

Appendix G1

**Dataset Received from S.C. Johnson & Son, Inc. in Support of
Cuellar et al. (2004) Poster Presentation**

[This Page Intentionally Left Blank]



A FAMILY COMPANY

S.C. Johnson & Son, Inc.
Worldwide Consumer Products, RD & E
Global Safety Assessment and Regulatory Affairs, Product Toxicology
MS 139 1525 Howe Street, Racine WI 53403

August 27, 2004

Christina Inhof, MSPH
Senior Project Coordinator/Technical Writer ILS, Inc.
NICEATM
P.O. Box 12233
NIEHS MD EC-17
Research Triangle Park, NC 27709

Christina,

Hi! How are you? I am happy to be submitting data on solvents and simple fragrance formulations, which were discussed in the poster citation listed below:

Cuellar, N., Lloyd, P.H., Swanson J.E., Merrill, J.C., Mun, G., Harbell, J.W. and Bonnette, K.L. (2004) Phase Two: Evaluating the eye irritancy of solvents in a simple fragrance mixture with the bovine corneal opacity and permeability (BCOP) assay. *The Toxicologist* 78(S-1): Abstract No. 1306.

Included with this submission are the following documents:

1. Cover letter
2. Poster text
3. Poster graphs
4. Histology slides
5. Coded formula spreadsheet

Study Protocols:

Modified Draize protocol was used for the in-vivo studies. Four animals were treated per sample (3 for histopathology and 1 animal for recovery). Each animal received a 0.1 ml dose of the formula in the conjunctival sac of the right eye. The left eye served as the untreated control. All animals were scored at 1, 4, and 24 hours after dosing. Histology was conducted on 3 of the 4 animals.

Standard BCOP protocol was used for the in-vitro work at IIVS. The first BCOP study required exposure times of 1 and 3 minutes with a post exposure of 20 hours to compliment the timing in the animal. The second BCOP study utilized a 3-minute exposure time with

post exposures of 2 to 4 hours to understand effects using standard post exposure times. Histology was conducted on all corneas. Since the 1-minute exposure did not produce extensive lesions, only the 3-minute exposure was evaluated in the second study. Only the 3-minute exposure data are reported in this poster.

Formula Spreadsheet:

The formulas listed in this spreadsheet are coded similarly to formulas listed in the poster. Test material number is the unique sample number and the group name denotes formula description. Raw materials are listed followed by their percentages in each formula.

Poster:

Fragrance poster not included. John Harbell previously sent it to you.

Poster Text:

A word document consisting of poster text and tables is included in this submission for ease of reading. The poster contained a wealth of information, thus limited visibility. This document highlights where the graphs and histology slides should be inserted for ease of understanding. Please note: Table one has improved coloring/formatting on the poster.

Poster Graphs:

Poster graphs should be referenced on page 10.

Histology Slides:

Histology slides should be referenced on page 12.

Data Worksheet:

The data worksheet is not included for this submission. GHS and EPA classification of results was not possible due to lack of animals. Only 1 of the 4 animals was carried out for recovery purposes. The remaining 3 animals were used for histopathology.

Summary:

Solvents have a major impact on the ocular irritation potential of fragrance mixtures. Both the degree and the time-course of the irritation can be impacted by the solvent. Over the three harvest times, the BCOP assay was able to identify histological changes that characterize the treatment groups into severe (ethanol alone), moderate (2-stages), and mild categories. One treatment group (fragrance + DPG) was over predicted by the BCOP compared to the in vivo assay. The time course of the tissue scores in vivo was similar to

August 30, 2004

the time course of the histological changes in BCOP. The BCOP model was more consistent in its response to a given treatment than the in vivo model.

If you have any questions or comments on this data set, please feel free to contact either Judith Swanson or myself at the following:

Nicole Cuellar
(262) 260-6916
ncuellar@scj.com

Judith Swanson
(262) 260-2688
jeswanso@scj.com

Sincere regards,

A handwritten signature in cursive script that reads "Nicole Cuellar".

Nicole Cuellar
Sr. Research Toxicologist

POSTER TEXT FOR S.C. JOHNSON SUBMISSION DATED AUGUST 27, 2004

TITLE:

PHASE TWO: EVALUATING THE EYE IRRITANCY OF SOLVENTS IN A SIMPLE FRAGRANCE MIXTURE WITH THE BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) ASSAY

N Cuellar¹, P H Lloyd², J E Swanson¹, J C Merrill³, G Mun³, J W Harbell³, and K L Bonnette⁴. ¹S.C. Johnson & Son, Inc. Racine, WI, USA; ²SCJ EURAFNE Ltd. Egham, Surrey, England; ³Institute for In Vitro Sciences, Inc. Gaithersburg, MD, USA; ⁴Charles River Laboratories, Inc. Spencerville, Ohio, USA.

ABSTRACT:

Fragrances are complex mixtures used in many consumer products. Organic solvents, such as ethanol, are major components of fragrance formulations functioning mainly as solubilizers and fragrance delivery mechanisms. In Phase One (Cuellar et al, 2003), the BCOP assay and primary eye irritation study (EPA-OPPTS 870.2400) were conducted using simple fragrance mixtures containing six commonly used solvents. The corneal depth of injury was assessed histologically both in vitro and in vivo. In the BCOP assay, corneas were exposed for 1 and 3 minutes, rinsed and incubated for 20 hours before the opacity and permeability endpoints were assessed. In vivo, animals were scored at 1, 4, and 24 hours. Individual solvents impacted the level of irritation of these formulations. Phase Two evaluated the time course of lesion development after exposure in the BCOP assay and determined those early lesion that would be predictive of damage observed after 20+ hours in vitro and in vivo. Bovine corneas were exposed for 3 minutes, rinsed, and incubated for 2 or 4 hours before the endpoints were assessed and tissue taken for histology. In vivo, certain solvents increased the rate of lesion development but not the overall intensity or duration compared to the fragrance alone. Other solvents decreased the overall intensity and duration. The BCOP assay showed a generally similar pattern of lesion development. Those combinations that showed opacity at 4 hours in vivo, showed epithelial and stromal lesion in the BCOP by 4 hours post-exposure. Fragrance alone was slower to develop opacity in vivo and required the 20 hour post-exposure to produce appreciable lesions in vitro. These data suggest that our standard post exposure (2 hour) can be predictive of irritation potential of fragrance/solvent mixtures.

INTRODUCTION:

The Bovine Corneal Opacity and Permeability (BCOP) Assay is routinely used by S.C. Johnson and Son as a tool for evaluating air fresheners for potential ocular irritation. Depending on the type of air freshener, other components may be added to solubilize a fragrance or to facilitate fragrance delivery mechanisms. In Phase I of this study (Cuellar et al., 2003), the BCOP assay and the primary eye irritation assay (EPA OPPTS 870.2400) were conducted using simple fragrance mixtures containing six commonly used solvents. As with Cuellar et al. (2002), Cuellar et al. (2003) found that solvents had an impact on the ocular irritation potential of fragrance mixtures. Based on this information, we examined the use of the BCOP at our standard post exposure times (2 and 4 hour) in comparison with Phase I post exposure times both in vitro (20 hour) and in vivo (24 hour) for rate, degree and intensity of irritation potential of the same fragrance mixtures.

Histological examination is necessary to understand the degree and depth of injury associated with the permeability and opacity measurements with diverse chemical classes or mixtures (Curren et al., 2000). Depth of injury has been shown to be a predictor of the degree and duration (reversibility) of ocular injury by Maurer et al. (2002) and Jester et al. (1998). Histological evaluation was used in Cuellar et al. (2002) to define the degree of injury to a reference sample and also to set the upper bound limit for other formulations of that type. Cuellar et al. (2003), demonstrated that permeability and opacity scores in the BCOP assay and the MAS scores in vivo paralleled the depth of injury and cellular changes seen histologically from both BCOP and in vivo samples. In Phase II of this study, we evaluated the progression of lesion development after exposure in the BCOP assay. Additionally, we determined specific early lesions that would be predictive of damage observed after 20+ hours in vitro and in vivo in the same fragrance mixtures.

MATERIALS AND METHODS:

Phase	In – vitro (BCOP)	In-vivo
One	3 & 10 minute exposure, 20 hour post-exposure	1, 4, & 24 hour scored
Two	3 minute exposure, 2 & 4 hour post-exposure	Not repeated

IN VITRO - BCOP:

Bovine Eyes

The BCOP assay was performed following the methods of Sina et al. (1995). Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals. The eyes were grossly examined for damage and those exhibiting defects were discarded. The corneas were excised such that a 2 to 3 mm rim of sclera was present around the cornea. The corneas were mounted in the holders and the two chambers filled with Minimum Essential Medium Eagle (MEM) without phenol red, supplemented with 1% fetal bovine serum (complete MEM). 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 medium replaced in both chambers and the opacity was determined for each cornea using a Spectro Designs OP-KIT opacimeter. Three corneas, whose opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The medium was then removed from the anterior chamber and replaced with either the test article, positive control, or negative control.

Testing Procedure

Each test article was administered neat to the BCOP test system. An aliquot of 750 μl of either the test article, positive control (100% ethanol), or negative control (deionized water) was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. Six corneas were used for each test article (three corneas per each post-exposure incubation period of 2 or 4 hours) were exposed for three minutes at $32 \pm 1^\circ\text{C}$. Six corneas were treated with the negative control (three corneas per each post-exposure incubation period of 2 and 4 hours) were exposed for ten minutes at $32 \pm 1^\circ\text{C}$. Three corneas were treated with the positive control for 10 minutes $32 \pm 1^\circ\text{C}$. After the exposure periods, the test or control article treatments were removed. The corneal surface was washed at least three times to ensure total removal of the test or control articles. The corneas exposed were returned to the incubator for approximately 2 hours (test articles, positive and negative controls) and 4 hours (test articles and negative controls). After this incubation (2 or 4 hours), the final measure of opacity was obtained. Corneas cultured for 4 hours were refeed immediately prior to the final measure of opacity. The values obtained at this second opacity measurement are presented in the report and were used in calculating the corneal opacity.

After the second opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was refilled with 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 $32 \pm 1^\circ\text{C}$. After the incubation, an aliquot of 360 μl from each chamber was placed into the designated well on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices *V*max kinetic microplate reader.

Opacity Measurement: The change in opacity for each cornea was calculated by subtracting the pre-treatment opacity readings from the final opacity readings. The corrected opacity value of each cornea was calculated by subtracting the average change in opacity of the negative control corneas from that of each treated cornea. The mean opacity values of each treatment group were then calculated.

Permeability Measurement: The corrected OD₄₉₀ was calculated by subtracting the mean OD₄₉₀ of the negative control corneas from the OD₄₉₀ value of each treated cornea. The mean OD₄₉₀ values of each treatment group were then calculated.

Histology

The corneas were placed in individual, prelabelled cassettes and fixed for at least 24 hours in 10% buffered formalin. The fixed corneas were transferred to Pathology Associates - A Charles River Company (Frederick, MD) for embedding, sectioning and staining. Each slide was then stained with hematoxylin and eosin. Slides were returned to the Institute for In Vitro Sciences, Inc. for evaluation. Photomicrographs and thickness measurements were prepared using a Spot Insight (Spot Diagnostic Instruments) digital camera and associated software.

IN VIVO:

The acute eye irritation study was conducted in accordance with the US EPA, Health Effects Test Guidelines (OPPTS 870.2400). Four New Zealand White rabbits were treated per sample (three animals for histopathology and one animal for recovery). Each animal received a 0.1mL dose of the appropriate test article in the conjunctival sac of the right eye. The left eye of each animal remained untreated and served as the control. Eyes were macroscopically scored at 1, 4, and 24 hours after dosing for both histopathology and recovery animals according to the Ocular Grading System based on Draize (1959). The group mean irritation score was then calculated for each scoring interval based on the number of animals initially dosed in each group. The calculated group mean ocular irritation scores for each interval were used to classify the test article according to the Ocular Evaluation Criteria of Kay and Calandra (1962).

Histology:

The test and control eyes were collected, identified, and placed in 10% neutral buffered formalin for fixation. The sections were processed histologically (embedded in paraffin, cut, and stained with hematoxylin and eosin). The histology was conducted by HistoTechniques (Powell, Ohio). The resulting slides were examined by a board certified pathologist (Dr. J. Dale Thurman, Senior Director of Pathology). No tissues were retained or examined for the recovery animals. Subsequently, slides were scored for cellular changes (paralleling those scored for bovine corneas) and photographed by one of us (JHW). These observations are reported in Table 1.

RESULTS:

The histological results of the BCOP and acute eye irritation assay for thirteen treatment groups are presented in Tables 1-3. The numerical scores of the BCOP and acute eye irritation assay for the thirteen treatment groups are graphically presented in Figures 1-6. The thirteen treatment groups include fragrance only, six solvents only, and six solvent/fragrance mixtures. The simple fragrance alone consists of 25% of each of the following fragrance components: benzyl acetate, linalool, dihydroxymyrcenol, and Verdox. The solvents consist of 100% of each of the following solvents: ethanol (ETOH), Dowanol DPM (DPM), Isopar M, dipropylene glycol (DPG), carbitol, and benzyl benzoate (BB). The solvent/fragrance mixtures (solvent + F, e.g. ETOH+F) consist of 80% fragrance mixture (20% of each fragrance component) and 20% of each solvent. Fragrance components and solvents were chosen because they are more frequently used in fragrance formulations.

Table 1 shows the depth of injury, cellular change and opacity score x area score for each animal for the thirteen treatment groups. Characteristic lesions are grouped together by severity of injury and cellular change from Group I (least irritating) to Group IV (most irritating). Days to clear for the recovery animal is listed below.

Table 1. In-Vivo Histological Summary

Group	Characteristic Lesions	Opacity Score x Area Score for each animal					
		2x4	2x3	2x2	2x1	1x1	0x0
IV	1) Complete or nearly complete loss of epithelium over the cornea 2) Marked inflammatory infiltrate extending well into the corneal stroma 3) Loss of keratocytes in the upper stroma 4) Increased frequency of enlarged keratocytes in the mid stroma	ETOH ETOH ETOH					
III	1) Focal, full thickness loss of epithelium 2) Marked inflammatory infiltrate restricted to the edge of the cornea 3) Increased frequency of enlarged keratocytes in the upper stroma under the epithelial lesion	Frag- rance ETOH+F DPM+F	DPG+F Carbitol +F	Frag- rance Carbitol+ F BB+F	Frag- rance Carbitol+F DPM DPM		
II	1) Small focus of epithelial loss or thinning 2) Limited inflammatory infiltrate 3) Slight increase in enlarged keratocytes		DPM Carbitol	Carbitol	ETOH+F DPM+F Carbitol	Isopar M+F BB+F	DPG+F DPG
I	1) Epithelium was intact or just slightly thinned 2) Little or no inflammatory infiltrate 3) No enlarged keratocytes						Isopar M Isopar M DPG DPG BB BB BB ETOH+F DPM+F Isopar M+F Isopar M+F DPG+F BB+F ETOH Isopar M

Days to clear (for the remaining animal in the treatment group)

>28 days - ETOH

7 Days - Fragrance, ETOH+F, DPM+F, Carbitol +F

3 Days – DPG+F, DPM, DPG, Carbitol

2 Days – Isopar+F, BB+F

1 Day – Isopar M, BB

Table 2 demonstrates the description of the characteristic lesions seen in the epithelial layer of the corneas of the 13 treatment samples in the BCOP assay at three different post-exposure times (2, 4, and 20 hours). Corneas are grouped by severity of the depth of injury and cellular changes from Group E (most irritating) to Group A (least irritating).

Table 2. Summary of the epithelial layer changes in the BCOP

In Vitro	Characteristic Lesions	2-hour post-exposure	4-hour post-exposure	20-hour post-exposure
Group E	Full thickness loss or separation on >50% of the corneal surface	ETOH	ETOH	ETOH DPM + F (2) Carbitol + F
Group D	Full thickness loss/separation on <50% of the corneal surface but substantial damage to wing and basal cell layers	ETOH+F(2) DPG+F Carbitol+F	ETOH+F Carbitol+F(2))	Fragrance ETOH+F DPG+F (2) DPM+F (1)
Group C	Little or no full thickness loss but damage well into the wing and basal cell layers (may include nuclear changes and cytoplasmic vacuolization)	ETOH+F(1) DPM+F DPM Carbitol	DPM+F DPG+F Carbitol+ F(1) DPM Carbitol	DPG+F(1) BB+F DPM Carbitol
Group B	Loss of the full squamous cell layer with occasional damage into the wing cell layer. No full thickness loss	Fragrance Isopar M+F	Fragrance	Isopar M+F
Group A	Similar to the time-matched negative control epithelium or with some loss/loosening of the surface squamous epithelial layers	BB+F Isopar M DPG BB	Isopar M+F BB+F Isopar M DPG BB	Isopar M DPG BB

Table 3 demonstrates the description of the characteristic lesions seen in the stroma of the corneas of the 13 treatment samples in the BCOP assay at three different post-exposure times (2, 4, and 20 hours). Corneas are grouped by severity of the depth of injury and cellular changes from Group E (most irritating) to Group A (least irritating).

Table 3. Summary of the Stromal Lesions in the BCOP.

In Vitro	Characteristic Lesions	2-hour post-exposure	4-hour post-exposure	20-hour post-exposure
Group E	1) Thickness: Appreciably thicker than the time-matched controls 2) Stromal matrix vacuolization: Marked to 50% depth 3) Keratocytes: a. Upper stroma: Marked cell loss and/or increase in nuclear changes (degeneration, pyknosis, vacuolization or abnormal chromatin condensation) to \leq 50% depth b. Nuclear enlargement/ cytoplasmic eosinophilia: Marked or Moderate to \geq 50% depth		ETOH	ETOH
Group D	1) Thickness: Thicker than the time-matched controls 2) Stromal matrix vacuolization: Some marked but moderate to 50% depth 3) Keratocytes: a. Upper stroma: Moderate cell loss and/or increase in nuclear changes (degeneration, pyknosis, vacuolization or abnormal chromatin condensation) to \leq 25% depth b. Nuclear enlargement/ cytoplasmic eosinophilia: Moderate/Marked to 50% depth		ETOH+F DPG+F Carbitol+F(2)	Fragrance ETOH+F DPM+F Carbitol+F
Group C	1) Thickness: Thicker than the time-matched controls 2) Stromal matrix vacuolization: Moderate to \leq 50% depth 3) Keratocytes: a. Upper stroma: Slight cell loss and/or increase in nuclear changes (degeneration, pyknosis, vacuolization or abnormal chromatin condensation) to \leq 25% depth b. Nuclear enlargement/ cytoplasmic eosinophilia: Moderate to \leq 50% depth	Carbitol+F DPM Carbitol	DPM+F DPM Carbitol	DPG+F Carbitol DPM
Group B	1) Thickness: Slightly thicker than the time-matched controls 2) Stromal matrix vacuolization: Moderate to \leq 30% depth 3) Keratocytes: a. Upper stroma: Normal (no cell loss or nuclear degeneration) b. Nuclear changes (enlargement)/ cytoplasmic eosinophilia: Moderate increase to \leq 30% depth	ETOH+F ETOH		BB+F
Group A	1) Thickness: Similar to the time-matched controls 2) Stromal matrix vacuolization: Slight or less increase to \leq 30% depth 3) Keratocytes:	Fragrance DPM+F Isopar M+F DPG+F	Fragrance Isopar M+F DPG+F Carbitol+F(1)	Isopar M + F Isopar M DPG BB

In Vitro	Characteristic Lesions	2-hour post-exposure	4-hour post-exposure	20-hour post-exposure
	a. Upper stroma: Normal (no cell loss or nuclear degeneration) b. Nuclear changes (enlargement)/ cytoplasmic eosinophilia: Slight or less increase to $\leq 20\%$ depth	BB+F Isopar M DPG BB	BB+F Isopar M DPG BB	

GRAPHICAL RESULTS: Appended Below

Figure 1. In-vivo Results – Opacity

Figure 2. In-vivo Results – Opacity X Area

Figure 3. In-vivo Results - MAS scores

Figure 4. BCOP Opacity Scores

Figure 5. BCOP Permeability Scores

Figure 6. BCOP In vitro Scores

RESULTS:

- Alone, the fragrance induced only slight corneal changes until 24 hours after treatment in vivo. In the BCOP assay, the similar time course was observed.
- Impact of Solvent:
 1. The addition of ethanol or DPM to the fragrance increased the in-vivo corneal scores (opacity and area) at 4 hours compared to the fragrance alone.
 2. The addition of ethanol, DPM, DPG, or carbitol to the fragrance increased the BCOP opacity and permeability scores (and histological changes) at 2 and 4 hours compared to fragrance alone.
 3. The addition of IsoPar M to the fragrance showed a strong mitigating effect on the overall irritancy of the fragrance in-vivo and BCOP assays.
 4. The addition of BB to the fragrance slowed the onset and reduced the overall irritation (in vivo) and BCOP opacity and permeability scores (and histological changes).
- The rapid onset of irritancy to the cornea (in-vivo) of ethanol, DPM, and carbitol was correctly predicted by the BCOP at 2 hours based on both the scores (opacity and permeability) and histological changes in the epithelium. The full expression of stromal changes took 4 hours in the BCOP (e.g., ethanol).
- Irritation Levels:
 1. Severe irritation was defined by sustained high Draize scores, high in-vitro scores, significant tissue damage (Group IV and E) and no recovery.
 2. Moderate irritation was defined at two levels with moderate tissue damage (Group III, II & D-B):
 - one level defined by moderate Draize and in-vitro scores, and seven days to clear and
 - second level defined by moderate Draize and in-vitro scores and three days to clear.
 3. Mild irritation was defined by mild Draize and in-vitro scores, minimal tissue damage (Group I & A) and rapid recovery (<3 days).
- Based on the histological changes over the three harvest times, the BCOP was able to distinguish a) the severe irritation potential of ethanol, b) the moderate irritation potentials of fragrance alone, fragrance + ethanol, fragrance + DPM, and fragrance + carbitol, c) the moderate but more rapidly clearing irritation of DPM and carbitol alone, d) the mild irritation of fragrance + Isopar M and fragrance + BB, and e) the very mild Isopar M, DPG, and BB alone. The exception is fragrance + DPG where the in vitro response was much more pronounced than the in vivo response.

In Vivo Histology : Appended below

Figures 7-8 show animal corneas treated with test substance.

Figure 7. In Vivo Group 1: BB & Isopar M

- (A) Center of the cornea, no changes observed (magnification 230x)
- (B) Area, away from limbus, showing separation of squamous epithelium (magnification 430x)

Figure 8. In Vivo Group 4: ETOH.

- (A) Central cornea showing loss of epithelium, inflammation, and marked increase in larger dark staining keratocyte nuclei in area of inflammatory infiltrate. Note - the cells were not in the upper 20% of the stroma (magnification 170x)
- (B) Area in denuded area showing keratocyte changes and swelling (magnification 430x)

BCOP Histology

Figures 9-12 show corneas treated for 3 minutes, at varying post exposure times with test substance in the BCOP.

Figure 9. BCOP Fragrance alone: 3-minute exposure, 2-hour post-exposure

- (A) Epithelium – Loss of surface squamous epithelium and some necrotic cells within the wing and basal layers (magnification 230x)(Epithelial Group B)
- (B) Stroma – Very similar to the time-matched negative control-treated corneas (magnification 430x)(Stromal Group A)

Figure 10. BCOP Fragrance alone: 3-minute exposure, 20-hour post-exposure

- (A) Epithelium – Loss of the squamous epithelium and marked nuclear pyknosis and cytoplasmic eosinophilia in the wing cell layer (magnification 230x) (Epithelial Group D)
- (B) Stroma – Marked collagen matrix vacuolization to 20% depth and keratocyte nuclear swelling and cytoplasmic eosinophilia (magnification 430x) (Stromal Group D)

Figure 11. BCOP ETOH alone: 3-minute exposure, 2-hour post-exposure

- (A) Epithelium – Marked cellular damage and separation between the basal cells and basal lamina (magnification 230x) (Epithelial Group E)
- (B) Stroma – Moderate collagen matrix vacuolization to mid depth and moderate increase in keratinocytes with nuclear pyknosis in the upper 25% of the stroma (magnification 430x) (Stromal Group B)

Figure 12. BCOP ETOH alone: 3-minute exposure, 4-hour post-exposure

- (A) Epithelium - Marked cellular damage and separation between the basal cells and basal lamina (magnification 230x) (Epithelial Group E)
- (B) Stroma – Marked collagen matrix vacuolization and a decrease in viable keratocytes extended to 30% depth. Marked keratocyte nuclear enlargement cytoplasmic eosinophilia was present at mid depth but is not shown in this micrograph (magnification 430x) (Stromal Group E)

CONCLUSIONS:

- Over the three harvest times, the BCOP assay was able to identify histological changes that characterize the treatment groups into severe (ethanol alone), moderate (2-stages), and mild categories. One treatment group (fragrance + DPG) was over predicted by the BCOP compared to the in vivo assay.
- Solvents have a major impact on the ocular irritation potential of fragrance mixtures. Both the degree and the time-course of the irritation can be impacted by the solvent.
- The time course of the tissue scores in vivo was similar to the time course of the histological changes in BCOP.
- When injury was significant enough, morphological changes in the keratocytes, specifically keratocyte nuclear enlargement (activation), were detectable in both the BCOP and in-vivo-treated corneas. Fini (1999) and collaborators have reported that certain morphological changes in keratocytes are associated with phenotypic changes (activation) and subsequent undesirable fibrotic scarring.
- BCOP model was more consistent in its response to a given treatment than the in vivo model (Table 1-3).

REFERENCES

- Cuellar, N., Lloyd, P.H., Swanson J.E., Merrill, J.C., Clear, M.L., Mun, G., Harbell, J.W. and Bonnette, K.L. (2003) Evaluating the eye irritancy of solvents in a simple fragrance mixture with the bovine corneal opacity and permeability (BCOP) assay. *The Toxicologist* 72(S-1): 312-313.
- Cuellar, N., Merrill, J.C., Clear, M.L. Mun, G., and Harbell, J.W. (2002) The application of benchmarks for the evaluation of the potential ocular irritancy of aerosol fragrances. *The Toxicologist* 66(1-S): 243-244.
- Curren, R., Evans, M., Raabe, H., Ruppalt, R., and Harbell, J. (2000) Correlation of histopathology, opacity, and permeability of bovine corneas exposed in vitro to known ocular irritants. *Veterinary Pathology* 37(5):557.
- Draize, J.H. (1959) Appraisal of the safety of chemicals in foods, drugs, and cosmetics. *The Association of Food and Drug Officials of the United States*: 49-51.
- Fini, E.M. (1999) Keratocyte and fibroblast phenotypes in the repairing cornea. *Progress in Retinal and Eye Research* 18(4):529-551.
- 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.
- Kay, J.H. and Calandra, J.C. (1962) Interpretation of eye irritation tests. *J Soc Cosmetics Chemists* 13: 281-289.
- 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(1):106-117.
- 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.

ACKNOWLEDGEMENTS

The authors wish to thank the general staffs at both the Institute for In Vitro Sciences, Inc. and the Charles River Laboratories, Inc. for their assistance on this project.

Figure 1. In Vivo Opacity Scores

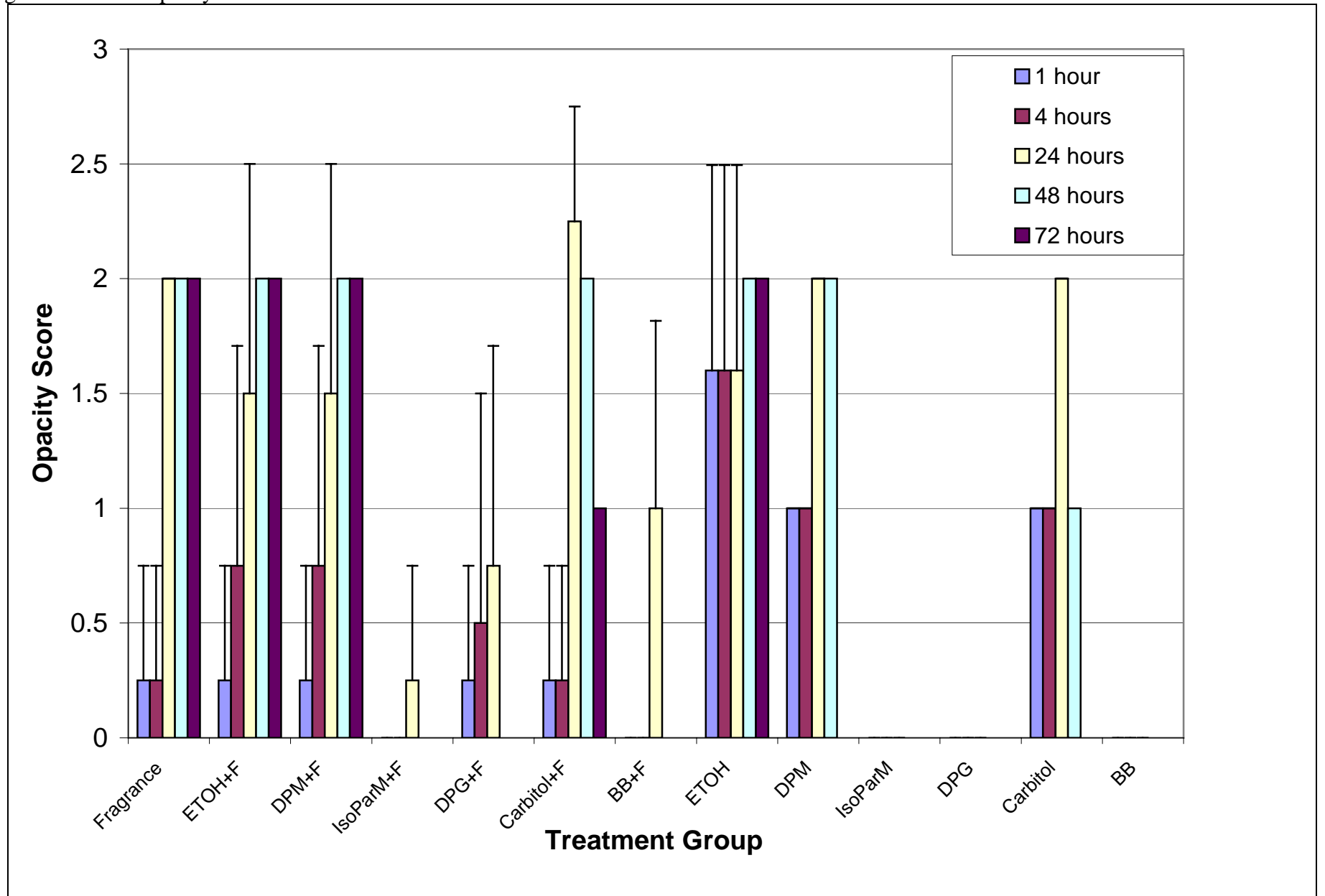


Figure 2. In Vivo Corneal Scores (Opacity x Area)

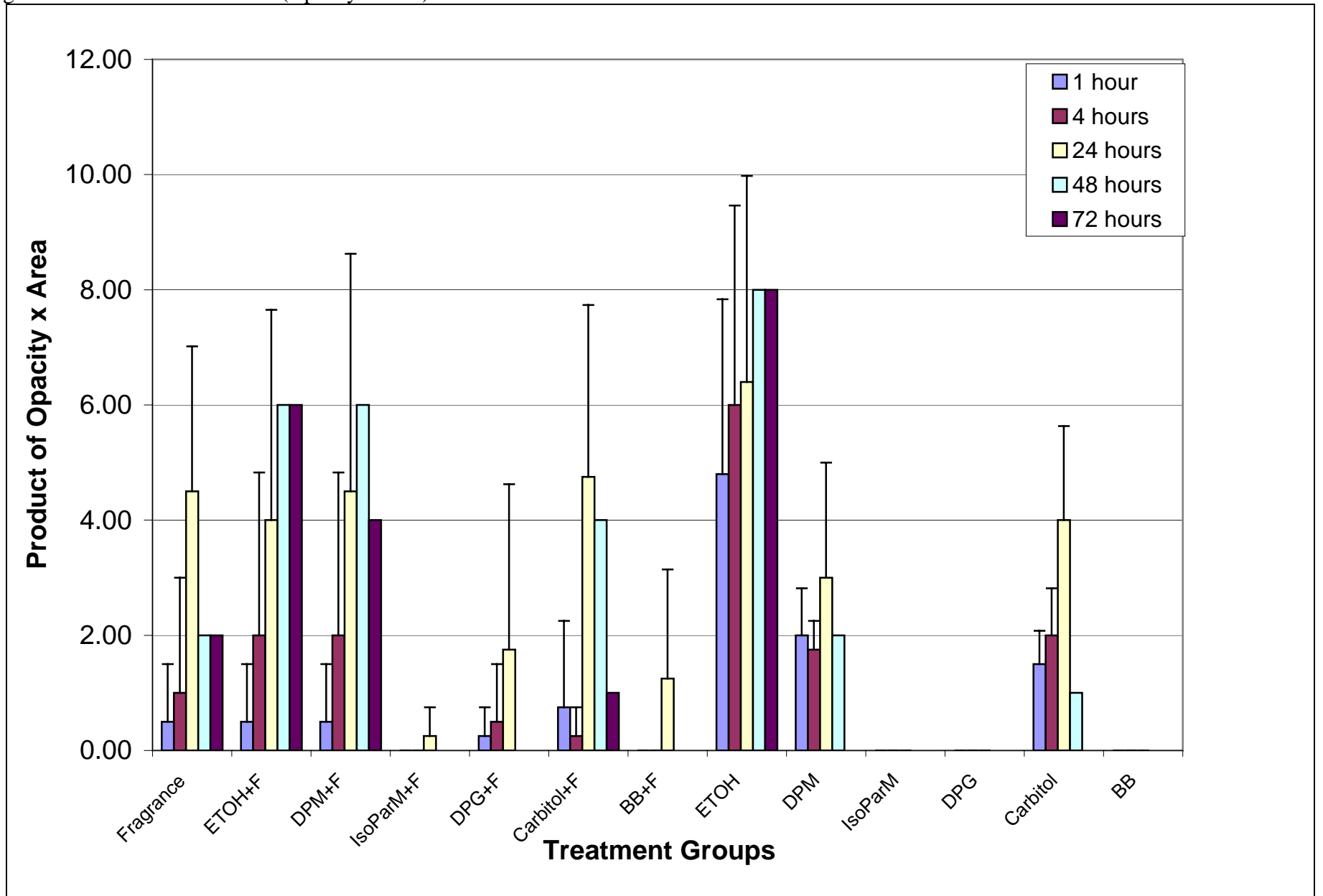


Figure 3. In Vivo MAS Scores

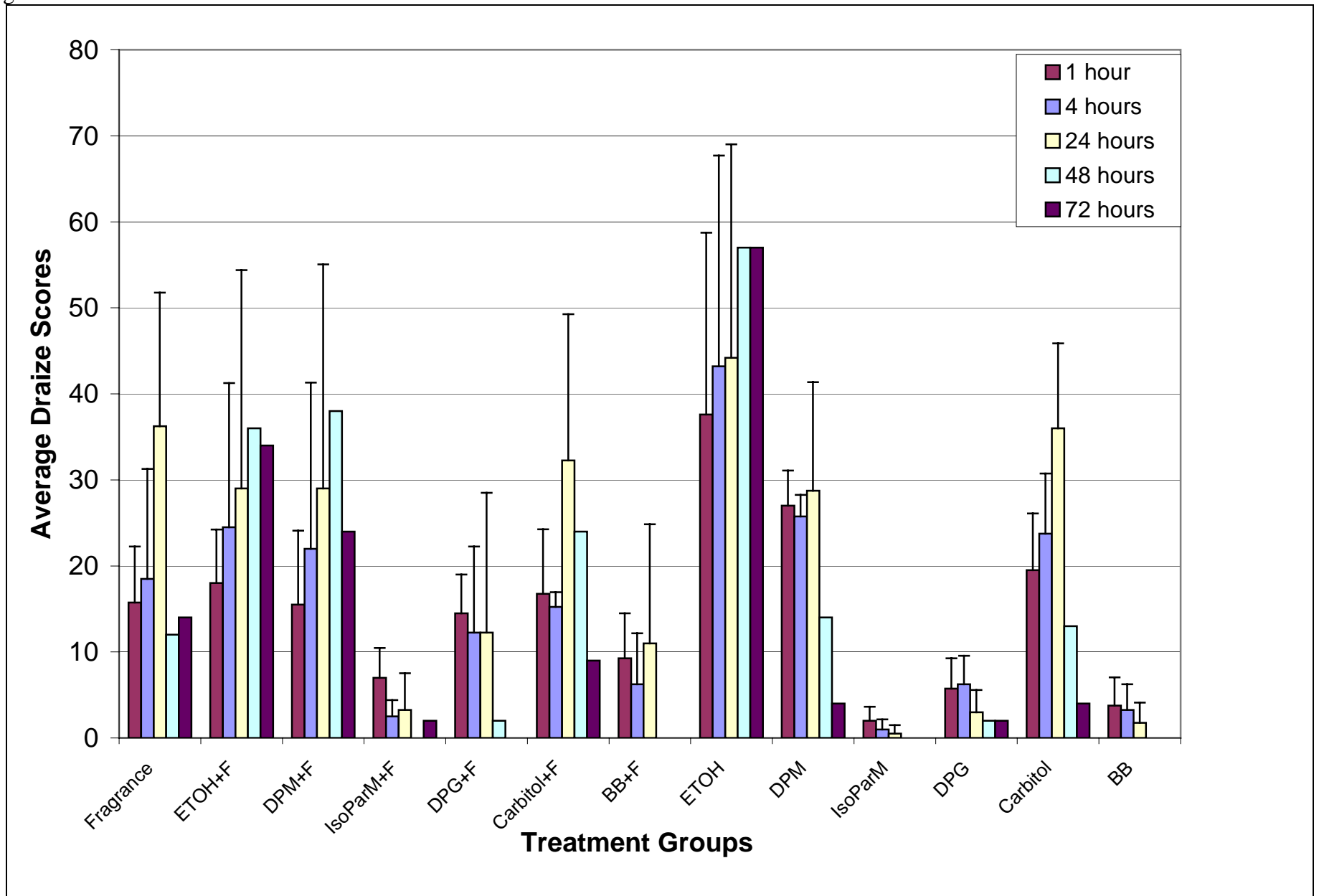


Figure 4. BCOP Opacity Scores

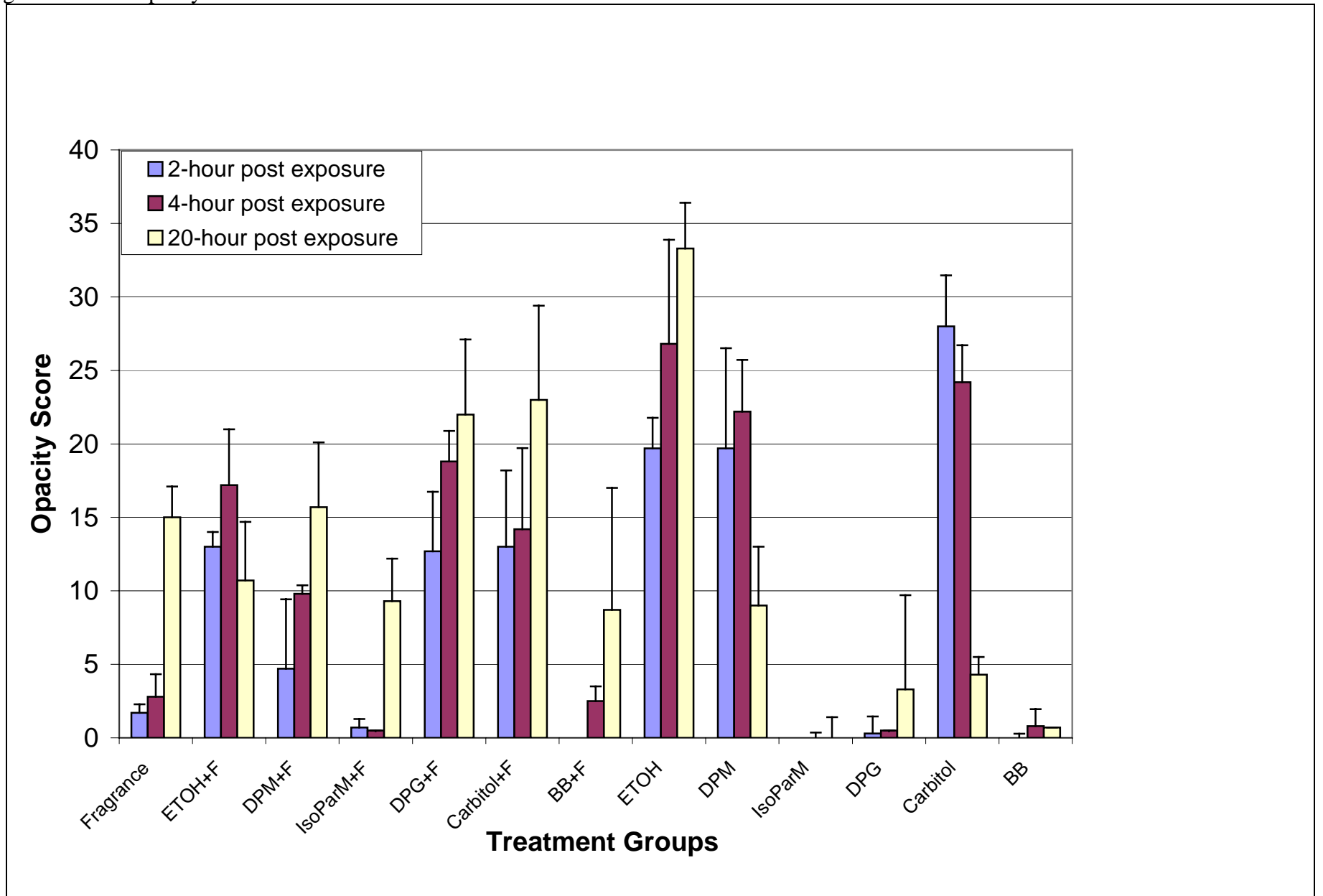


Figure 5. BCOP Permeability Scores

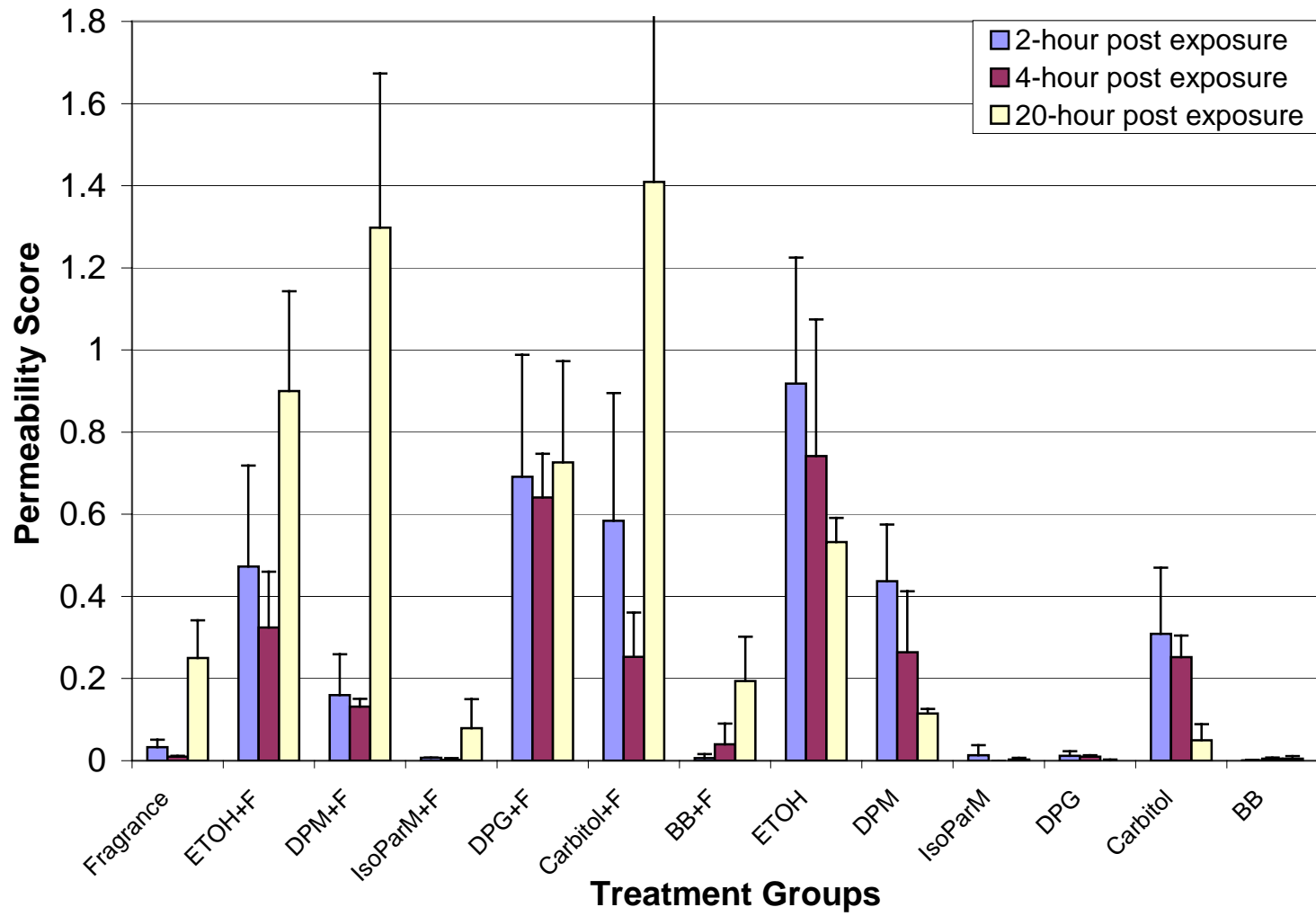


Figure 6. BCOP In Vitro Scores

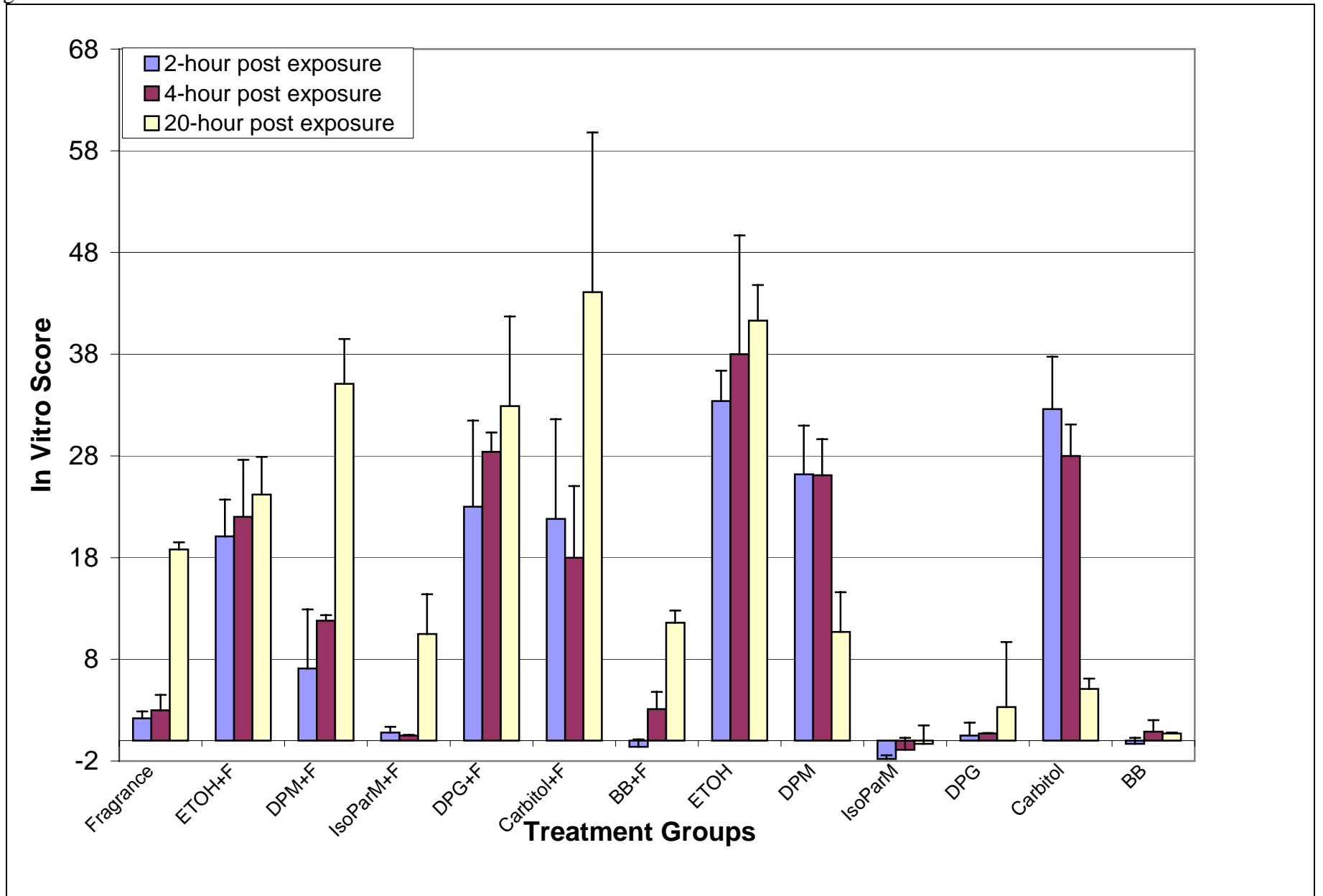
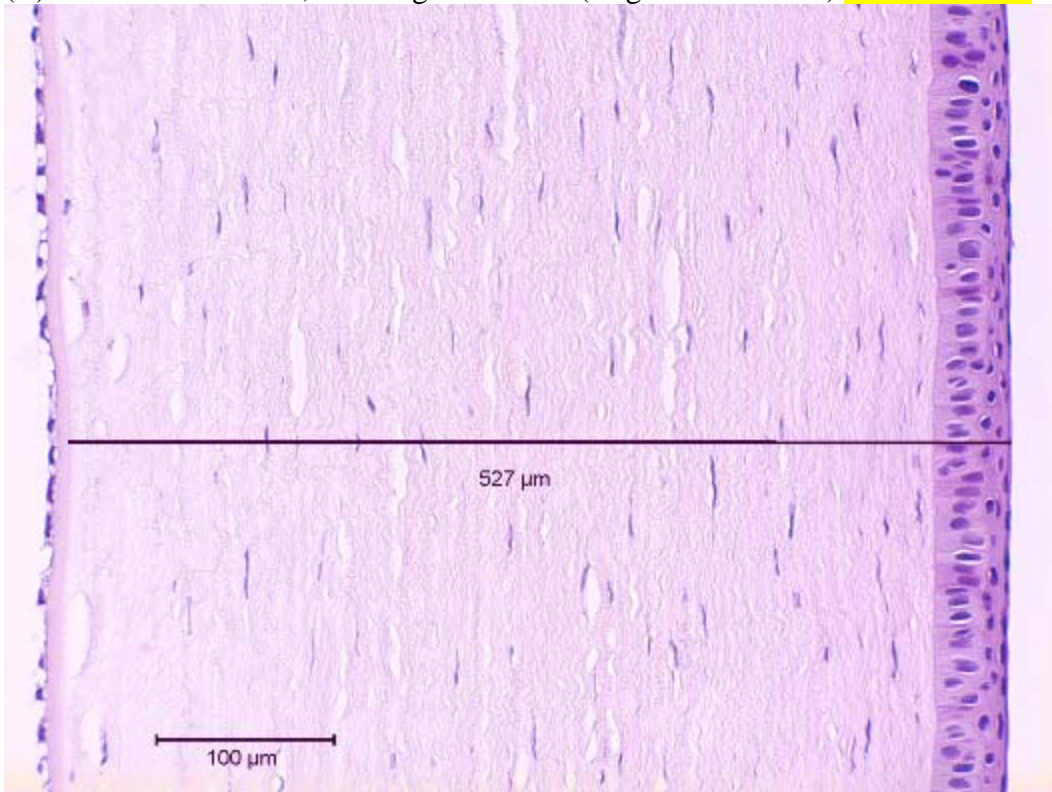


Figure 7. In Vivo Group 1: BB & Isopar M

(A) Center of the cornea, no changes observed (magnification 230x) [File 100902-77](#)

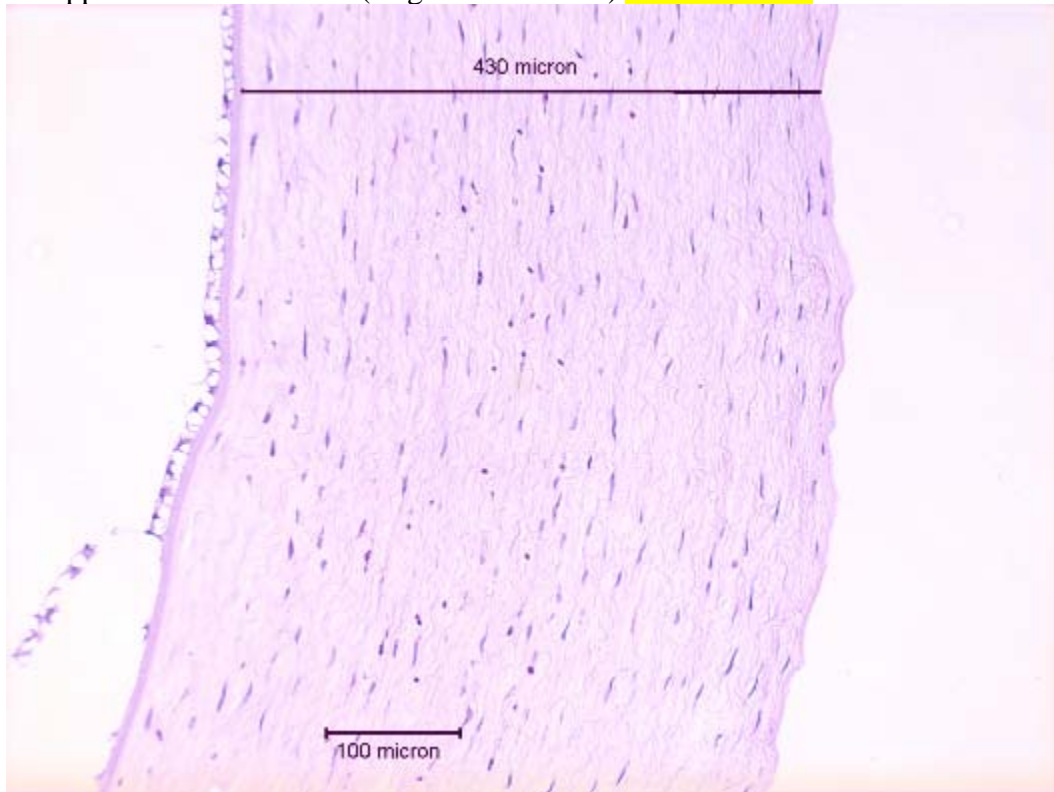


(B) Area, away from limbus, showing separation of squamous epithelium (magnification 430x) **File 100902-61**



Figure 8. In Vivo Group 4: ETOH.

(A) Central cornea showing loss of epithelium, inflammation, and marked increase in larger dark staining keratocyte nuclei in area of inflammatory infiltrate. Note - the cells were not in the upper 20% of the stroma (magnification 170x) [File 100902-41](#)



(B) Area in denuded area showing keratocyte changes and swelling (magnification 430x) File 100902-42

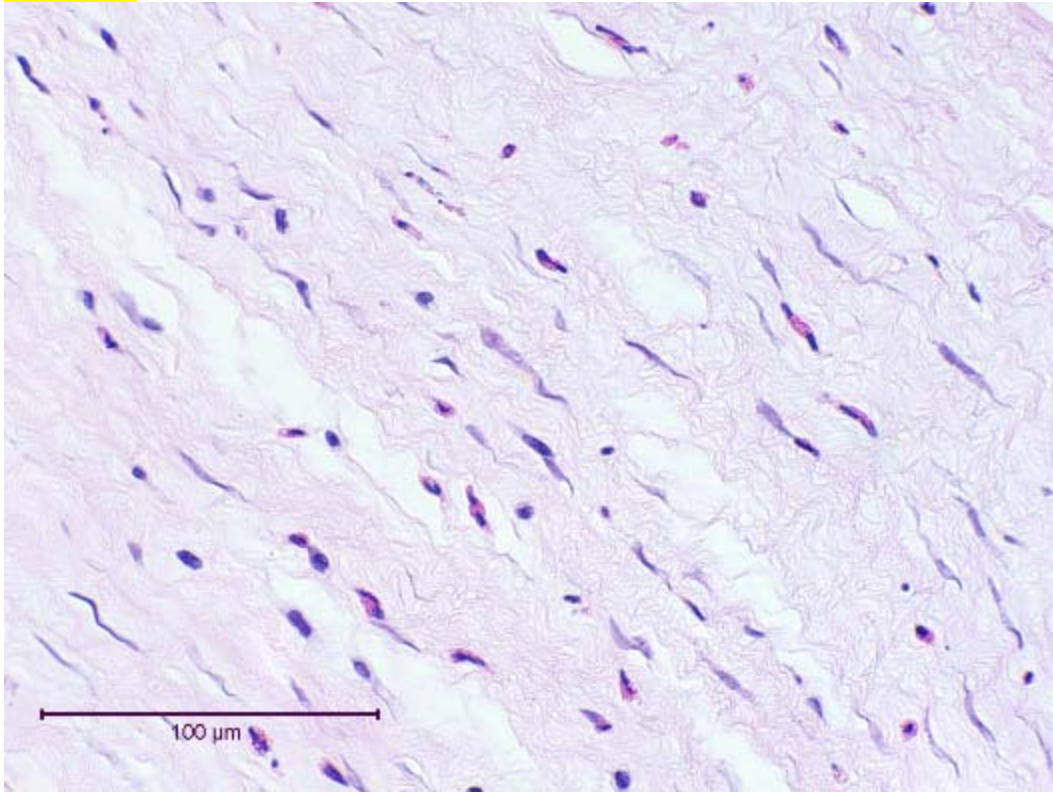
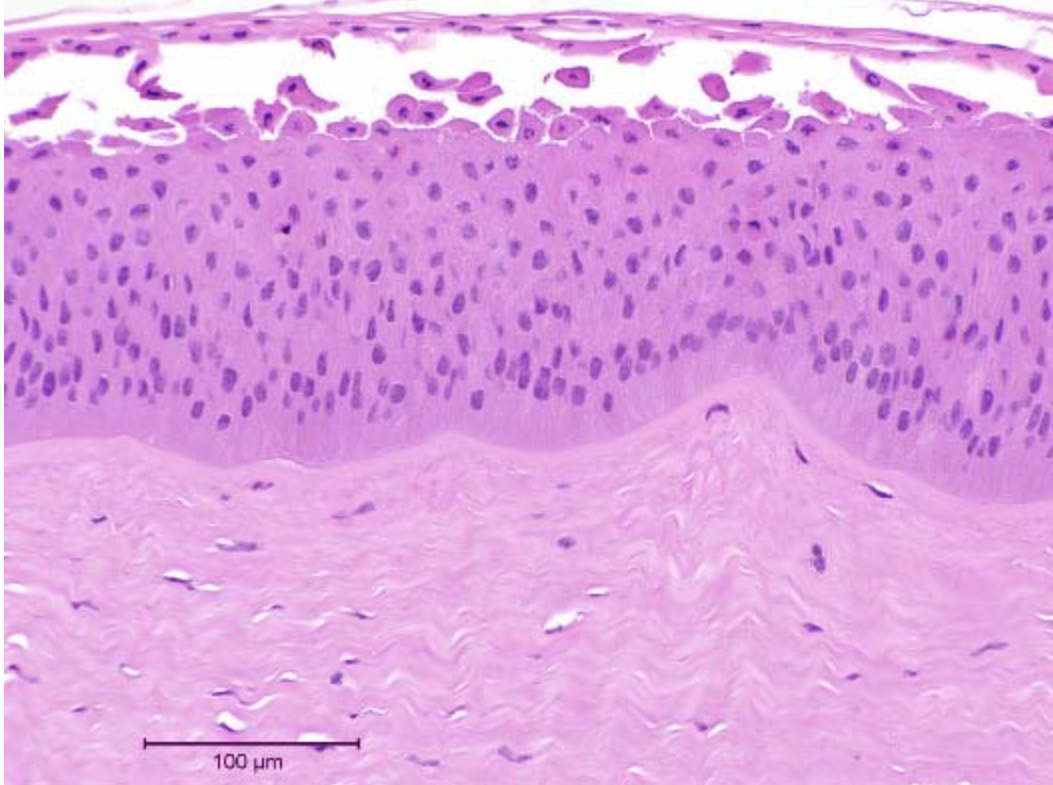


Figure 9. BCOP Fragrance alone: 3-minute exposure, 2-hour post-exposure

(A) Epithelium – Loss of surface squamous epithelium and some necrotic cells within the wing and basal layers (magnification 230x)(Epithelial Group B) [File 082903-08](#)



(B) Stroma – Very similar to the time-matched negative control-treated corneas (magnification 430x)(Stromal Group A) [File 082903-10](#)

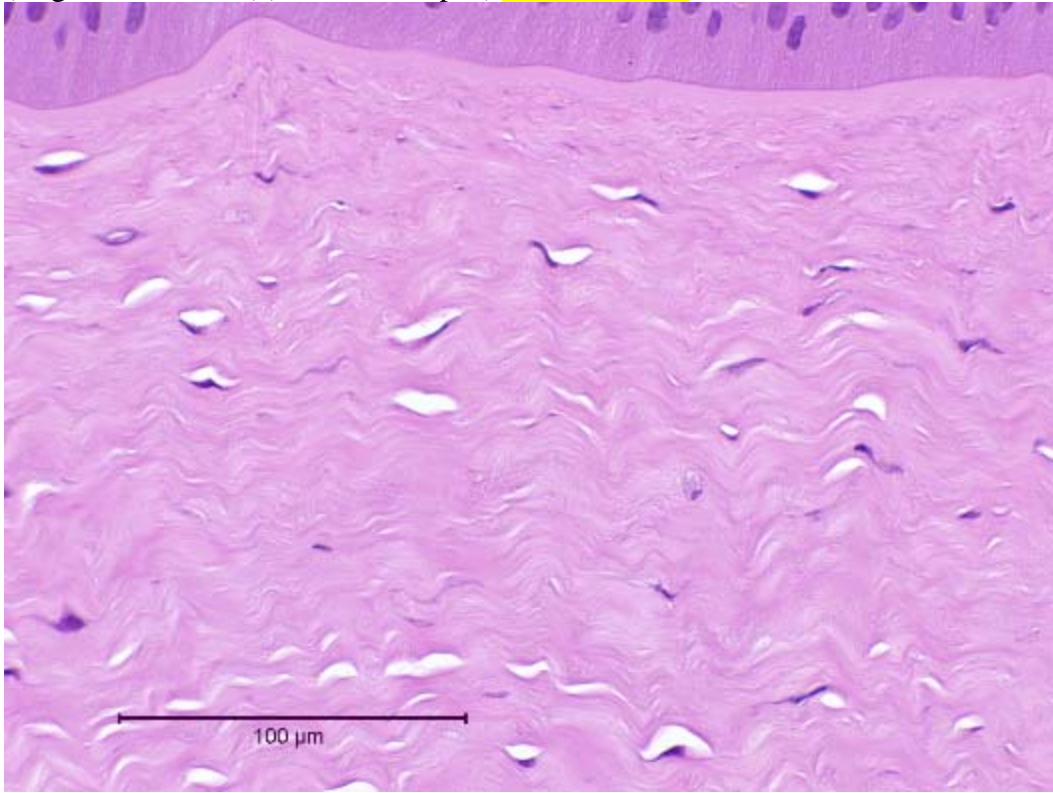
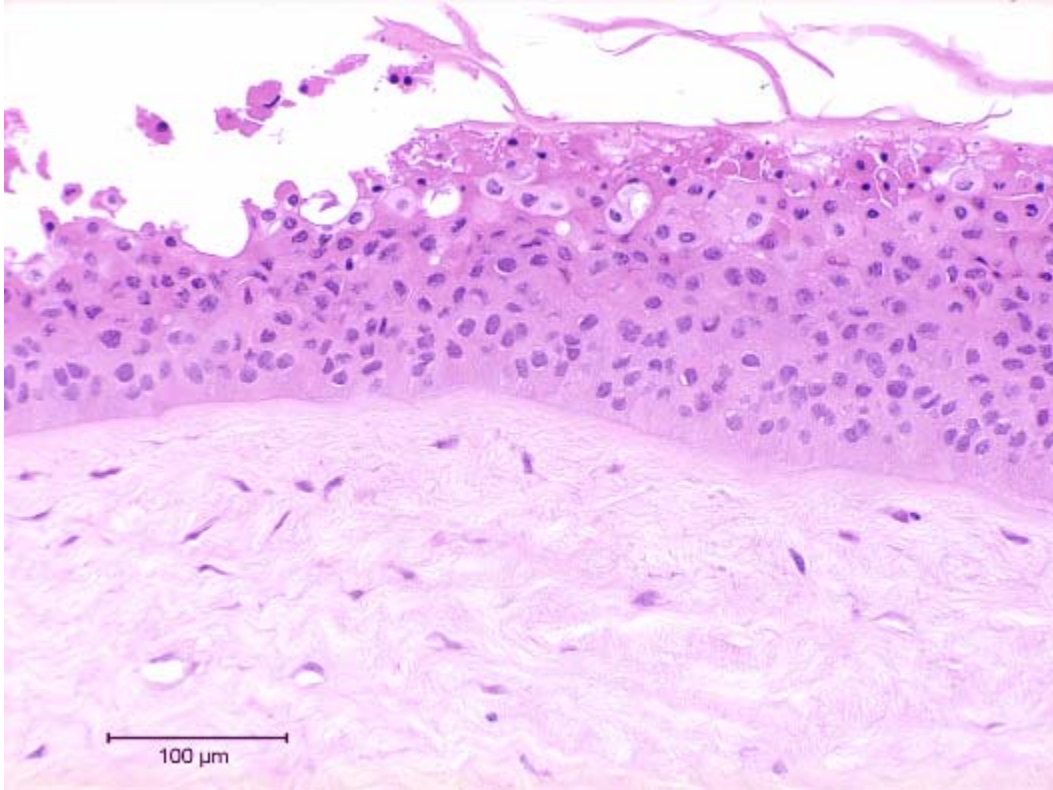


Figure 10. BCOP Fragrance alone: 3-minute exposure, 20-hour post-exposure

(A) Epithelium – Loss of the squamous epithelium and marked nuclear pyknosis and cytoplasmic eosinophilia in the wing cell layer (magnification 230x) (Epithelial Group D) [File 111702-12](#)



(B) Stroma – Marked collagen matrix vacuolization to 20% depth and keratocyte nuclear swelling and cytoplasmic eosinophilia (magnification 430x) (Stromal Group D) **File 111702-14**

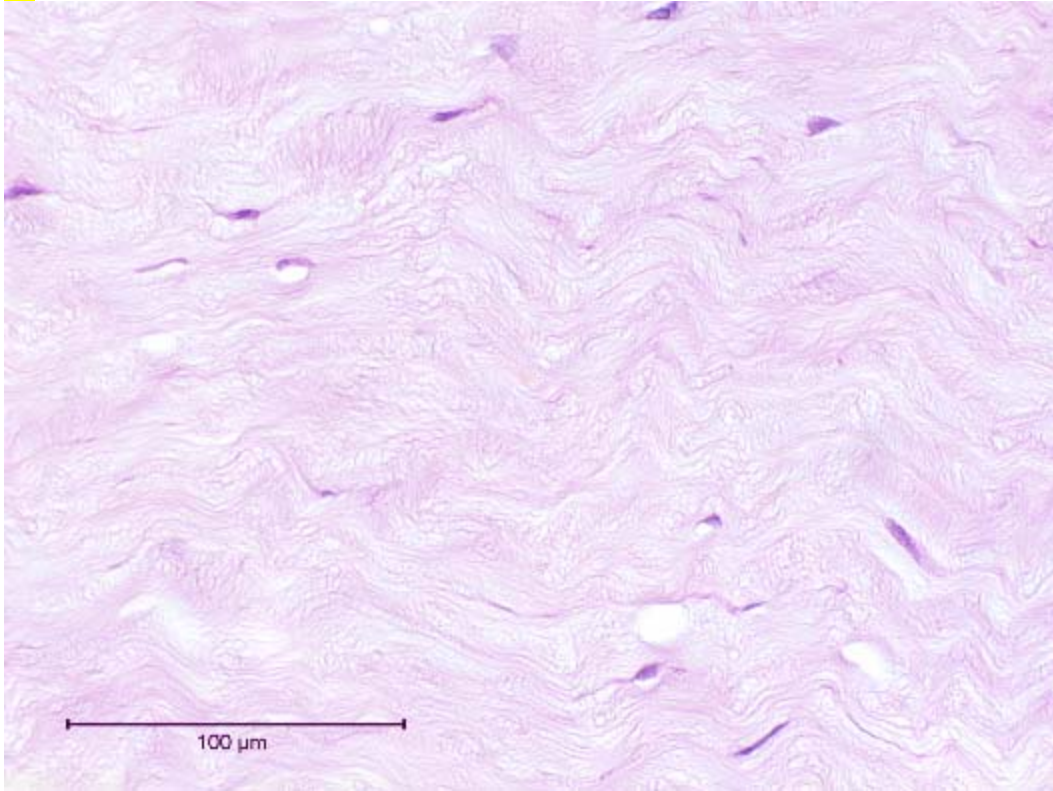
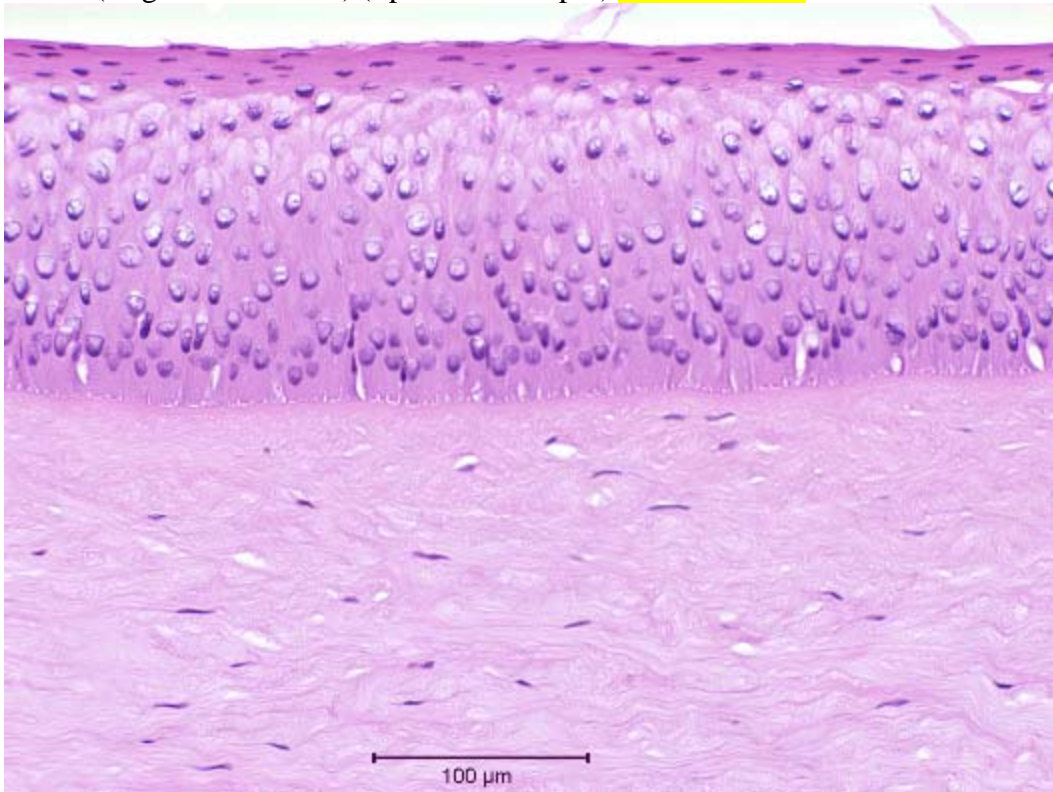


Figure 11. BCOP ETOH alone: 3-minute exposure, 2-hour post-exposure

(A) Epithelium – Marked cellular damage and separation between the basal cells and basal lamina (magnification 230x) (Epithelial Group E) [File 082903-35](#)



(B) Stroma – Moderate collagen matrix vacuolization to mid depth and moderate increase in keratinocytes with nuclear pyknosis in the upper 25% of the stroma (magnification 430x)
(Stromal Group B) [File 082903-37](#)

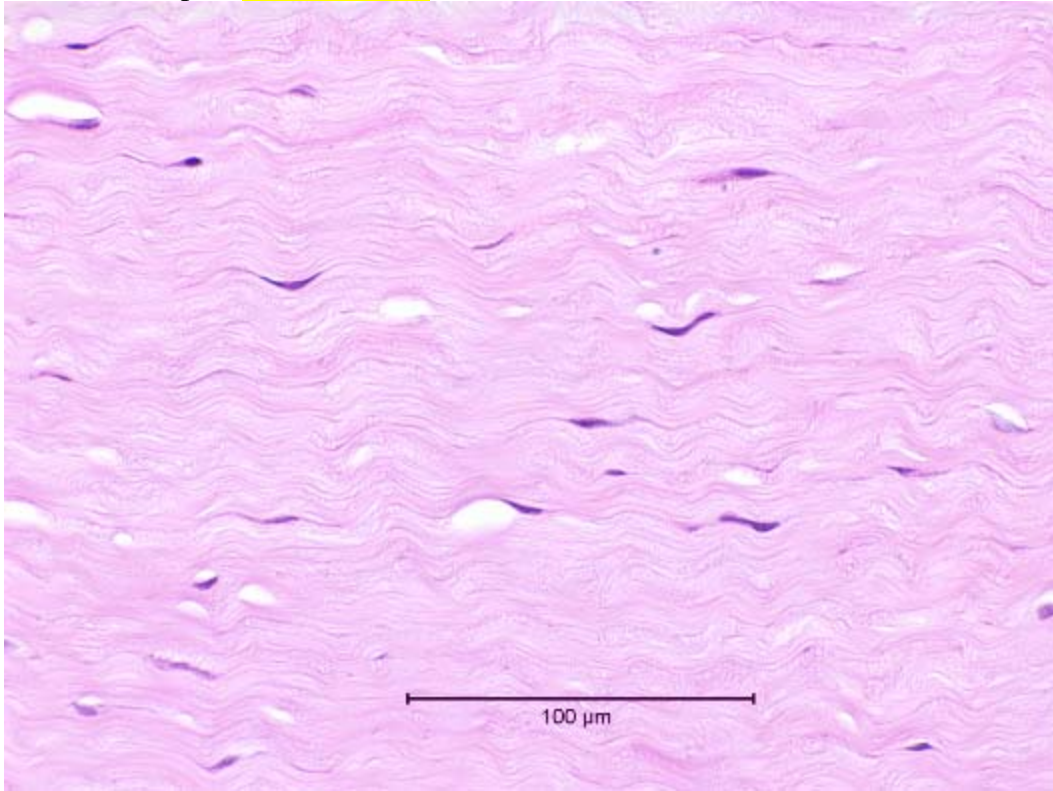
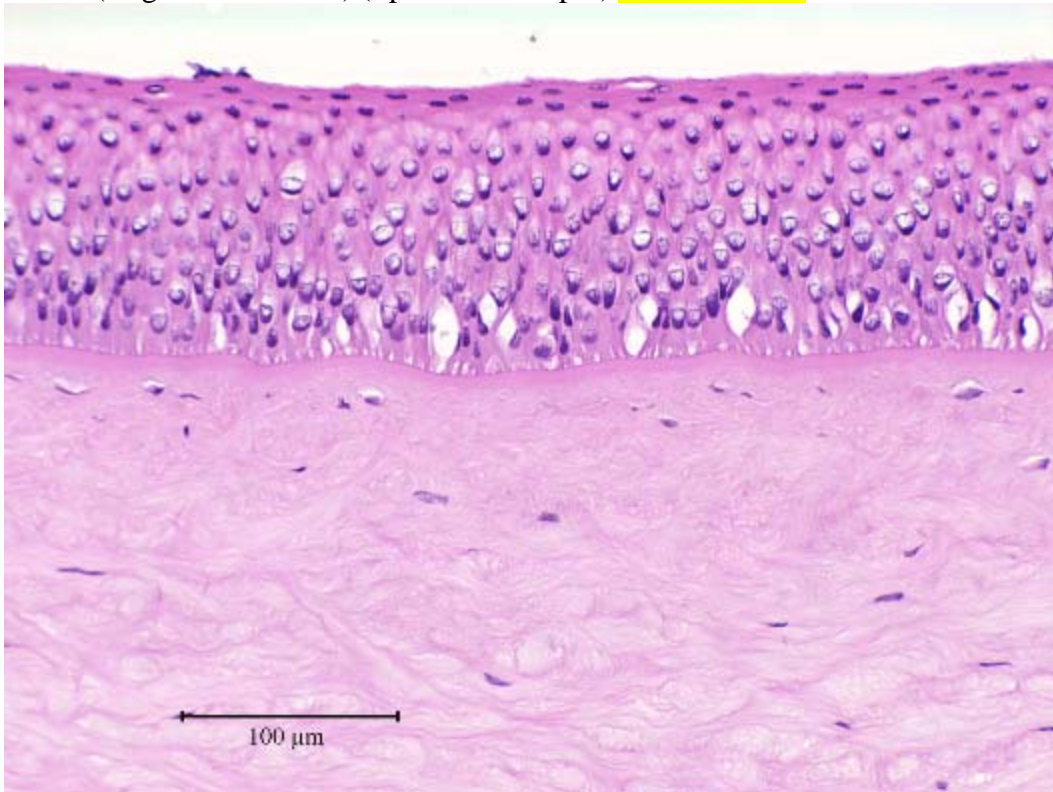
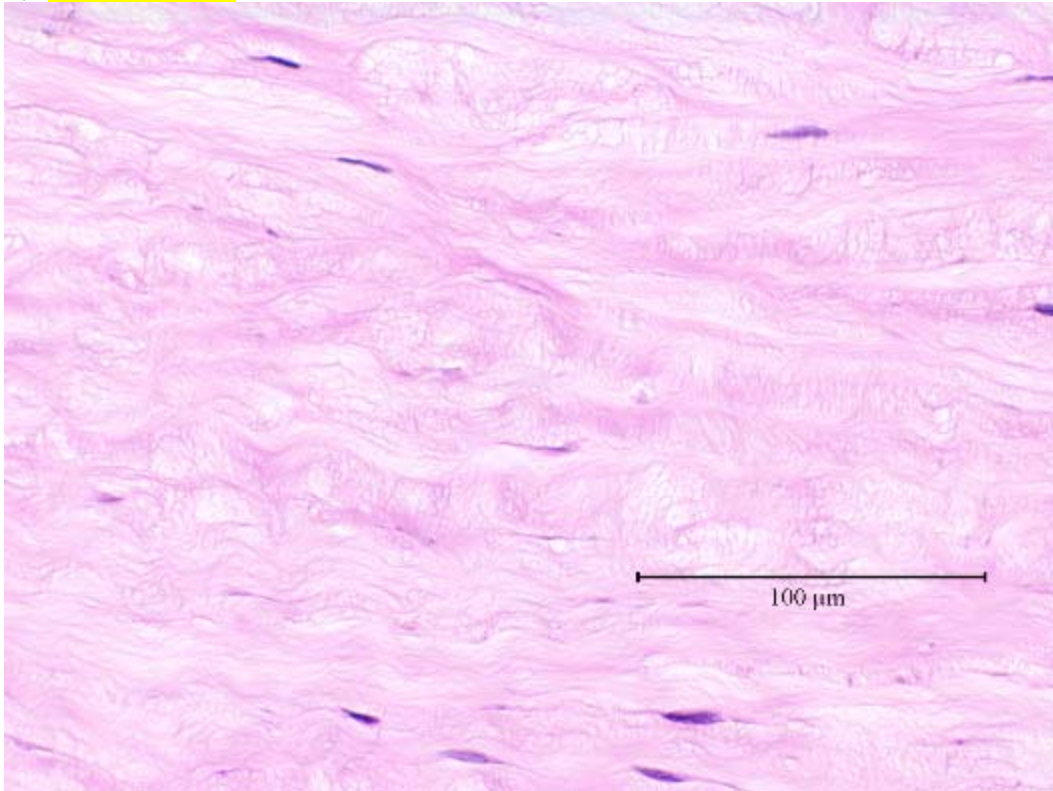


Figure 12. BCOP ETOH alone: 3-minute exposure, 4-hour post-exposure

(A) Epithelium - Marked cellular damage and separation between the basal cells and basal lamina (magnification 230x) (Epithelial Group E) [File 091503-43](#)



(B) Stroma – Marked collagen matrix vacuolization and a decrease in viable keratocytes extended to 30% depth. Marked keratocyte nuclear enlargement cytoplasmic eosinophilia was present at mid depth but is not shown in this micrograph (magnification 430x) (Stromal Group E) [File 091503-45](#)



FORMULAS

Test Material #	Group	Raw Material	Percentage
1	Fragrance	Benzyl acetate	25
		linalool	25
		Dihydroxymyrcenol	25
		Verdox	25
2	Ethanol	Ethanol	100
3	Dowanol DPM	Dowanol DPM	100
4	Isopar M	Isopar M	100
5	Dipropylene glycol	Dipropylene glycol	100
6	Carbitol	Carbitol	100
7	Benzyl benzoate	Benzyl benzoate	100
8	Ethanol + Fragrance	Ethanol	20
		Benzyl acetate	20
		linalool	20
		Dihydroxymyrcenol	20
		Verdox	20
9	Dowanol DPM + Fragrance	Dowanol DPM	20
		Benzyl acetate	20
		linalool	20
		Dihydroxymyrcenol	20
		Verdox	20
10	Isopar M + Fragrance	Isopar M	20
		Benzyl acetate	20
		linalool	20
		Dihydroxymyrcenol	20
		Verdox	20
11	Dipropylene glycol + Fragrance	Dipropylene glycol	20
		Benzyl acetate	20
		linalool	20
		Dihydroxymyrcenol	20
		Verdox	20
12	Carbitol + Fragrance	Carbitol	20
		Benzyl acetate	20
		linalool	20
		Dihydroxymyrcenol	20
		Verdox	20

FORMULAS

Test Material #	Group	Raw Material	Percentage
13	Benzyl benzoate + Fragrance	Benzyl benzoate	20
		Benzyl acetate	20
		linalool	20
		Dihydroxymyrcenol	20
		Verdox	20