rare mitotic figures are seen in the basal layer. The squamous layer is flattened with limited cytoplasm and highly condensed nuclei.

Stroma: The stromal elements begin with the Bowman's Layer and are composed of well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei show 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 visible, is moderately basophilic. Rarely, cells with eosinophilic cytoplasmic staining may be observed. Collagen bundles are generally parallel and well ordered. The Descemet's membrane is prominent and forms the bottom of the stroma. The overall thickness of the stroma is approximately one 20x field when a good cross section is obtained.

Endothelium: The endothelium is a single layer of flattened cells attached to the basal surface of the Descemet's Membrane. Nuclei are elongated and flattened. In a cross section, little cytoplasm is visible. Generally, the cells are firmly attached to the Descemet's Membrane but in some areas (or fields), they may be detached or lost through mechanical damage.

The overall thickness of the fixed cornea is generally between 850 and 1000 μ.

Evaluating the Corneal Lesions

The goal in scoring the corneal lesions is to record the nature, degree and depth of the lesion in each tissue layer. In most cases, the individual corneas in a treatment group will not be reported separately but rather they will be "averaged" to highlight the predominant lesions. The opacity and permeability values should be reviewed before scoring the slides. If there are wide variations among the corneas in either the indirect measures or histological changes, it may be necessary to report on some individual corneas within the treatment group.

Epithelium: Characteristic lesions observed in the epithelium are cell loss, cell coagulation (especially in the squamous epithelial layer), nuclear vacuolization (swelling), nuclear condensation (pyknosis or precipitation of nuclear proteins and/or DNA), cytoplasmic vacuolization, cytoplasmic precipitation (leaving only the cytoskeleton), and separation of the cells from the Bowman's layer. The degree of the lesion may be somewhat subjective. It may be the fraction of the cells showing a lesion or the magnitude of the lesion within the cells or cell layer (e.g., degree of cytoplasmic vacuolization). The depth of the lesion relates to the number of cell layers impacted. Damage to or loss of the Bowman's Layer will be reported with the epithelium.

Stroma: The lesions of the stroma are reported in two parts, those of the extracellular collagen matrix and those of the keratocytes. The predominant lesion seen in the extracellular collagen matrix is swelling (loss of the normal ordered array of the fibers). 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. The depth (see below) and degree of vacuolization are reported. The degree refers to the relative number and size of the holes in the matrix. As the degree and/or depth of vacuolization increases, the overall thickness of the stroma would be expected to increase.

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 the depth is not always a straight-forward 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 the isolated corneas, 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 increase stromal thickness. Thus, measurements of the depth of a stromal lesion can be complicated by the change in overall stromal thickness. 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). However, 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 Layer) did not show vacuolization.

An exception to the anterior to posterior progression of stromal swelling is caused by the loss of the endothelial cell layer. Since the endothelium is responsible for maintaining balanced hydration in the lower stroma, its loss (either through mechanical damage or test article toxicity) can lead to appreciable deep stromal swelling. It is important to differentiate between endothelial damage and swelling caused by the test article exposure and damage from other sources (e.g., mechanical). In the case of mechanical damage, the deep swelling can occur in the absence of swelling in the anterior stroma. Test article-induced damage should progress through the cornea and be manifested in both the anterior and posterior stroma. Sections or portions of sections where the endothelium is lost and posterior stromal swelling (collagen matrix vacuolization) is observed without similar anterior stromal swelling are likely the result of mechanical damage to the endothelium that occurred early in the assay (incubation). An effort should be made to score corneal sections that do not show such damage.

Lesions in the keratocytes are manifested in both the cytoplasm and nucleus. Rapid necrotic cell degeneration, as might follow exposure to a strong alkaline, organic solvent or surfactant, is quite apparent because the cellular components rapidly breakdown. Oxidative damage or DNA alkylation might produce more subtle damage (initially) but could also lead to cell death (delayed) and release of inflammatory mediators. Nuclear changes (pyknosis or karyorrhexis) are signs of this process. Progressive nuclear pyknosis or complete destruction are also signs of this process. Cytoplasmic changes include vacuole formation or loss of basic elements (mRNA for example) that 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 change is termed keratocyte eosinophilia.

Endothelium: Lesions in this layer include cell loss and cytoplasmic degeneration (vacuolization). Since this layer is only one cell thick, mechanical damage has the potential to confound the evaluation. Where there is endothelial cell loss, it is important to evaluate surrounding fields for the presence of normal endothelium. Since the whole corneal surface is treated, a lack of a uniform changes to most of the endothelium would suggest mechanical damage to isolated patches rather than test article-induced damage. When mechanical damage occurs late in the assay or after fixation (e.g., during processing), little or no deep stromal swelling is expected.

Application of histological assessment to the determination of ocular irritation potential

The results of a histological evaluation may be used in several ways depending on the needs of the study sponsor. In some cases, it is used to confirm the absence of tissue damage beyond that detected by the opacity and permeability scores. In other cases, it is helpful to understand the types of lesions that a material might induce. In some studies, we have compared the depth of injury induced by a product concentrate and then the mitigation produced by dilution. This approach can produce the ocular irritation potential for proper end-use dilutions (as specified by the label) as well as the ocular irritation potential for more concentrated solutions (produced by those who do not follow the instructions). The most common application, in our experience, is to compare depth and degree of injury in a benchmark formulation(s) with the changes in the new formulation(s). In this way, all three endpoints are used to make the final assessment of ocular irritancy potential. This is how much of final product safety assessment is conducted and how studies submitted to the Office of Pesticide Programs, USEPA for product registration have been performed.

It should be understood that a stand-alone, quantitative prediction model for the translation of depth of injury observations (by themselves) to a predicted degree and duration of ocular irritation in the rabbit has not been completed. Where a prediction of irritation potential in the rabbit is desired by the study sponsor, the depth and degree of injury induced by the test material is compared to the depth and degree of injury to the benchmark(s) materials.

Certain lesions are characteristic of severe irritants. These include necrosis or pyknosis of the keratocytes in the deep stroma (below mid depth) and loss of functional endothelium across the majority of the cornea. The loss of functional endothelium is reflected in the presence of collagen matrix vacuolization directly above Descemet's Membrane.

Not all of the BCOP studies conducted at IIVS include histological evaluation. Where the mode of action of the test article on the cornea is well understood (e.g., surfactant formulations), the sponsor may well rely on the opacity and permeability measurements alone. However, since the end of 1999, over 375 studies have been conducted with histology where over 5500 slides were evaluated.

A short compendium of micrographs to illustrate negative control-treated (normal) and select lesions in bovine corneal tissue

The following series of photomicrographs is intended to illustrate normal bovine corneal morphology and provide examples of the types of lesions that might be observed in the epithelium,

stroma and endothelium. It is by no means a complete listing of all lesions but is intended to illustrate the types of changes mentioned in the discussion of corneal lesions. In some figures, the chemical and exposure are provided. When photomicrographs have been taken from unpublished client studies, test material information is omitted.

Negative Control-Treated Cornea (Treated for 10 minutes with sterile, deionized water)

Figure 1. Negative Control Cornea - Full thickness

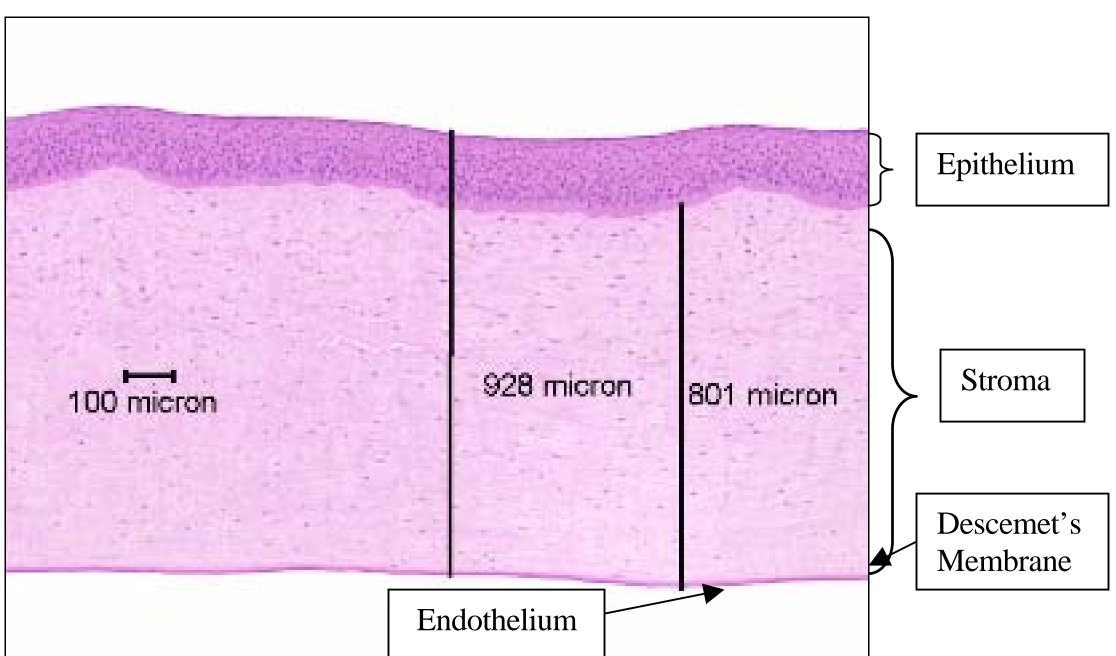


Figure 2. Negative Control Cornea - Epithelium

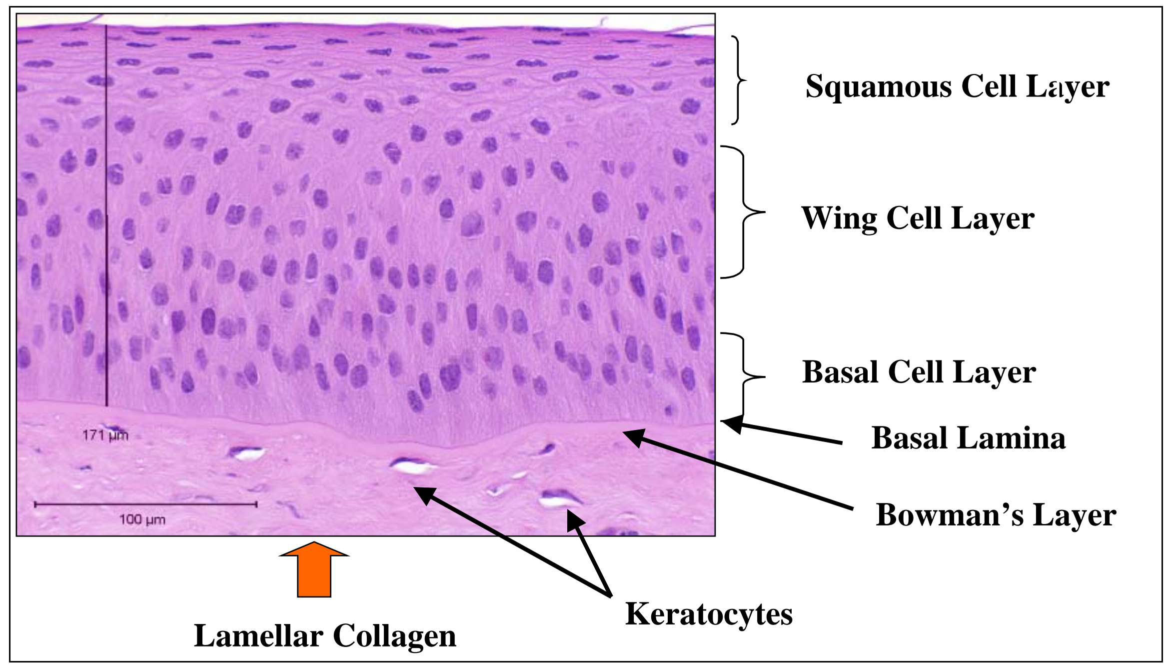


Figure 3. Negative Control Cornea - Upper stroma showing normal collagen matrix organization and keratocyte morphology

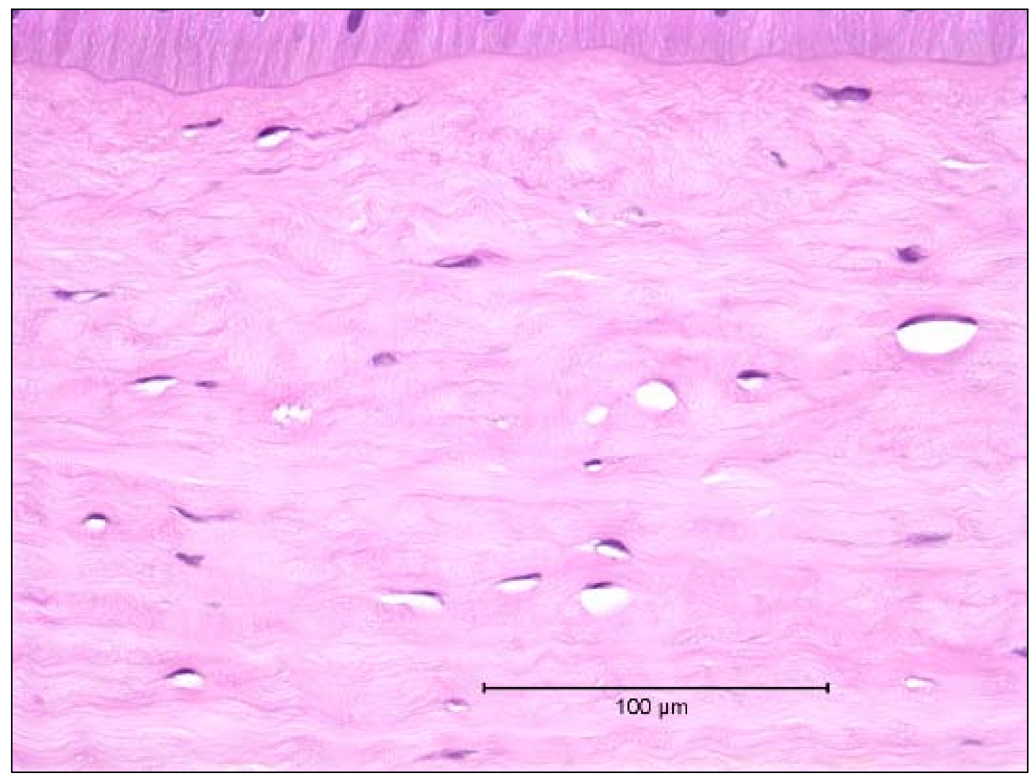
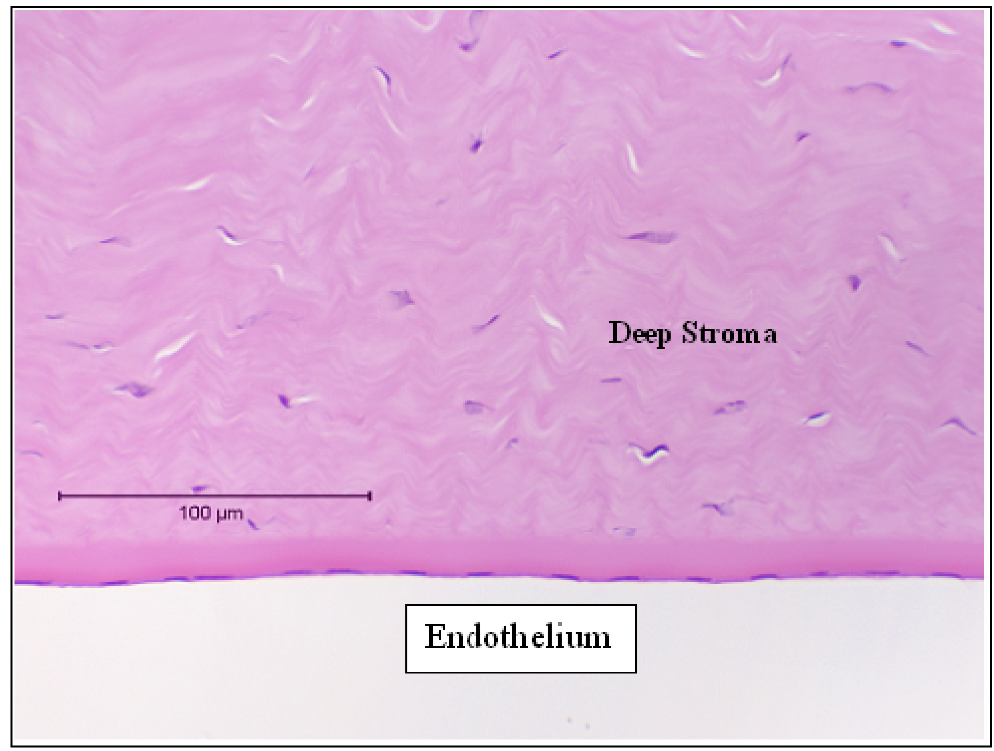


Figure 4. Negative Control Cornea - Deep stroma and endothelium



Overview of Corneal Lesions

Figure 5. Epithelial cell loss induced by surfactant exposure

a) 1% SLS 30-minute exposure OD_{490}=0.77	b) 5% SLS 30-minute exposure OD_{490}=2.54

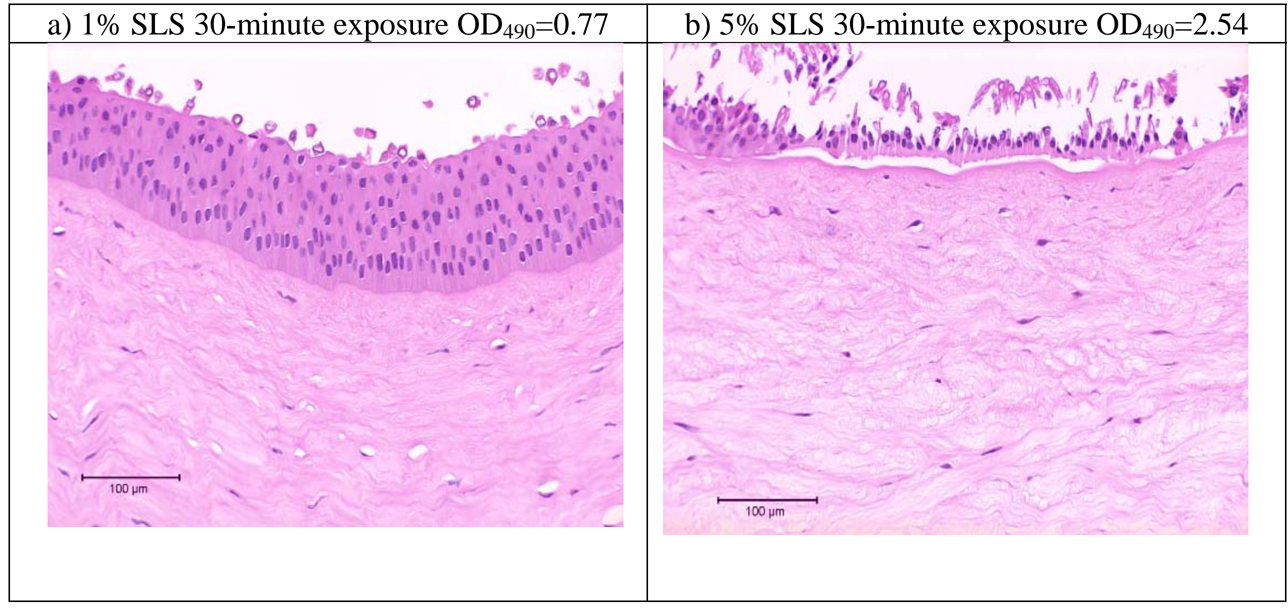
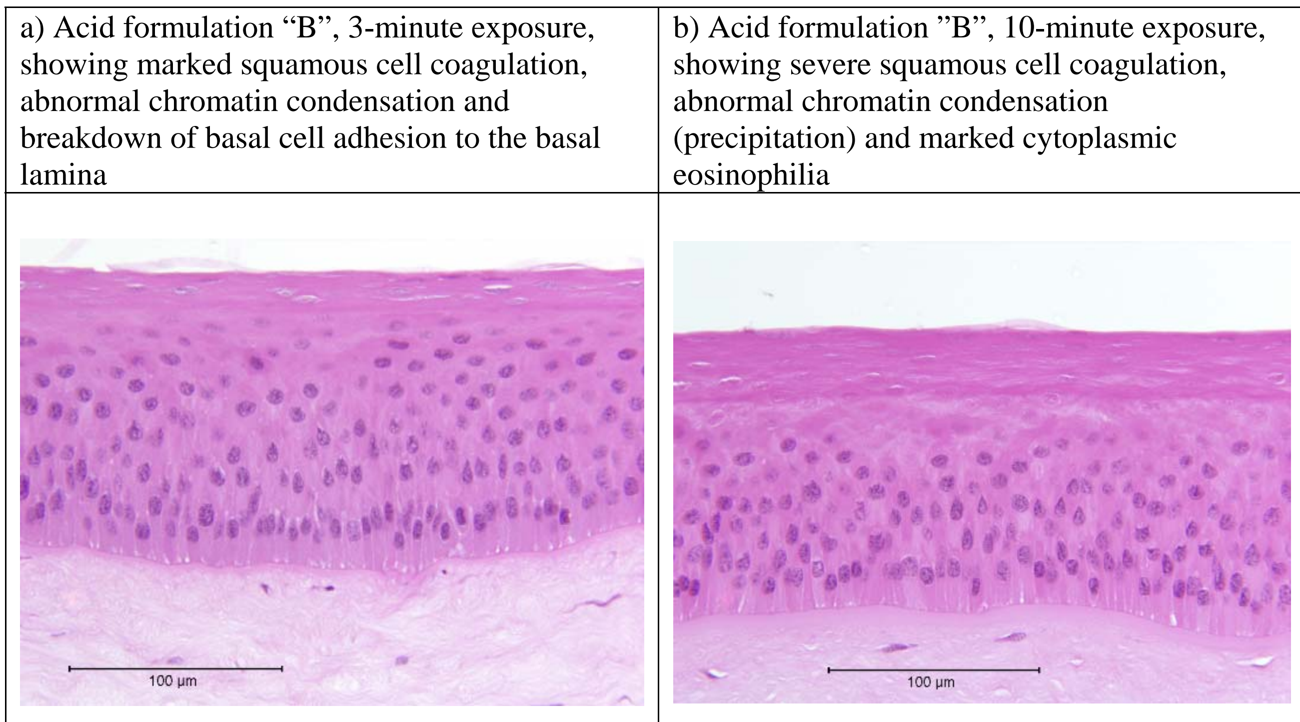


Figure 6. Cell coagulation, nuclear vacuolization, and cytoplasmic vacuolization

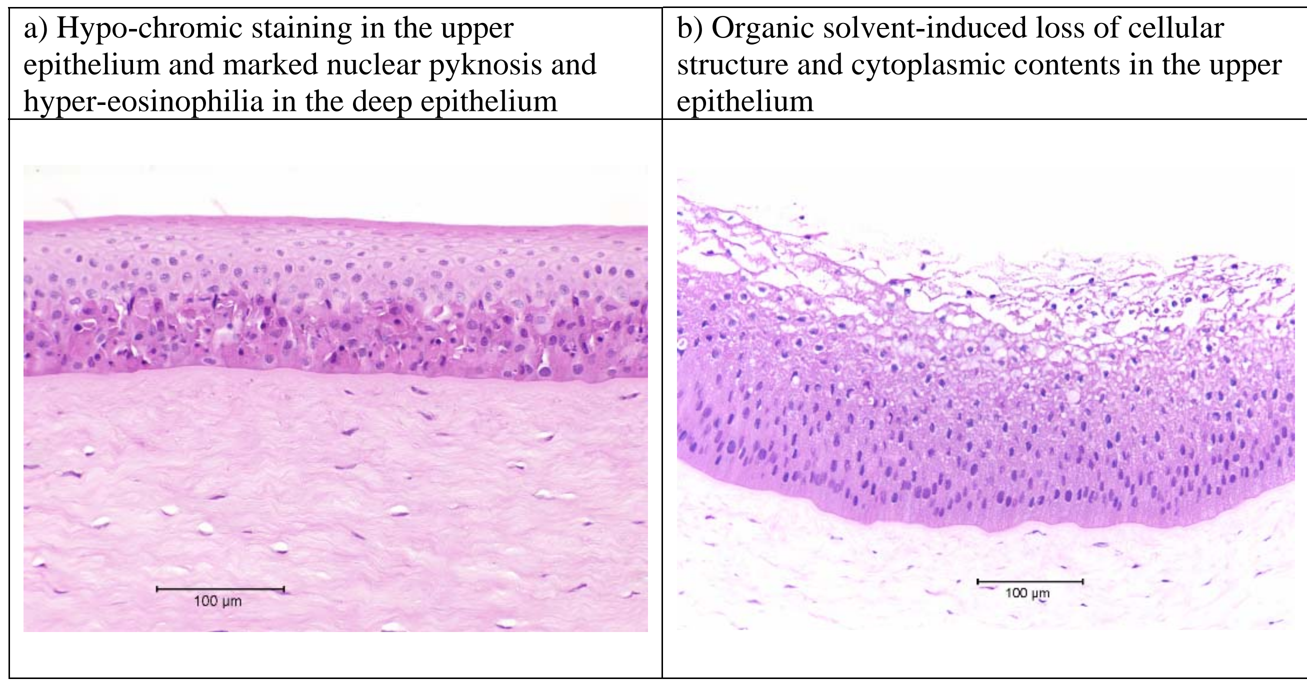
a) Ethanol, 10-minute exposure, showing squamous layer coagulation and cytoplasmic and nuclear vacuolization in the wing and basal layers	b) Acid formulation "A", 3-minute exposure, showing moderate coagulation, abnormal chromatin condensation and cytoplasmic vacuolization in the squamous and upper wing cell layers

Figure 7. Cell coagulation, abnormal chromatin condensation, and cytoplasmic eosinophilia



a) Acid formulation “B”, 3-minute exposure, showing marked squamous cell coagulation, abnormal chromatin condensation and breakdown of basal cell adhesion to the basal lamina	b) Acid formulation ”B”, 10-minute exposure, showing severe squamous cell coagulation, abnormal chromatin condensation (precipitation) and marked cytoplasmic eosinophilia

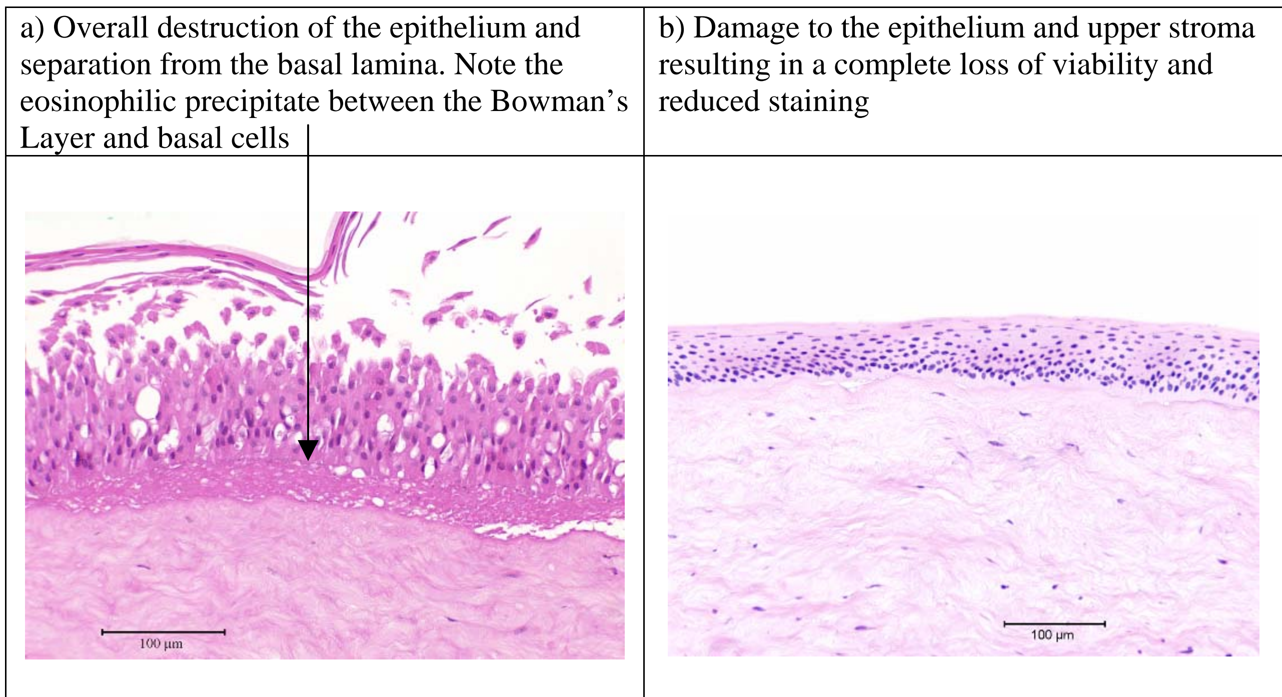
Figure 8. Nuclear pyknosis and loss of contents



a) Hypo-chromic staining in the upper epithelium and marked nuclear pyknosis and hyper-eosinophilia in the deep epithelium	b) Organic solvent-induced loss of cellular structure and cytoplasmic contents in the upper epithelium

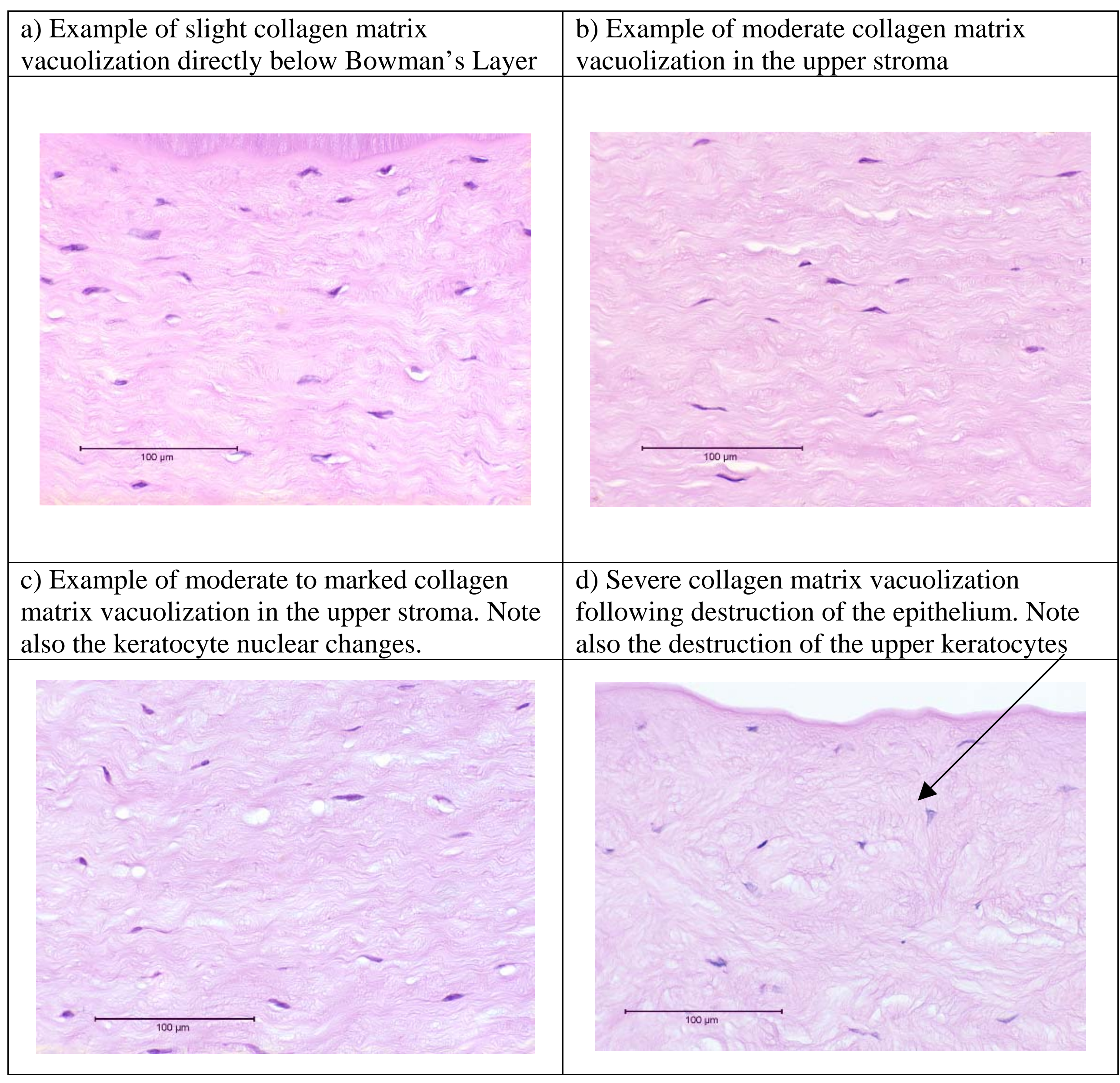
Figure 9. Nonviable Epithelium

a) Overall destruction of the epithelium and separation from the basal lamina. Note the eosinophilic precipitate between the Bowman's Layer and basal cells	b) Damage to the epithelium and upper stroma resulting in a complete loss of viability and reduced staining



Overview of Stromal Lesions

Figure 10. Collagen matrix swelling (vacuolization)



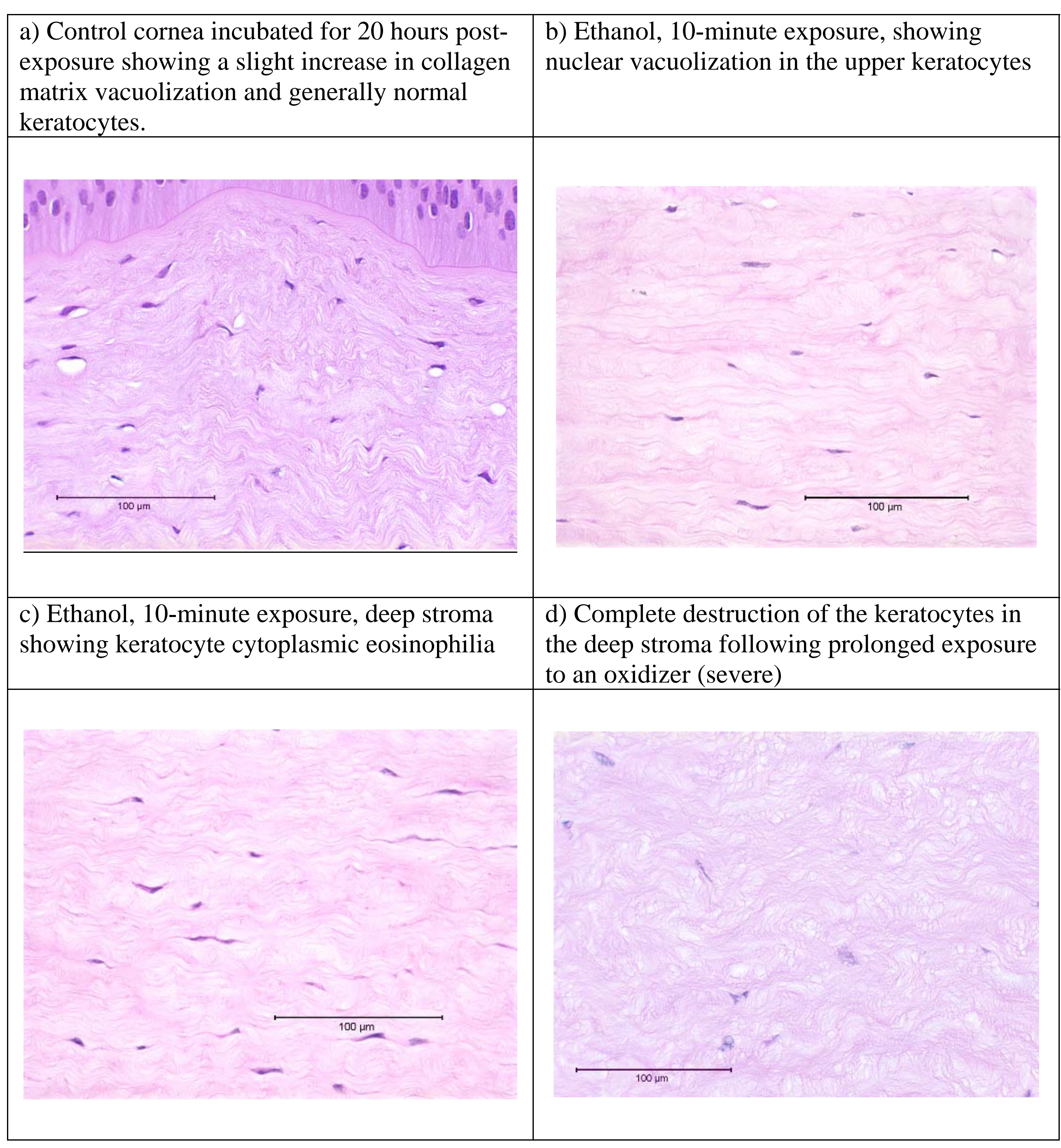
a) Example of slight collagen matrix vacuolization directly below Bowman's Layer

b) Example of moderate collagen matrix vacuolization in the upper stroma

c) Example of moderate to marked collagen matrix vacuolization in the upper stroma. Note also the keratocyte nuclear changes.

d) Severe collagen matrix vacuolization following destruction of the epithelium. Note also the destruction of the upper keratocytes

Figure 11. Keratocyte Changes



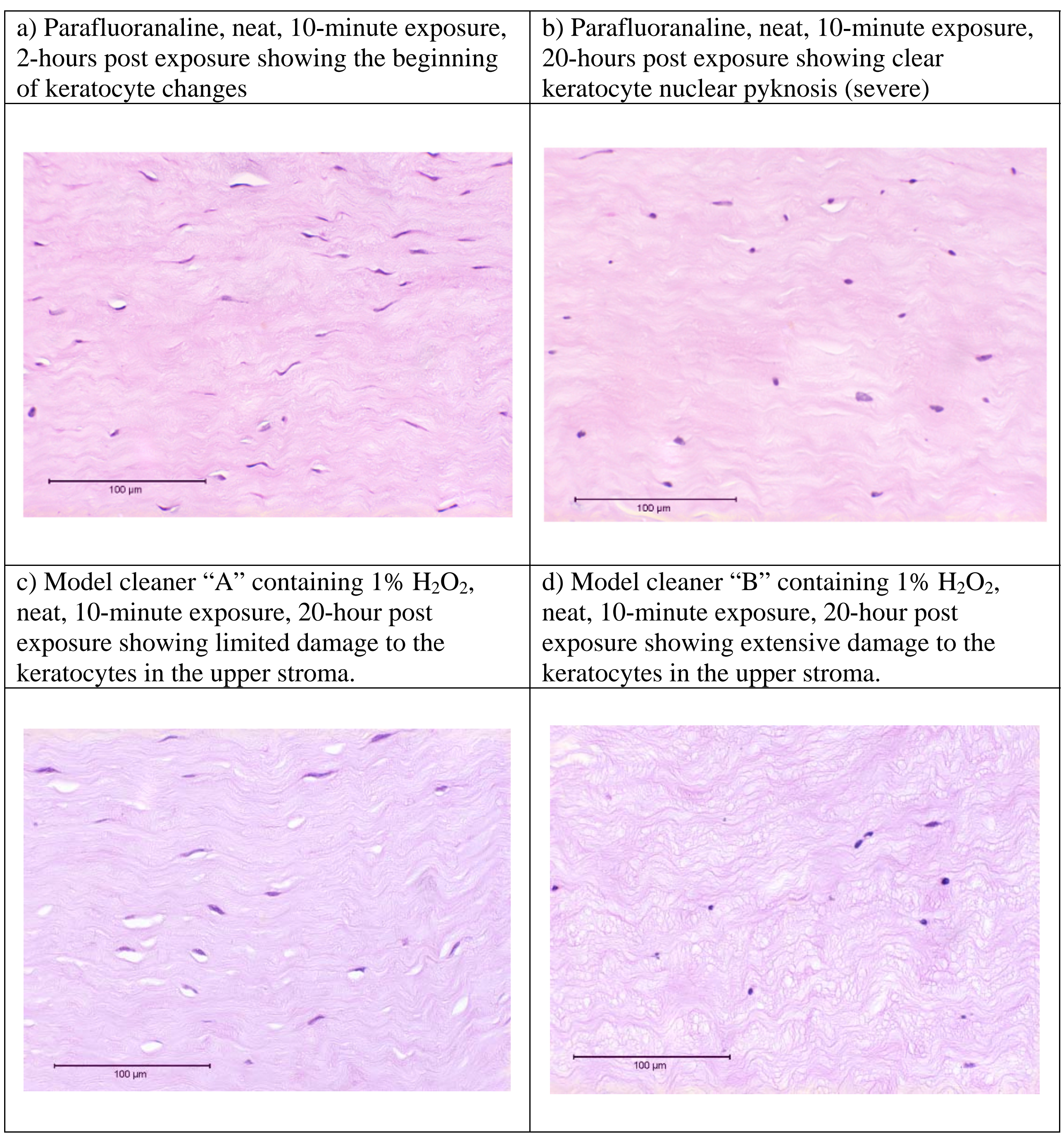
a) Control cornea incubated for 20 hours post-exposure showing a slight increase in collagen matrix vacuolization and generally normal keratocytes.

b) Ethanol, 10-minute exposure, showing nuclear vacuolization in the upper keratocytes

c) Ethanol, 10-minute exposure, deep stroma showing keratocyte cytoplasmic eosinophilia

d) Complete destruction of the keratocytes in the deep stroma following prolonged exposure to an oxidizer (severe)

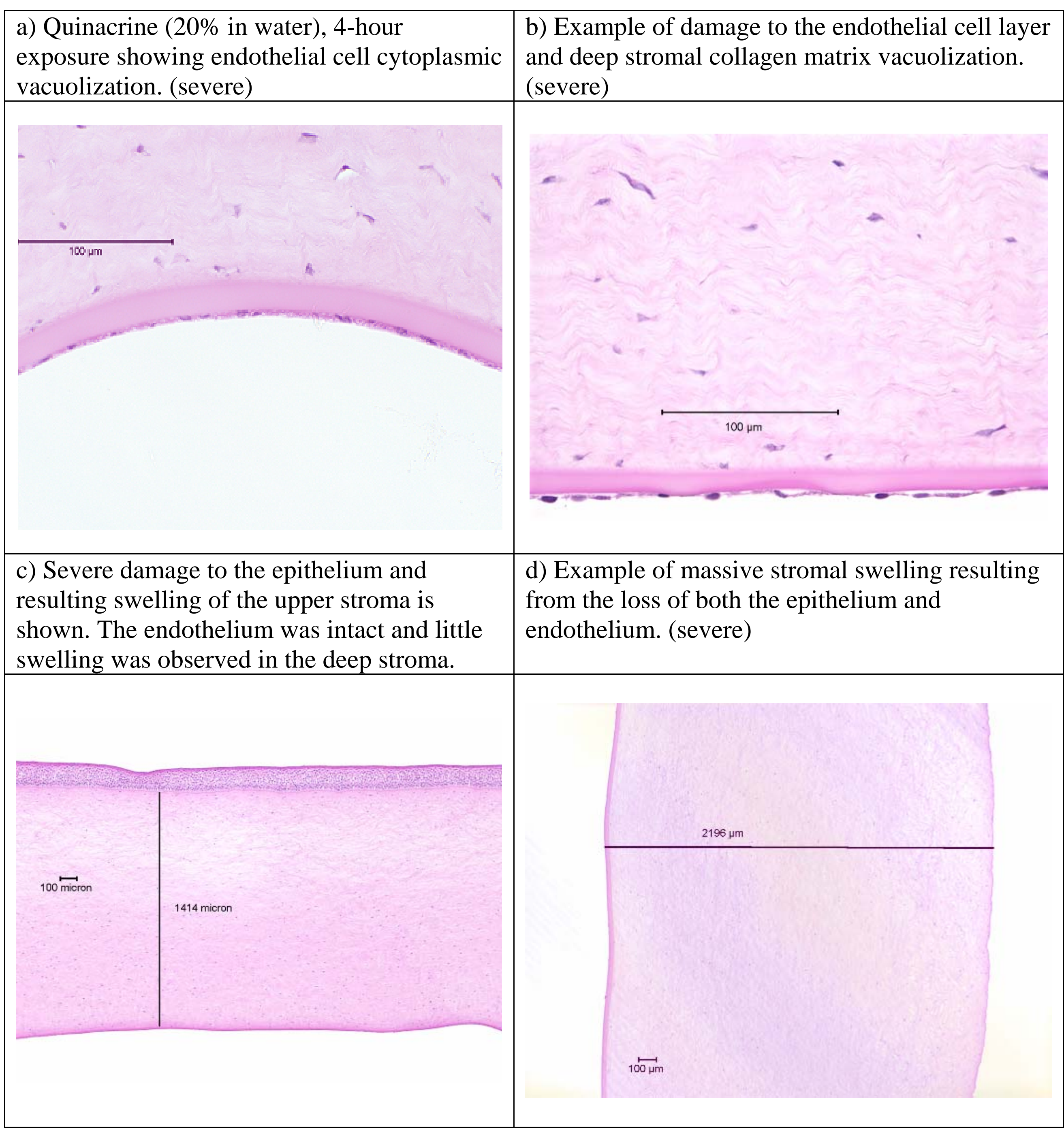
Figure 12. Progressive changes in keratocytes with time after exposure



a) Parafluoranaline, neat, 10-minute exposure, 2-hours post exposure showing the beginning of keratocyte changes	b) Parafluoranaline, neat, 10-minute exposure, 20-hours post exposure showing clear keratocyte nuclear pyknosis (severe)
c) Model cleaner "A" containing 1% H_2O_2, neat, 10-minute exposure, 20-hour post exposure showing limited damage to the keratocytes in the upper stroma.	d) Model cleaner "B" containing 1% H_2O_2, neat, 10-minute exposure, 20-hour post exposure showing extensive damage to the keratocytes in the upper stroma.

Overview of Endothelial Cell Lesions

Figure 13.



a) Quinacrine (20% in water), 4-hour exposure showing endothelial cell cytoplasmic vacuolization. (severe)	b) Example of damage to the endothelial cell layer and deep stromal collagen matrix vacuolization. (severe)
c) Severe damage to the epithelium and resulting swelling of the upper stroma is shown. The endothelium was intact and little swelling was observed in the deep stroma.	d) Example of massive stromal swelling resulting from the loss of both the epithelium and endothelium. (severe)