The combined effects of benzalkonium chloride and ultraviolet radiation on the bovine lens

in vitro

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis,

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Abstract

Purpose: To determine the combined effects of benzalkonium chloride (BAK) and ultraviolet radiation (UV) using the primary culture of intact bovine lenses.

Methods: Abattoir-provided lenses were dissected from the eyes of cattle age 2 to 3 years. Lenses received one of three treatments: BAK, UV, or BAK followed by UV exposure. BAK-treated lenses received 10 minutes of exposure to a test solution prepared at 0.01%, 0.005%, or 0.001% concentration with PBS. Irradiated lenses were treated for 1.5h at a measured irradiance of 11.32411 $W \cdot m^{-2}$ (280-400 nm). Lenses receiving the combined treatment underwent the BAK treatment condition followed by UV irradiation. The effects were measured using the alamarBlue metabolic activity assay (n=8), and the ScanTox laser-scanning system to assess optical quality (n=6). PBS exposure served as the control treatment. Assessments of metabolic activity were performed on days 0, 2, and 7 following exposure. Optical quality was assessed on days 0, 2, 7, 14, 16, 18, and 20.

Results: BAK-induced toxicity was concentration-dependent. Treatment with BAK 0.01% and 0.005% resulted in reduced lens metabolic activity on day 2 and day 7, respectively (p<0.05). Optical quality was significantly reduced for 0.01% BAK-treated lenses on day 7 (p<0.05). For UV-treated lenses, reduced metabolic activity was observed on day 2, while optical quality was significantly diminished on day 14 (p<0.05). Combined treatment reduced lens metabolic activity and optical quality (p<0.05). Effects were demonstrated earlier with the increasing concentration of BAK. However, the combined effects of BAK and UV were not significantly different from BAK alone for metabolic activity, or from UV alone for optical quality (p>0.05).

Conclusion: The combined effects of BAK and UV were not significantly different from the independent exposures. Reductions in metabolic activity were detected prior to changes in optical quality.

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List of Abbreviations

AGEs .... advanced glycation end products

ATP .... adenosine triphosphate

BAK .... benzalkonium chloride

BVD .... back vertex distance

DNA .... deoxyribonucleic acid

FBS .... fetal bovine serum

HCEC .... human corneal epithelial cells

HEPES .... N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) hemisodium salt

IL .... interleukin

MTT .... 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazonium bromide

PBS .... phosphate buffered saline

ROS .... reactive oxygen species

SDS .... sodium dodecyl sulfate

SE .... standard error

UV .... ultraviolet

I. Background

1.1 The eye and the lens

The vertebrate eye is a sensory organ specially adapted for the perception of light to facilitate navigation in the physical environment. In humans, the eye is organized into three layers: the outermost fibrous tunic, the middle vascular tunic, and the inner nervous tunic.[1] The continuous outer tunic is comprised of the densely fibrous sclera, the limbus, and the transparent cornea. The vascular tunic includes the iris, ciliary body, and the choroid. The nervous tunic consists of the retina.[1] The eye is additionally structured into three compartments; anterior, posterior, and vitreous. The anterior chamber is formed by the anterior boundary of the cornea, and by the iris and lens at the posterior. The posterior chamber is defined as the space between the back of the iris and surrounding the lens.[1] Aqueous humor circulates through the anterior and posterior compartments as a means of distributing nutrients and removing waste for nearby structures. The vitreous chamber, located behind the lens, and extending to the retina, contains the vitreous humor.[1]

Incident light rays passing into the eye are converted to electrical signals by the photoreceptors which are subsequently interpreted by the central nervous system. The passage of light through the eye begins at the cornea, a transparent structure which focuses light toward the retina.[1] Light travels through the aqueous anterior chamber and its intensity is controlled by the iris, which modifies the size of the pupil opening. Light is further focused by the lens through the vitreous toward the retina.[1] The vertebrate retina is inverted, such that the photoreceptors are located beyond the other sensory retinal layers. Photoreceptors are responsible for the conversion of the light to an electrical signal.[1] The signal is carried

through the visual pathway from the retina to the optic nerve for processing by the lateral geniculate nucleus, as well as the primary visual cortex in the occipital lobe and other association areas.[1]

The ocular lens is a biconvex structure. Its molecular composition is largely protein, both insoluble and soluble.[2] The insoluble proteins are primarily located in the cellular membrane and cytoskeleton. The soluble proteins, namely alpha, beta, and gamma crystallin proteins, are densely concentrated in the lens and consequently contribute to lens properties.[2] The cellular composition of the lens is comprised mainly of elongated fiber cells. Fiber cells are compactly organized into concentric layers, with ends extending toward the anterior and posterior surfaces. The ends of the cells meet at the anatomical sutures of each surface.[2] Fiber cells are further distinguished into the primary fiber cells, which are present from gestation and contained within the central nucleus, and secondary fiber cells, which form the surrounding cortex. Secondary fiber cells differentiate from the equatorial region of the lens and are continually produced as additional layers for life.[2] The anterior epithelial monolayer sheathes the anterior face of the lens. The lens capsule, the outer boundary of the lens and basement membrane, provides attachment for the zonules of Zinn.[2]

Optically, the lens functions with the cornea to focus incident light to the retina.[3] It additionally alters its shape to adjust to the changing focus necessary to produce a clear retinal image, known as accommodation.[3] To maintain its optical performance, the lens must remain transparent and minimize light scatter.[2] A number of conditions contribute to lens transparency. The lens is avascular, and is largely devoid of organelles, which would contribute to light scatter. Only the lens epithelium and superficial cortical fiber cells contain

organelles; consequently these areas are primarily responsible for homeostatic mechanisms, including cellular metabolism.[2] The lens corrects for differences between the refractive index of cell membranes and cytoplasm by having increased densities of protein in the cytoplasm of centrally located cells.[4] The resulting property is a gradient refractive index, decreasing from the center to the periphery.[5] This is made possible by the tight, regular arrangement of soluble intracellular crystallin proteins. Small intercellular spaces further contribute to lens transparency, which are narrower than incident wavelengths.[4] Gap junctions between adjacent fiber cells enable the distribution of metabolites and ions from metabolically active cells to secondary fiber cells.[2]

1.2 Embryological development of the lens

Embryological development of the eye in humans is initiated from the primitive forebrain.[2] After approximately 4 weeks development, the neural tube closes at its rostral end, and two optic pits form bilaterally as bumps. Developmental events for the two optic pits occur in synchrony.[2] Cells forming the optic pit undergo proliferation, producing an increasingly large, hollow, and spherical opening: the optic vesicle. The optic stalk forms; a hollow, single-layer tube of epithelial cells which connect the optic vesicle to the neural tube.[2] Cell proliferation of the optic vesicle continues until it reaches the peripheral surface ectoderm. This step is critical for the induction of the lens from the surface ectoderm.[2] The surface ectoderm overlying the optic vesicle thickens, and is referred to as the lens placode. Next, both tissues invaginate, producing an ectodermal lens vesicle and progression of the optic vesicle to the optic cup.[2] Once the lens vesicle completely pinches off from the surface ectoderm, it is first referred to as the lens. Development of the optic cup progresses

simultaneously; a deep fissure (the choroidal fissure) develops, allowing a blood vessel to arise between the optic stalk toward the lens vesicle.[2] This blood vessel becomes the hyaloid artery, supporting early lens development and eventually contributing to the formation of the central retinal artery. Next, the choroidal fissure of the optic cup closes.[2] The cells of the posterior lens elongate, forming primary lens fiber cells and filling the hollow lumen of the lens. These cells eventually lose their organelles to maintain lens transparency.[2] At this point, lens cells begin the process of differentiation through the synthesis of crystallin proteins. The anterior lens cells undergo differentiation as part of continuous lifelong growth of the lens.[2] Some will additionally contribute to the hollow center of the lens, and the rest form secondary lens fiber cells. Throughout life the differentiation of the anterior epithelial cells into fiber cells adds approximately 20 µm in diameter to the lens per year, or about five new shells.[2]

1.3 Age-related changes in the lens

As previously mentioned, the lens grows continuously throughout life. Several changes are observed in the lens with increasing age, at both the molecular level and in overall properties such as size, shape, and mass.[2] Presbyopia, the loss of accommodation, is theorized to be age-related due to the accumulation of lens growth.[2] The ongoing addition of cell layers results in increased lens diameter, thickness, and mass. These changes consequently render the lens less capable of inducing curvature.[2] Similarly, since the distance from the anterior pole to the point of attachment of the zonules does not change over time, as a consequence of lens gradual diameter increase, the points of attachment are said to shift anteriorly with respect to the equator.[2] The lens capsule undergoes age-related changes in thickness

distribution, increasing in thickness anteriorly with age.[2] The aging lens is observed to have a steepening radius of curvature. Optically, a shift toward myopia would be expected.[2, 6] Paradoxically, this is not the case; it is theorized that changes in the refractive index occur concurrently to account for the change in power resulting from the surface change.[2, 6, 7] Over time, oxidative stress or other sources of damage may produce modifications in lens crystallins.[8] This results in crystallin cross-linking and protein insolubility in the lens. The nucleus is particularly subject to these changes.[8] Fluorescent chromophores additionally form in the lens as a result of accumulated UV exposure, and the production of advanced glycation end products (AGEs).[8] These changes cumulatively produce reduced transparency and coloration of the lens, most notably after age 50.[8] Changes of this nature may progress to the level of cataract.

1.4 Cataract

Cataract is the primary cause of blindness in the world, affecting over 20 million people.[9, 10] The condition is defined by the opacification of the ocular lens, resulting in blurred vision or loss of vision.[2] Treatment of cataract involves the surgical replacement of the ocular lens.[2] Cataracts are typically classified based on the location of the opacity in the lens; they may be cortical, nuclear, and posterior subcapsular.[2] Cortical cataracts develop in the cortex, and may be additionally categorized as a central or equatorial cataract.[2] Nuclear cataracts occur within the central core of the lens.[2] Posterior subcapsular cataracts develop in the superficial cortex at the posterior pole.[2] Cataracts additionally vary in size, shape, density, and colour, introducing uncertainty as to which characteristics are significant to the etiology.[2]

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The etiology of cataract is complicated as a result of the large number of common risk factors associated with the condition, of both genetic and environmental origin. There are six general categories of risk factors for cataract: social and personal factors, diabetes, diarrhea, antioxidants, drugs, and UV radiation.[2] Examples of social and personal risk factors include hypertension, gender, smoking, and alcohol consumption. It is likely that interaction effects between risk factors contribute to the cause of cataract.[2] Consequently, cataract is regarded as a multifactorial condition.[2] The most common cause of cataract is age-related change.[2] As previously mentioned, many age-related changes in the lens are attributable to the lifetime accumulation of UV exposure. It follows that UV exposure likely accelerates the mechanisms involved in age-related cataract.

1.5 Ultraviolet radiation

The solar light spectrum includes UV radiation (100-400 nm), the visible light spectrum (400-700 nm), and infrared radiation (700-10 000 nm). The atmospheric ozone layer effectively filters UVC radiation (100-280 nm), while some UVB (280-315 nm), and UVA radiation (315-400 nm) passes through.[11, 12] Recently, the amount of UVB penetration to the Earth's surface due to stratospheric ozone layer depletion has been of concern.[13]

Along with the cornea and retina, the lens is one of the most important ocular structures vulnerable to incident optical radiation damage.[14] A study of UV-irradiated lens, corneal, and retinal cell lines suggests that lens epithelial cells are particularly vulnerable to UV.[14] As previously mentioned, ultraviolet radiation exposure is an established risk factor for cataract formation. The lens is reported to absorb much of the radiation from 300 to 400 nm.[15, 16] UV-induced damage to the lens can be produced from either direct or indirect

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mechanism.[17] Direct cataract induction involves the absorption of UV radiation by cellular chromophores, such as nucleic acids and proteins.[13, 17] For example, alteration and aggregation of lens crystallins has been noted as a direct impact of UVB irradiation contributing to lens opacities.[18] In addition, UV-induced deoxyribonucleic acid (DNA) damage has been confirmed through chromatin condensation and fragmentation, as well as the ill-timing of DNA synthesis and repair mechanisms.[18, 19] The indirect mechanism involves the generation of reactive oxygen species (ROS) and free radicals.[13, 17] It is reported that UVB damages DNA through the direct pathway, while UVA damages DNA through the indirect pathway.[14]

1.6 Benzalkonium chloride

Ophthalmic formulations, including solutions and ointments, remain the preferred method for treating ocular diseases. A preservative is included in order to reduce the risk of microbial contamination of the solution and resulting introduction of a potentially sight-threatening infection.[20, 21] Benzalkonium chloride is the most commonly employed preservative, and is typically found in solution within a concentration range of 0.004% to 0.025%.[20] Its chemical composition is usually a combination of 12 and 14 carbon tail homologs with a cationic head group.[20] BAK is reported to possess favourable allergic tolerance, solution stability, and antimicrobial efficacy as compared to other preservatives.[11, 20-23] Its partially hydrophobic nature allows BAK to disrupt the cell walls of pathogenic microorganisms and induce cell lysis. While BAK is a useful preservative, the compound has been linked to ocular irritation and cytotoxicity.[23]

The toxicity of BAK to the ocular surface has been well reported. *In vivo*, long-term exposure to BAK has been shown to disrupt the tear film, cornea, and conjunctiva.[20, 21, 24] The toxic effects of BAK have been notably observed from long-term use of preserved formulations.[21-23, 25] The discovery that BAK can penetrate to deep ocular structures is relatively recent.[20] BAK toxicity to the lens has been examined in epidemiological and *in vitro* research, though the specific molecular mechanism of action is yet to be defined. Several clinical trials have followed patients treated with preserved anti-glaucoma medication and a general increased incidence of cataract occurred against control.[26] The toxicity of BAK is known to be both concentration and dose-dependent.[11, 22] The toxicity of BAK to the eye is additionally enhanced with the administration of multiple doses, due to sequestering in tissues.[20]

1.7 Mechanisms of combined toxicity

Exposure to multiple sources of toxicity occurs regularly as part of daily living. Two agents which produce independent effects in the body may additionally interact with one another. When two exposures do interact, the nature of the reaction may be additive, synergistic, potentiating, or antagonistic.[16] An additive interaction occurs when the outcome of combination is summative of independent toxicity.[16] Synergistic reactions are said to occur when combined toxicities produce consequences which are significantly greater than their sum.[16] Potentiation occurs when one exposure whose effects are expected to be inconsequential are greatly increased with the introduction of another substance.[16] Antagonism is when one substance impedes the action of another.[16]

1.8 Comparison of bovine and human lenses

The bovine lens shares several anatomical and physiological characteristics with the human lens. Both are asymmetric, elliptical structures that attach via zonules to the muscular ciliary body.[27] The incremental change in the gradient refractive index from center to periphery is additionally similar for these species.[27] Other work has shown that bovine and human total and bound water content in the lens is similar.[28] As in other mammalian vertebrates, the bovine lens develops from the embryological surface ectoderm.[2, 22] One study found that the embryonic and early fetal fiber cells of the nucleus were comparable in size and arrangement between these species.[29] At birth, the sutures of the human lens are Y-shaped, as is seen in the bovine lens.[30] However, as the human lens develops, the sutures become further branched, forming a stellar appearance.[30] The sutures in bovine and human lenses have implications for accommodation.[31] Human lenses are known to perform accommodation. In bovine lenses, fiber arrangement at the sutures does not permit overlap, and consequently bovine lenses are generally not thought to undergo accommodation.[31] One study performed *in vitro* determined with manipulation of the bovine lens that it could theoretically produce accommodation of approximately 2 diopters.[32] One clear difference between bovine and human eyes is that the bovine eye is significantly larger; this is additionally true for lenses. The equatorial diameter of bovine lenses is approximately 17 mm,[33] as compared to 10 mm for the aged human lens.[2] Bovine and human lenses are also known to exhibit differences in spherical aberration.[34] Bovine lenses exhibit no spherical aberration, while in humans positive and negative spherical aberration is observed.[34]

II. Introduction

Cataract is the primary cause of blindness in the world.[10] The treatment for the condition is surgical replacement of the ocular lens, and represents a significant burden to health care systems. This has prompted considerable effort in research to determine the underlying causes of the condition. The onset of cataract is abstract because of the large number of risk factors and lifelong accumulation of lens damage from sub-threshold exposures.[16] Exposure to preservatives in ophthalmic formulations and UV radiation are common experiences, and each is independently associated with cataract.[15, 35]

The Draize test is the most common method for assessing ocular irritancy and toxicity.[36] In this method, the animal, typically a rabbit, is exposed to the test substance and the toxicity on the skin or the eye is observed over time.[36] However, there are important criticisms of this method to consider. For example, it has been shown to produce variable results, and it demonstrates low sensitivity.[37, 38] Moreover, the associated animal discomfort and pain raises ethical concerns. *In vitro* assessment of the lens has been suggested as an alternative method to test for ocular toxicity.[39] The bovine lens has been proposed as a suitable *in vitro* model to assess ocular irritancy due to the similarities in embryology, morphology, and physiological properties as the human cornea and lens.[22, 27]

Our group has previously developed an *in vitro* method for assessing the effects of BAK toxicity and UVB radiation on the bovine lens.[22] The results indicate that, at sufficient exposure, BAK and UVB can independently induce lens damage and reduce optical quality.[22] We have also looked at the combined effects of BAK and UV exposure on human corneal

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epithelial cells (HCEC), which showed that additive or synergistic effects may occur as a result of a combined mechanism.[11] To our knowledge, no studies have reported the combined effects of BAK and UV on the bovine lens. The results of this study may provide valuable insights on the underlying mechanisms leading to cataract formation and prevention, with significant consequences for public health and health systems.
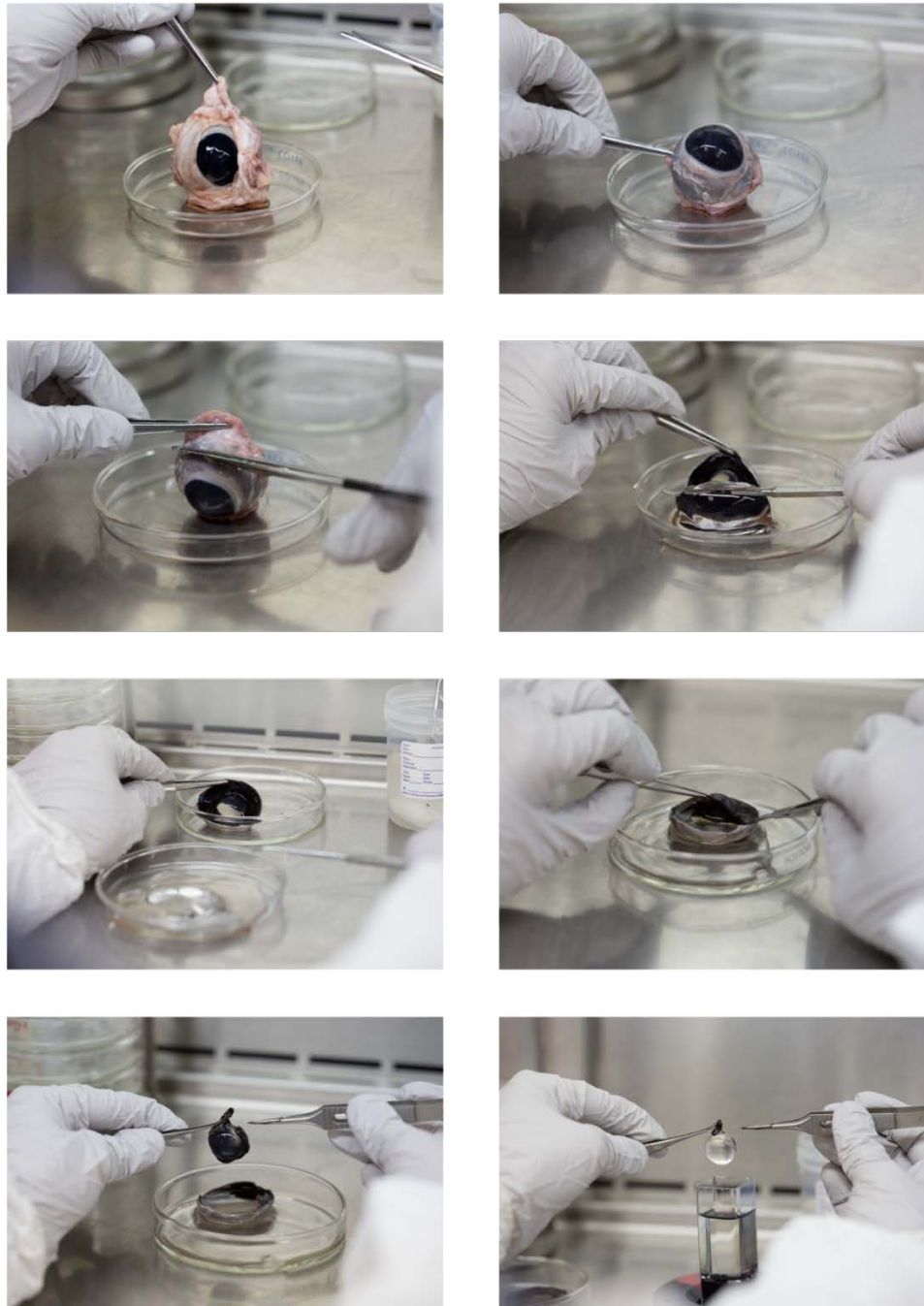
III. Methodology

3.1 Ocular dissection and tissue culture

Bovine eyes, from cows 2-3 years of age, were obtained through a local abattoir (Cargill Meat Solutions, Guelph, ON). The eyes were prepared for dissection by immersion in a diluted iodine solution. The dissection of the bovine lens (Figure III-1), performed under sterile conditions in a flow hood, has been previously reported.[22] The dissections were completed approximately 2-5 hours after obtaining the eyes. Eyes with corneal damage or scleral puncture as a result of the extraction process were not dissected. First, excess muscle, fat, and connective tissue were removed. The posterior half of the eye was then removed, followed by the excision of the vitreous humour. The anterior half of the eye was transferred to another petri dish and dissected with a separate set of instruments to reduce the risk of microbial or fungal contamination of the lens. Subsequently, the iris and ciliary attachments were separated from the anterior portion of the globe along with the lens. The zonule connections to the lens were then carefully cut, with the final cuts taking place over the culture chamber to avoid possible damage to the lens. Lenses were rinsed with culture medium to prevent pigmented tissue fragments from adhering to the lens and to reduce exposure of the lens to oxygen. Dissection instruments were briefly soaked in 70% ethanol between new dissections. A minimum of 18 dissected lenses were cultured to ensure that 12 samples would meet the standards for dissection quality and to prevent reduction of sample size due to contamination or culture chamber leaks.

The dissected crystalline lenses were cultured in custom two-compartment culture chambers, with 21 mL of culture medium. The medium was prepared from 9.4g/L M-199

(Sigma-Aldrich, St. Louis, MO), 3% fetal bovine serum (FBS) (Life Technologies, Burlington, ON), 1% penicillin/streptomycin (Life Technologies), 0.1g/L L-glutamine (Sigma-Aldrich), 7mL/L 1M NaOH (Sigma-Aldrich), 5.96g/L HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) hemisodium salt) (Sigma-Aldrich), and 2.2g/L sodium bicarbonate (Sigma-Aldrich). The medium was sterile-filtered through a 0.2 µm vacuum filtration system (VWR, Mississauga, ON) prior to the addition of FBS. The lenses were incubated at 37°C, in an atmosphere of 5% CO_2 and 95% air. Lenses were cultured for 48 hours post-dissection and assessed for damage prior to experimental use. Lenses damaged due to dissection or contamination were excluded from experimental use. The culture medium was aspirated and replenished after lens treatment, and every 48 hours during experimentation with the ScanTox assay. During the experiments with alamarBlue, culture medium was replaced after the assay was performed, and 48 hours after the previous medium replacement. The culture medium was stored at 2-4°C in a refrigerator between uses and was warmed to 37°C prior to lens culture. Lenses were inspected at the time of medium replacement for contamination and integrity.

Figure III-1 The dissection of the bovine eye, performed under sterile conditions.

Depicted from left to right and top to bottom.

3.2 Exposure methodology

The study evaluated and compared the effects of three treatments on the bovine lens: (1) BAK (2) UV irradiation (3) BAK followed by UV irradiation. The following procedure has been adopted from Youn et al.[22]

3.2.1 Exposure to BAK

BAK for this study was diluted from a purchased 50% solution (Sigma-Aldrich) with phosphate buffered saline (PBS) (Lonzo, Walkersville, MD). The preparation of BAK solution was performed while protecting BAK from direct light. BAK solutions were prepared at concentrations of 0.001%, 0.005%, and 0.01% with PBS. These concentrations were chosen based on the current clinical use of BAK in ophthalmic formulations. In addition, exposure to BAK 0.01% solution for 10 min was determined in preliminary experiments to produce approximately 20% reduction in metabolic activity compared to control after 7 days. Lenses were oriented in their chambers with the posterior surface resting level on the circular beveled washer. Exposure to BAK was performed under sterile conditions in a flow hood at room temperature. The culture medium was aspirated from the chamber cells, and replaced with 6.5 mL of a BAK test solution. The lenses were exposed to the BAK test solution for 10 min. Control lenses were exposed to PBS for 10 min. After the treatment period, all lenses were rinsed three times with FBS-free culture medium. The chambers were then replenished with 21 mL of culture medium. The lenses were then evaluated using the alamarBlue assay (t = 0d, 2d, 7d), or the laser scanner (t = 0d, 2d, 7d, 14d, 16d, 18d, 20d). After treatment with BAK, lenses were replenished with culture

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medium, and incubated for 2.5 hours to acclimatize the lens to a change in osmolarity and temperature during the experimental procedure, prior to the assessment of optical quality. Experiments were performed in triplicate, with n = 3 per treatment condition.

3.2.2 Exposure to UV

UV radiation (280-400 nm) exposure was accomplished using a unique UV irradiation chamber.[11] The UV source used for this study was two UVA and two UVB fluorescence tubes (Microlites Scientific, Toronto, ON). The UV source was located approximately 30 cm above the sample stage. The UV source was switched on for approximately 15 min prior to experimental use. Lenses were irradiated for 1 h 30 min. The irradiance was measured at 11.32 $W \cdot m^{-2}$ using a USB 2000+ fiber optic spectrometer (Ocean Optics, Inc., Dunedin, FL). The exposure conditions were determined based on pilot experiments. These conditions were shown to produce an approximate 20% reduction in metabolic activity as compared to control after 7 days. The lenses were irradiated within their culture chambers, oriented such that the anterior surface was level and facing toward the UV source. Culture medium was aspirated from the culture chamber and replaced with approximately 6.5 mL of FBS-free culture medium. A thin layer of medium (approximately 1 mm) was left above the anterior surface of the lens left to prevent air exposure and to reduce absorption of UV radiation by the medium. The lens chambers were covered with quartz cover slips to allow the passage of UV, and to reduce both evaporation of the medium and the potential for contamination of the sample. At the half-time point of exposure, the samples were reordered in the irradiation chamber to ensure similar exposure to the spectral irradiance for all lenses.

The temperature of the irradiation chamber gradually increased from starting room temperature, approximately from 24°C to 38°C. The atmosphere of the irradiation chamber was ambient air. Control lenses were placed in a separate incubator with ambient air and whose temperature was gradually increased from 24°C to 38°C. After the irradiation interval, the lenses were evaluated using the alamarBlue assay (t = 0d, 2d, 7d) and the laser scanner (t = 0d, 2d, 7d, 14d, 16d, 18d, 20d). Following irradiation with UV, lenses were replenished with culture medium, and incubated for 2.5 hours to acclimatize the lens to a change in medium osmolarity and temperature during the experimental procedure, prior to the assessment of optical quality. Experiments were performed with n = 6 in each experimental group.

3.2.3 Combined exposure to BAK and UV

The crystalline lenses were exposed first to a BAK solution, followed by UV irradiation using the same procedures detailed above. Control lenses were exposed to the previously outlined control conditions for BAK and UV exposure, consecutively. The lenses were evaluated using the alamarBlue assay (t = 0d, 2d, 7d) and the laser scanner (t = 0d, 2d, 7d, 14d, 16d, 18d, 20d). Following the treatment protocol, lenses were replenished with culture medium, and incubated for 2.5 hours to acclimatize the lens to a change in osmolarity and temperature during the procedure, prior to the assessment of optical quality. Lenses were assessed for optical quality prior to medium replacement. Experiments were performed in triplicate, with n = 3 per treatment condition.

3.3 Analysis of cellular activity

AlamarBlue (resazurin) is a nontoxic colorimetric and fluorometric assay of cellular activity. Viable cells can metabolize the indicator through a redox mechanism to produce an absorbance or fluorescence change, proportional to metabolic activity. A higher fluorescence reading correlates to higher metabolic activity. Metabolism of the indicator produces a colour change from blue to pink (resazurin to resorufin) (Figure III-2).

For evaluation of metabolic activity, lenses were placed into a sterile clear-bottom 12-well plate containing 3.8 mL of alamarBlue solution (Life Technologies), prepared at 8% with clear FBS-free culture medium. The assay solution was freshly prepared to prevent precipitation of the indicator, and protected from direct light, as alamarBlue is light-sensitive. The lenses were incubated in the solution for 5h, after which the end-point fluorescence was measured using the SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA).[22] The alamarBlue solution preparation and duration of incubation time were determined from previous experiments using porcine and bovine lenses.[22, 40] Fluorescence was measured at excitation and emission wavelengths at 560 nm and 590 nm, respectively. Following incubation, the lenses were replaced into their respective culture chambers and rinsed with FBS-free medium warmed to 37°C. Next, lenses were replenished with fresh medium and cultured until the next time point measurement. For the fluorescence reading, 100 µL was sampled from the 12-well plate for each lens and transferred into a sterile clear-bottom 96-well plate for measurement. Fluorescence was measured at t = 0d, 2d, 7d (d = days). The t = 0d time point was defined as the time immediately following exposure to the test condition.

All procedures were completed under sterile conditions. A total of 88 lenses were included in

the metabolic activity study, with n = 8 per experimental condition.

Figure III-2 End-point appearance of the alamarBlue assay for control and BAK-treated lenses. AlamarBlue (blue) is reduced to fluorescent alamarBlue (red) proportional to cellular metabolic activity.

3.4 Analysis of optical quality

The assessment of lens optical quality used a custom designed laser scanning system (ScanTox, University of Waterloo, Waterloo, ON).[22] During the assessment, the lenses remained within their two-compartment culture chambers. The lenses were oriented with the anterior surface resting on the plastic beveled washer (diameter = 14 mm). The laser system projects light onto a mirror, mounted at a 45 degree angle, which then reflects the light through the lens (Figure III-3). The mirror position is altered using a computer-aided mechanism, such that the system captures the light passing through the lens at multiple positions. A digital video camera, mounted within the scanner, captures the position and slope of the refracted beam. Software analysis of this data produces measurements of back vertex distance (BVD) variation for each lens.[22] Lenses were scanned at 20 eccentricities at increments of 0.5 mm, with a range of 10 mm along one axis. Each lens was scanned twice along perpendicular axes; single measurements of BVD variability generated by ScanTox II software were based on 40 quantitative measurements. The ScanTox experiments consisted of 60 lenses, or 6 lenses per experimental group, cultured for 22 days. Individual beams passing through the central zone were excluded from the BVD measurement calculations to exclude beams passing through lens sutures and provide the most conservative measurement of BVD variability. The optical quality was assessed for t = 0d, 2d, 7d, 14d, 16d, 18d, 20d. The t = 0d time point was defined as the time 2.5 hours after the exposure period. This time was chosen to allow lenses to stabilize following changes in medium osmolarity and temperature as a result of the experimental procedure.
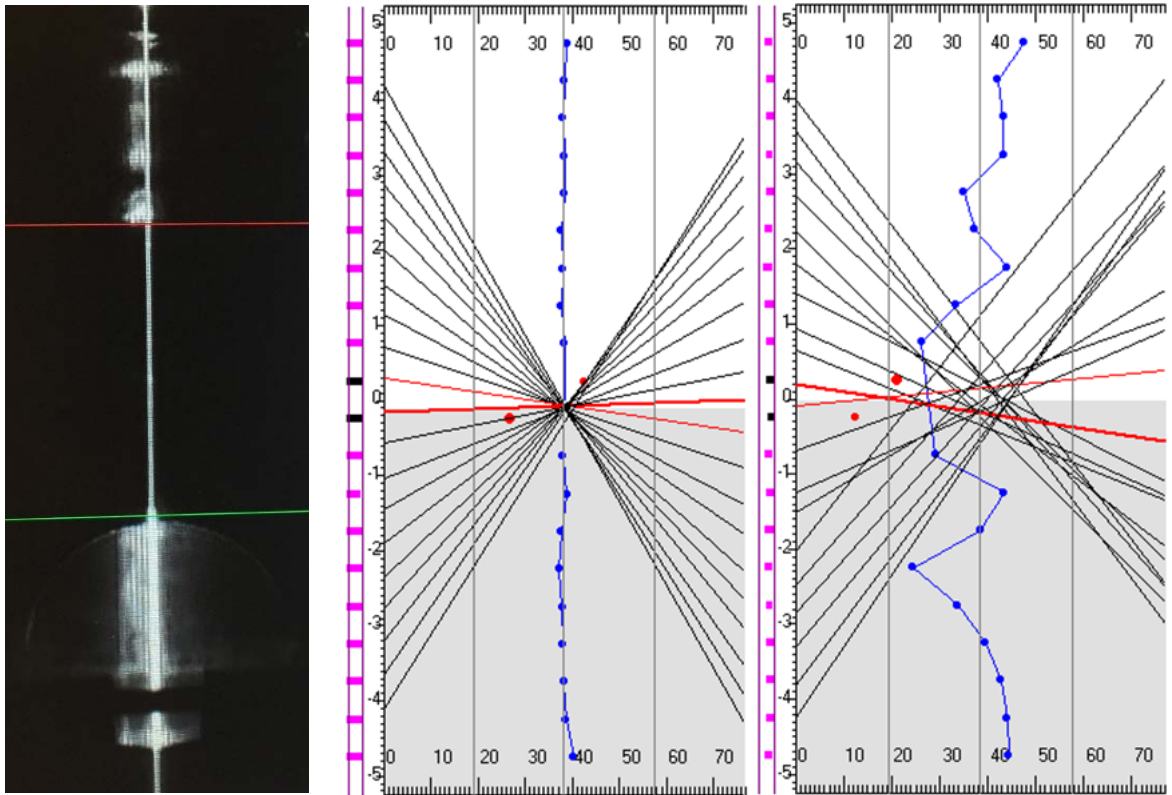
Figure III-3 Measurement of optical quality. The ScanTox reflects incident beams of light through the lens (left) at different eccentricities and calculates the BVD mean and error based on the distances behind the lens where the refracted rays intersect with a central axis. Optimal optical quality is produced with minimal BVD variability (center), and optical quality is increasingly poor as BVD variability is increased (right).

3.5 Statistical analysis

Experimental data was analyzed using two-way repeated measures analysis of variance using the GraphPad Prism 7® statistical analysis software program. Analysis was performed between experimental groups to determine the effect of different treatments against control conditions, as well as within experimental groups to determine changes in lens performance over time for a particular treatment. The Bonferroni multiple comparisons *post-hoc* test was performed for both alamarBlue and ScanTox results. Statistical significance was determined based on a p value less than or equal to 0.05.

IV. Results

4.1 Metabolic activity

The metabolic activity of the cultured bovine lenses following treatment was measured using the alamarBlue assay. The assay results produced measurements of fluorescence for each lens sample. The fluorescence measurements were proportional to cellular metabolic activity within the lens. One of the main challenges of this project was to identify doses of UV and BAK that caused intermediate toxicity. Using previous studies[11, 22] and pilot studies, it was determined that the best preparations of BAK solution were 0.01%, 0.005%, and 0.001% and an exposure time of 10 minutes, and a UV exposure time of 90 minutes with a measured irradiance of 11.32411 $W \cdot m^{-2}$. Exposure times that are too extensive would mask the toxicity of the second agent and toxicity of little consequence may not provide enough toxicity to measure individual toxicities and then the additive effects. Therefore, the results from this study look at toxicity of intermediate effects of the both UV and BAK tested.

As shown in Figure IV-1, treatment of the lenses with BAK, UV, or combined BAK and UV resulted in decreased metabolic activity compared to control on day 7. Table IV-1 additionally shows the decreased activity from the first measurement to the end point measurement for each lens. The control (no BAK) lenses that were tested with the BAK samples did not experience a significant change in metabolic activity for the experimental duration ($p > 0.9999$). BAK toxicity to the lens demonstrated a concentration-dependent response. Exposure to a BAK 0.01% solution resulted in significantly lower metabolic activity on day 7 compared to exposure to BAK 0.005% and BAK 0.001% ($p = 0.0006$; $p < 0.0001$). Lenses treated with a 0.01% BAK solution had significantly lower cellular

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metabolic activity compared to control ($p < 0.0001$). The damage caused by the BAK 0.01% resulted in metabolic activity 71% ± 9% of the control. The metabolic activity of lenses treated with a BAK 0.005% was statistically similar to control on day 0 and day 2, and was significantly lower than control on day 7 ($p = 0.0115$). The damage produced by BAK 0.005% was 88% ± 6% of control. Lenses treated with a 0.001% BAK solution had metabolic activity that was not significantly different from the control at all time points, with metabolic activity 91%±6.5% of control ($p > 0.05$). Additionally, the observable toxicity of BAK was time-dependent, such that reduction in cellular metabolic activity compared to control was greater at each subsequent time point. For example, lenses treated with 0.005% BAK solution, the metabolic activity of the lenses progressed from 93%±5.4% of control on day 0, to 91%±7.9% on day 2, to 88%±6% on day 7. Reductions in lens metabolic activity persisted through the study period.

Table IV-1 shows the mean metabolic activity for UV-irradiated lenses (n = 12) as a percent of the control (n = 12). The lenses used as a control (no UV) for the UV experiment did not demonstrate significant changes in metabolic activity for the experimental duration ($p > 0.9999$). The UV-treated lenses demonstrated significant reductions in mean metabolic activity at day 2 ($p < 0.0002$), and day 7 ($p < 0.0001$) as compared to control. The metabolic activity was reduced to 80%±9% on day 7. The decline in cellular activity also became greater with each subsequent time point following exposure.

Table IV-1 shows the mean metabolic activity of the combined treatment groups as a percent of the control. As compared to control, all combined treatment groups showed significantly decreased metabolic activity at the day 2 measurement. The adjusted p values

on day 2 for the combined treatment groups BAK 0.01% + UV, BAK 0.005% + UV, and BAK 0.001% + UV at day 2 were $p < 0.001$, $p = 0.0012$, and $p = 0.0276$, respectively. The mean metabolic activity for these groups on day 2 were 72%±12%, 82%±9%, and 87%±11% of control, respectively. At day 7, BAK 0.01% + UV, and BAK 0.001% + UV were significantly different from control, with metabolic activity 71%±16% and 85%±9% of control, respectively. The BAK 0.005% + UV treatment group at day 7 was statistically similar to the control ($p = 0.0522$); the metabolic activity was 88%±12% of the control.

A two-way analysis of variance was performed to compare independent BAK or UV exposure against the combined treatment group (Table IV-2 and Table IV-3, respectively). Data analysis was performed with the raw data and additionally normalized to a control. The normalized data are presented here. The analyses produced similar results; no significant difference was determined between the combined treatment and both individual exposures for any treatment during the experiment ($p > 0.05$).

There was no statistical difference observed between single exposure BAK-treated lenses and combined treatment lenses at any concentration for any time point ($p > 0.05$) (Figures IV-2, IV-3, IV-4). A significant difference in metabolic activity was observed between the UV-treated lenses and the BAK 0.01% plus UV-treated lenses at day 2 ($p = 0.0014$) (Figure IV-5).

Table IV-1 Mean metabolic activity as a percent of the control for all treatment groups.
All data are reported as mean ± standard deviation. Each experimental condition had an independent control. The exposure duration for BAK-treated lenses was 10 min. UV-treated lenses were irradiated for 1.5h. Statistical significance was determined based on a p value less than 0.05. Statistical difference from control is indicated by *, and † indicates statistical difference from the day 0 measurement.

Alamar blue / Normalized	Time		
Experimental Group	Day 0 (post-treatment)	Day 2	Day 7
Control (no BAK) (n = 8)	100.0 ± 6.11	100.0 ± 8.12	100.0 ± 8.39
BAK 0.01% (n = 8)	95.90 ± 8.41	78.50 ± 8.34*†	71.74 ± 9.02*†
BAK 0.005% (n = 8)	93.0 ± 5.40	91.21 ± 7.87*	88.01 ± 5.99*
BAK 0.001% (n = 8)	92.73 ± 10.30	94.59 ± 10.39	91.96 ± 6.59
Control (no UV) (n = 12)	100.0 ± 5.39	100.0 ± 5.30	100.0 ± 5.88
UV (n = 12)	98.36 ± 5.91	88.35 ± 7.91*†	80.15 ± 9.06*†
Control (no BAK or UV) (n = 8)	100.0 ± 4.39	100.0 ± 7.21	100.0 ± 10.35
BAK 0.01% + UV (n = 8)	93.17 ± 5.77	72.77 ± 11.75*†	70.71 ± 16.41*†
BAK 0.005% + UV (n = 8)	93.12 ± 5.70	82.21 ± 8.76*†	88.31 ± 12.41
BAK 0.001% + UV (n = 8)	96.28 ± 5.80	87.15 ± 10.67*†	84.86 ± 9.18*†

Table IV-2 Comparison of BAK-treated lenses and BAK plus UV-treated lenses. All data are reported as mean metabolic activity as a percent of the respective control ± standard deviation.

Alamar blue / Normalized	**Time**		
Experimental Group	Day 0 (post-treatment)	Day 2	Day 7
BAK 0.01% (n = 8)	95.90 ± 8.41	78.50 ± 8.34	71.74 ± 9.02
BAK 0.01% + UV (n = 8)	93.17 ± 5.77	72.77 ± 11.75	70.71 ± 16.41
BAK 0.005% (n = 8)	93.0 ± 5.40	91.21 ± 7.87	88.01 ± 5.99
BAK 0.005% + UV (n = 8)	93.12 ± 5.70	82.21 ± 8.76	88.31 ± 12.41
BAK 0.001% (n = 8)	92.73 ± 10.30	94.59 ± 10.39	91.96 ± 6.59
BAK 0.001% + UV (n = 8)	96.28 ± 5.80	87.15 ± 10.67	84.86 ± 9.18

Table IV-3 Comparison of UV-treated lenses and BAK plus UV-treated lenses. All data are reported as mean metabolic activity as a percent of the respective control ± standard deviation.

| Alamar blue / Normalized	**Time**		
Experimental Group	Day 0 (post-treatment)	Day 2	Day 7
UV (n = 12)	98.36 ± 5.91	88.35 ± 7.91	80.15 ± 9.06
BAK 0.01% + UV (n = 8)	93.17 ± 5.77	72.77 ± 11.75*	70.71 ± 16.41
BAK 0.005% + UV (n = 8)	93.12 ± 5.70	82.21 ± 8.76	88.31 ± 12.41
BAK 0.001% + UV (n = 8)	96.28 ± 5.80	87.15 ± 10.67	84.86 ± 9.18
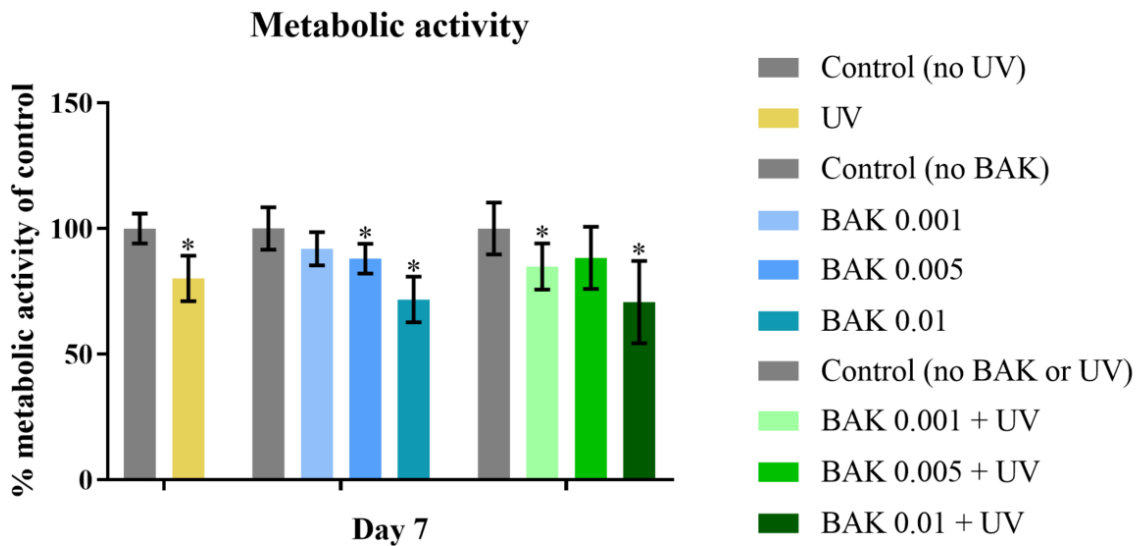
Figure IV-1 Mean metabolic activity as a percent of the control for all treatment groups. All data are reported as mean ± standard deviation. Each experimental condition had an independent control. Statistical significance was determined based on a p value less than 0.05.
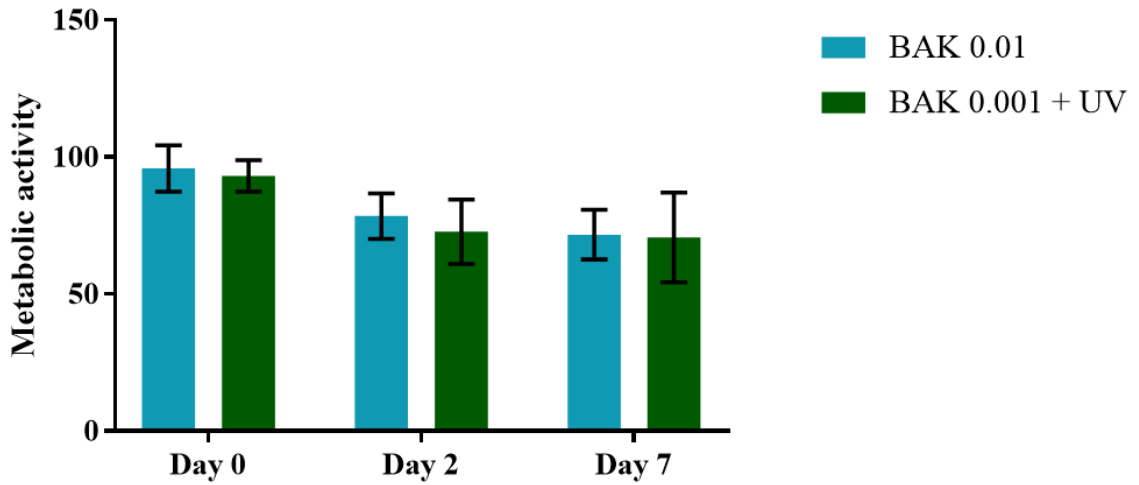
Figure IV-2 Mean metabolic activity for independent BAK 0.01% exposure and combined treatment with BAK 0.01% plus UV.
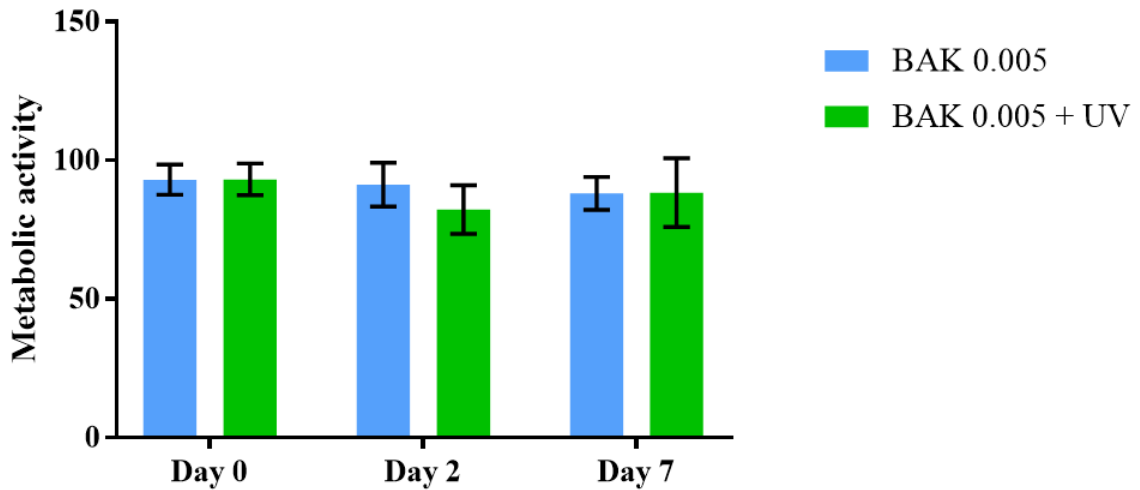
Figure IV-3 Mean metabolic activity for independent BAK 0.005% exposure and combined treatment with BAK 0.005% plus UV.
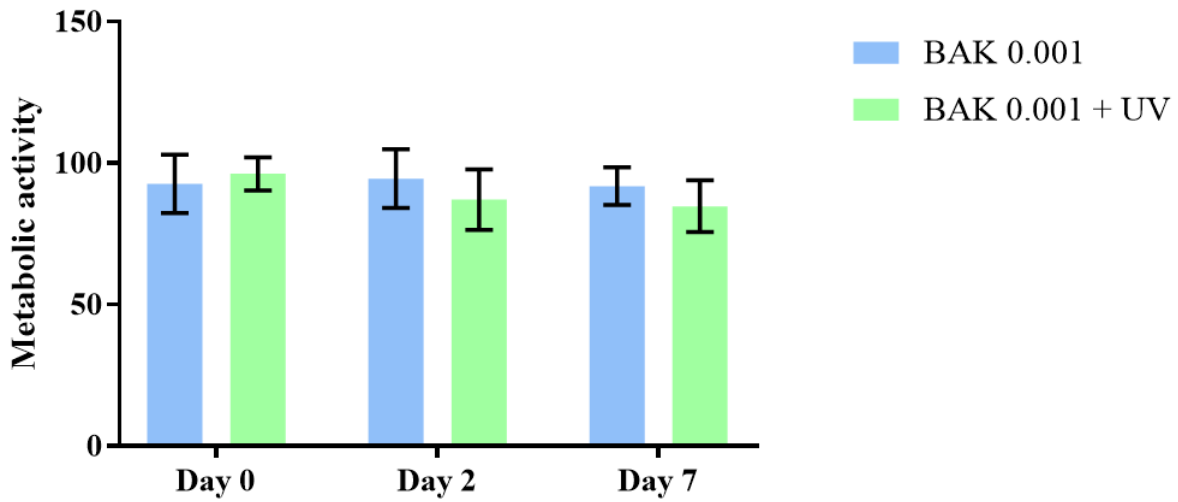
Figure IV-4 Mean metabolic activity for independent BAK 0.001% solution and combined treatment with BAK 0.001% plus UV.

Figure IV-5 Mean metabolic activity for independent UV exposure and combined treatment with BAK plus UV.

4.2 Optical performance

The concept of applying intermediate toxicity was also used for a second endpoint evaluated using the ScanTox method. The doses of BAK and UV administered in the experiments for optical quality were adopted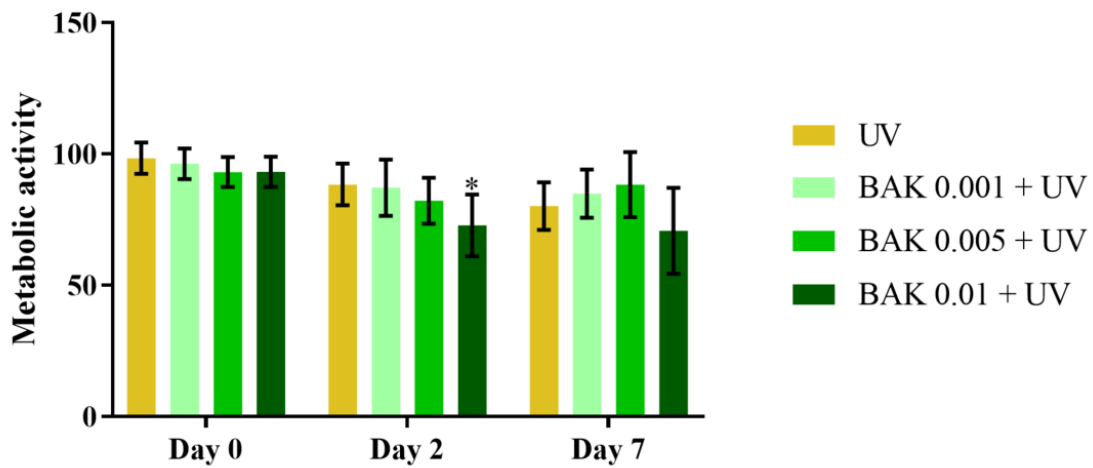 from previous work by our group,[11, 22] and are consistent with those evaluated in the metabolic activity assay. The optical performance of the cultured bovine lenses was measured using the ScanTox laser scanning system. The ScanTox produces measurements of BVD standard error (SE) to indicate the ability of the lens to focus light to a single point behind its posterior surface along a central axis. An increase in BVD error indicates reduced optical quality, or the reduced functional ability of the lens to focus light. Treatment with BAK, UV, or BAK plus UV generally produced lens damage, resulting in increased BVD error (Figure IV-6).

The toxicity of BAK was observed in the highest experimental concentration of BAK (Figure IV-8). Lenses exposed to BAK 0.001% and BAK 0.005% demonstrated BVD variability statistically similar to the control group (p > 0.9999). A significant difference was apparent in lenses exposed to BAK 0.01% at the day 7 time point (p = 0.0225). The damage produced by BAK 0.01% was associated with time such that at each subsequent time point the BVD variability increased and optical quality was increasingly poor. The BVD variability of lenses between groups and over time is shown in Table IV-4.

The control lenses (no UV) in the UV experiment did not show a significant change in optical quality over the duration of the experiment (p > 0.9999). The effect of the UV treatment on optical quality was significantly different from control data at the day 14 time point (p < 0.0001). Within this group, the decline in optical quality was also apparent on day

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14, as compared to day 0 ($p < 0.0001$). Maximum damage due to UV treatment was observed at the day 16 time point (BVD SE = 2.638, $p < 0.0001$) (Figure IV-5). The lenses showed some recovery of BVD variability after day 16. The BVD variability for the UV experiment lenses is reported in Table IV-4.

All lenses receiving combined treatment of BAK and UV demonstrated a significant increase in BVD error as compared to controls ($p < 0.05$) (Figure IV-9). There was no significant change in the optical quality in the control lenses during the experiment ($p > 0.9999$). The damage produced by combined treatment followed a dose-response, such that lenses exposed to BAK 0.01% plus UV, showed significantly reduced optical quality earlier than BAK 0.005% plus UV and BAK 0.001% plus UV. Additionally, the BAK 0.001% plus UV-treated lenses showed some recovery at the final time point; BVD variability was not significantly greater than the control ($p = 0.0780$). Lenses treated with BAK 0.01% plus UV showed significant reductions in optical quality at day 7 against control ($p = 0.0379$), which persisted to the final time point. Lenses treated with BAK 0.005% plus UV and BAK 0.001% plus UV showed significant changes in BVD variability at day 14 ($p < 0.0001$, $p = 0.0001$).

No statistical difference in optical quality was observed between UV-treated lenses and the combined treatment groups at any time point ($p > 0.05$); shown in Table IV-5. Similarly, as shown in Table IV-6, lenses treated with BAK 0.01% were statistically comparable to the combined group BAK 0.01% plus UV across all time points ($p > 0.05$). As compared to lenses treated with BAK 0.005%, the effect of a combined treatment BAK 0.005% plus UV produced significantly greater BVD variability from day 14 ($p < 0.05$). This was also true between the treatments of BAK 0.001% and BAK 0.001% plus UV ($p < 0.05$).

A significant difference between treatment groups was observed only between BAK alone and UV alone, at day 14 between BAK 0.001 and UV, and at day 16, between all concentrations of BAK and UV ($p < 0.05$). There was no significant difference between the independent and combined treatment groups ($p > 0.05$) (Figure IV-10, IV-11, IV-12, and IV-13).

Table IV-4 Back vertex distance variability for all groups. All data are reported as mean ± standard deviation. Statistical significance was determined based on a p value less than 0.05. Statistical difference from control is indicated by *, and † indicates statistical difference from the day 0 measurement. The reported mean and standard deviation values are produced from the average of 6 lenses evaluated at each time point.

| ScanTox	Time						
Experimental Group (n = 6)	Day 0 (post-treatment)	Day 2	Day 7	Day 14	Day 16	Day 18	Day 20
Control (no BAK)	0.340 ± 0.11	0.344 ± 0.07	0.265 ± 0.04	0.314 ± 0.06	0.321 ± 0.07	0.329 ± 0.07	0.364 ± 0.08
BAK 0.01%	0.365 ± 0.11	0.501 ± 0.07	0.928 ± 0.44*†	1.089 ± 0.82*†	1.214 ± 0.88*†	1.376 ± 1.04*†	1.534 ± 1.41*†
BAK 0.005%	0.338 ± 0.07	0.318 ± 0.08	0.294 ± 0.06	0.424 ± 0.23	0.376 ± 0.09	0.439 ± 0.18	0.510 ± 0.29
BAK 0.001%	0.323 ± 0.09	0.343 ± 0.09	0.322 ± 0.11	0.331 ± 0.11	0.336 ± 0.11	0.335 ± 0.08	0.356 ± 0.10
Control (no UV)	0.340 ± 0.05	0.302 ± 0.04	0.288 ± 0.05	0.281 ± 0.09	0.314 ± 0.08	0.280 ± 0.04	0.349 ± 0.07
UV	0.262 ± 0.09	0.317 ± 0.12	0.497 ± 0.31	1.536 ± 0.62*†	2.638 ± 1.15*†	1.338 ± 0.46*†	1.472 ± 0.56*†
Control (no BAK or UV)	0.307 ± 0.08	0.345 ± 0.07	0.305 ± 0.08	0.320 ± 0.07	0.336 ± 0.07	0.330 ± 0.09	0.345 ± 0.09
BAK 0.01% + UV	0.414 ± 0.14	0.850 ± 0.31	1.503 ± 1.13*†	2.062 ± 1.42*†	1.607 ± 0.93*†	2.420 ± 2.38*†	1.574 ± 0.47*†
BAK 0.005% + UV	0.344 ± 0.06	0.325 ± 0.07	0.746 ± 0.74	2.382 ± 1.92*†	1.658 ± 0.85*†	1.665 ± 0.83*†	1.587 ± 0.68*†
BAK 0.001% + UV	0.391 ± 0.35	0.377 ± 0.18	0.440 ± 0.03	2.307 ± 0.98*†	1.822 ± 0.83*†	2.130 ± 0.81*†	1.412 ± 0.29

Table IV-5 Combined treatment groups compared to UV treatment group. Data are reported as mean ± standard deviation.

ScanTox	Time						
Experimental Group (n = 6)	Day 0 (post-treatment)	Day 2	Day 7	Day 14	Day 16	Day 18	Day 20
UV	0.262 ± 0.09	0.317 ± 0.12	0.497 ± 0.31	1.536 ± 0.62	2.638 ± 1.15	1.338 ± 0.46	1.472 ± 0.56
BAK 0.01% + UV	0.414 ± 0.14	0.850 ± 0.31	1.503 ± 1.13	2.062 ± 1.42	1.607 ± 0.93	2.420 ± 2.38	1.574 ± 0.47
BAK 0.005% + UV	0.344 ± 0.06	0.325 ± 0.07	0.746 ± 0.74	2.382 ± 1.92	1.658 ± 0.85	1.665 ± 0.83	1.587 ± 0.68
BAK 0.001% + UV	0.391 ± 0.35	0.377 ± 0.18	0.440 ± 0.03	2.307 ± 0.98	1.822 ± 0.83	2.130 ± 0.81	1.412 ± 0.29

Table IV-6 Comparisons of combined treatment groups against individual exposure to BAK for each concentration. All data are reported as mean ± standard deviation. Statistical significance, indicated with *, was determined based on a p value less than 0.05.

ScanTox	Time						
Experimental Group (n = 6)	Day 0 (post-treatment)	Day 2	Day 7	Day 14	Day 16	Day 18	Day 20
BAK 0.01%	0.365 ± 0.11	0.501 ± 0.07	0.928 ± 0.44	1.089 ± 0.82	1.214 ± 0.88	1.376 ± 1.04	1.534 ± 1.41
BAK 0.01% + UV	0.414 ± 0.14	0.850 ± 0.31	1.503 ± 1.13	2.062 ± 1.42	1.607 ± 0.93	2.420 ± 2.38	1.574 ± 0.47
BAK 0.005%	0.338 ± 0.07	0.318 ± 0.08	0.294 ± 0.06	0.424 ± 0.23	0.376 ± 0.09	0.439 ± 0.18	0.510 ± 0.29
BAK 0.005% + UV	0.344 ± 0.06	0.325 ± 0.07	0.746 ± 0.74	2.382 ± 1.92*	1.658 ± 0.85*	1.665 ± 0.83*	1.587 ± 0.68*
BAK 0.001%	0.323 ± 0.09	0.343 ± 0.09	0.322 ± 0.11	0.331 ± 0.11	0.336 ± 0.11	0.335 ± 0.08	0.356 ± 0.10
BAK 0.001% + UV	0.391 ± 0.35	0.377 ± 0.18	0.440 ± 0.03	2.307 ± 0.98*	1.822 ± 0.83*	2.130 ± 0.81*	1.412 ± 0.29*
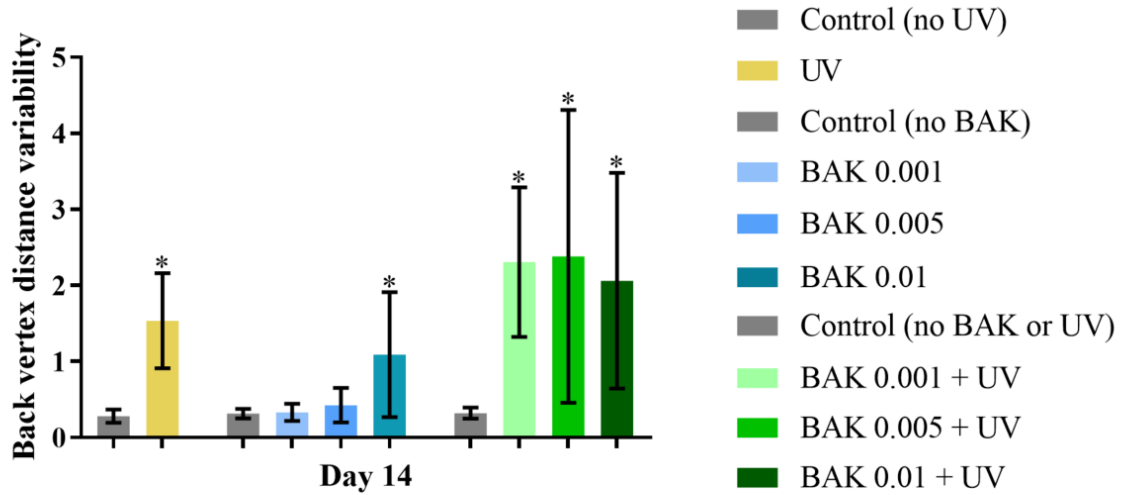
Figure IV-6 Back vertex distance variability for all experimental conditions. All data are reported as mean ± standard deviation. Statistical significance* was determined by a p value less than 0.05 when compared to respective experimental control.
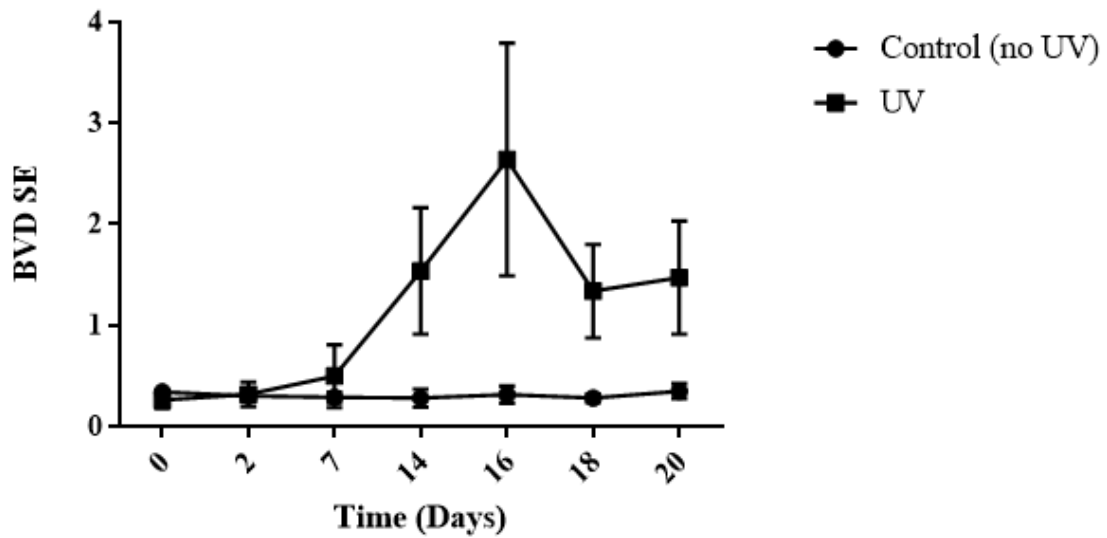
Figure IV-7 Back vertex distance variability for UV-treated lenses as compared to control (no UV).
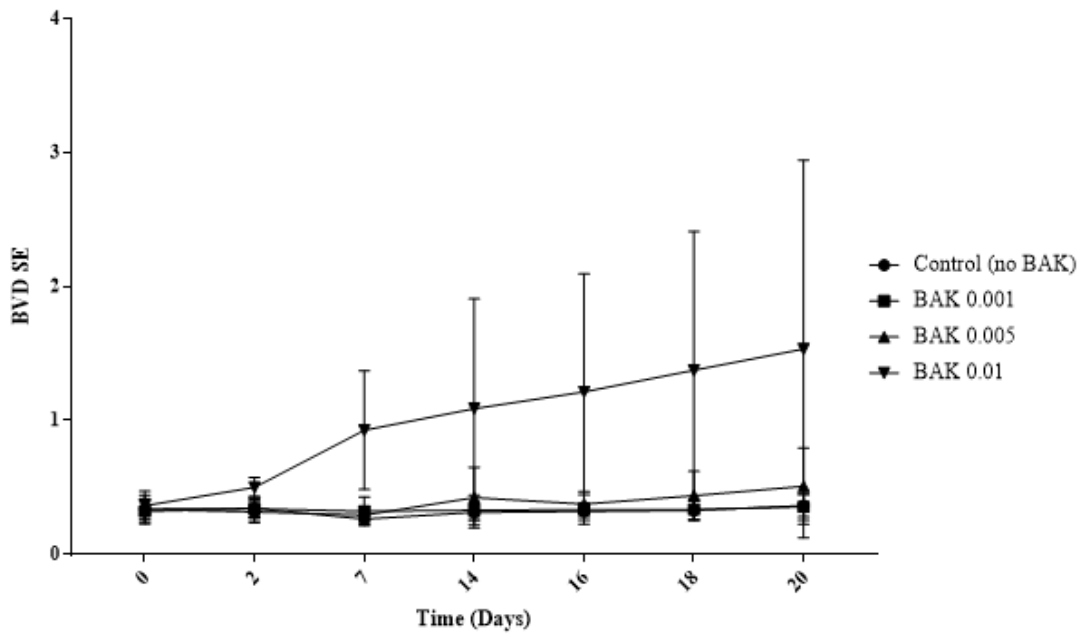
Figure IV-8 Back vertex distance variability for BAK-treated lenses as compared to control (no BAK).
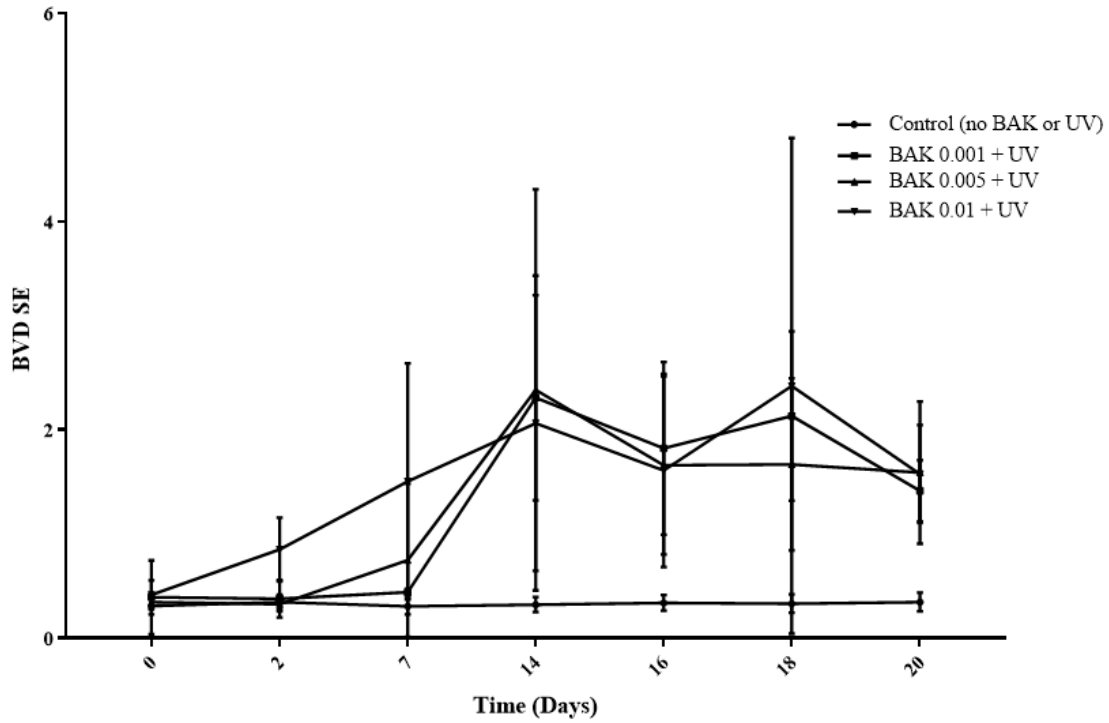
Figure IV-9 Back vertex distance variability for combination BAK plus UV-treated lenses, as compared to control (no BAK or UV).
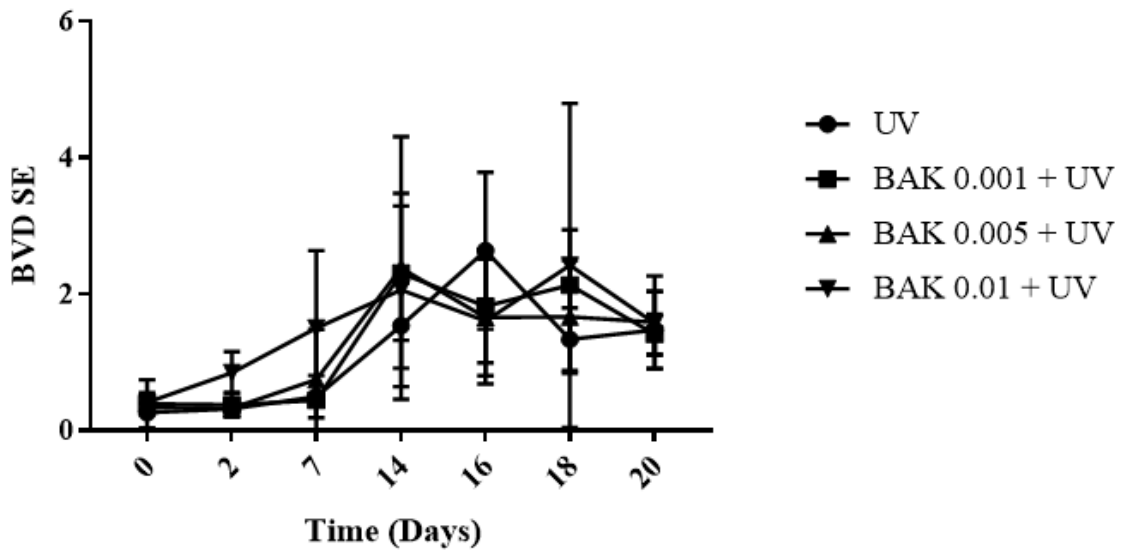
Figure IV-10 Back vertex distance variability for combination BAK plus UV-treated lenses, as compared to UV-treated lenses.
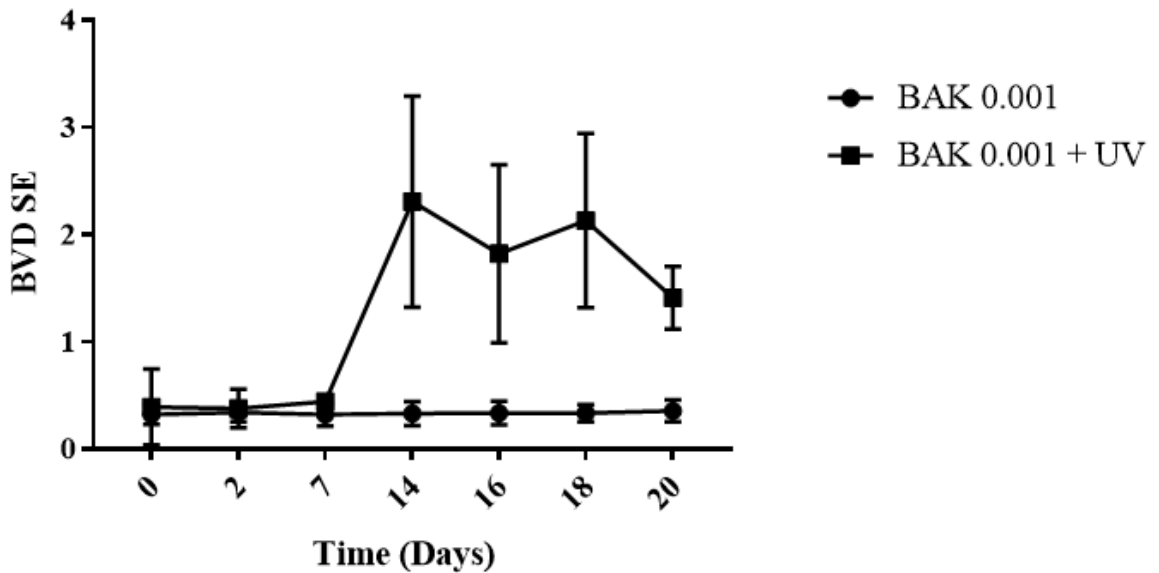
Figure IV-11 Back vertex distance variability for lenses treated independently with BAK 0.001% solution or a combination treatment with BAK 0.001% plus UV.
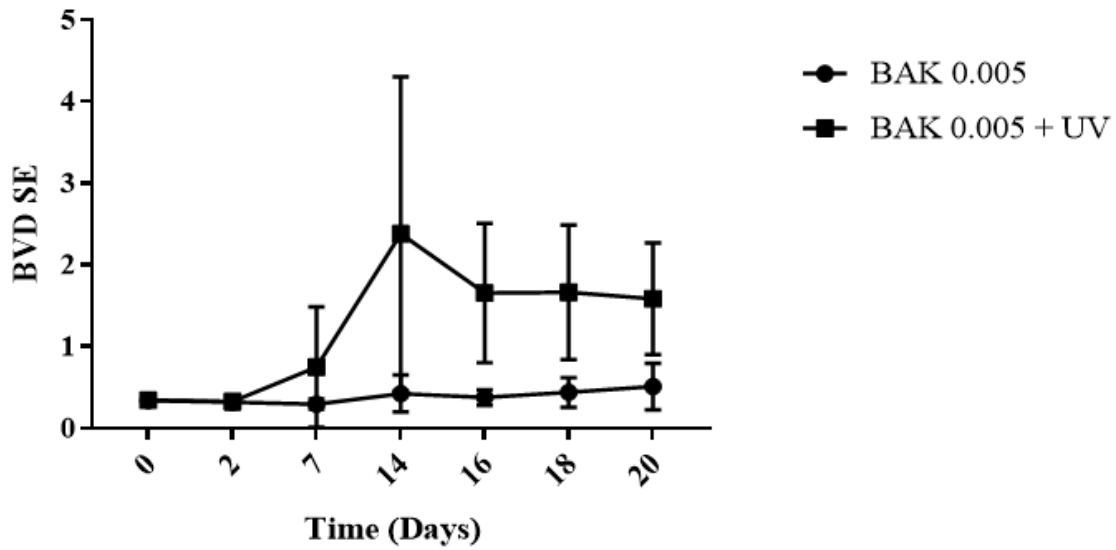
Figure IV-12 Back vertex distance variability for lenses treated independently with BAK 0.005% solution or a combination treatment with BAK 0.005% plus UV.
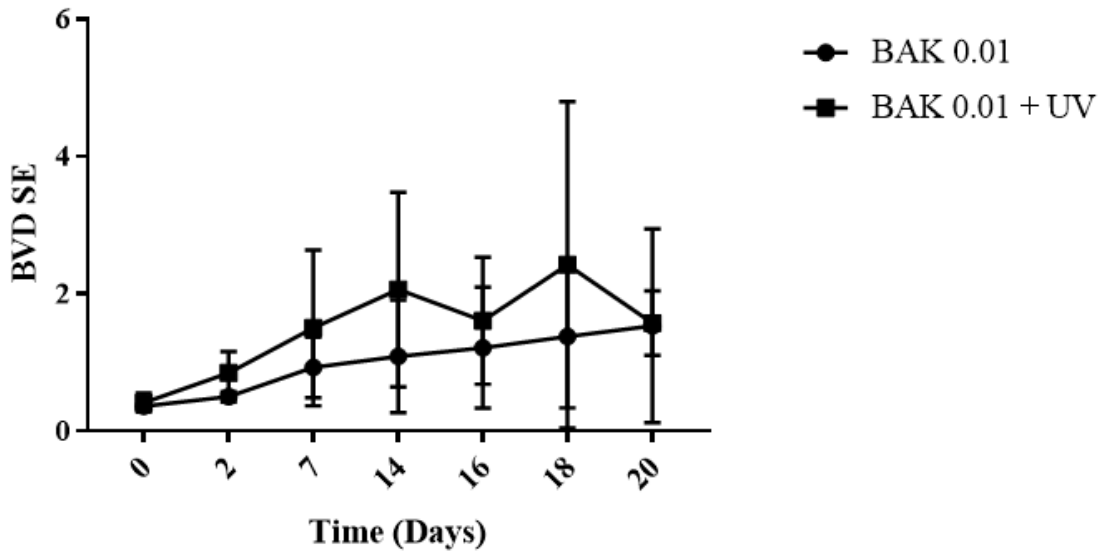
Figure IV-13 Back vertex distance variability for lenses treated independently with BAK 0.01% solution or a combination treatment with BAK 0.01% plus UV.

V. Discussion

5.1 Key findings

This study evaluated the combined toxicity of BAK and UV radiation on the bovine lens. The research was prompted by recent work demonstrating that that BAK is able to penetrate the eye as deep as the anterior lens,[23] and that the combined effects of BAK and UV on HCEC lines can be additive or synergistic.[11] Our results confirmed those of previous work, that treatment with a BAK solution or UV radiation independently reduced both cellular metabolic activity and optical quality. The response to BAK exposure was concentration-dependent. Treatment with a 0.01% BAK solution significantly reduced both optical quality and metabolic activity. Exposure to a 0.005% BAK solution produced toxicity to lens metabolism, while optical function was conserved. Treatment with a 0.001% BAK solution did not produce lens damage in these experiments. The combined treatment of BAK and UV produced toxicity to the lens at all concentrations of BAK, and demonstrated a dose-response relationship in that reductions in optical quality were detected earlier with the highest concentration of BAK. However, the metabolic activity of lenses treated with both BAK and UV was not significantly different from those exposed to BAK alone, and similarly BVD variability for the combined treatment group was not statistically different from UV-treated lenses. The assays together indicated that significant reductions in metabolic activity preceded changes in optical quality.

The results of this study replicated in part those obtained from previous research by our group evaluating the individual toxicity of BAK and UV on the bovine lens. For the effects of BAK, Youn et al. also demonstrated the concentration-dependent response of the

lens.[22] The study found similar timing for the onset of lens metabolic activity damage from exposure to BAK 0.01% solution at 24 hours post-exposure, as compared to the day 2 time point of the current study. Youn et al. also noted that BAK 0.001% exposure did not significantly decrease optical quality as compared to control.[22] In this study, BAK 0.001% did not produce significant toxicity to lens metabolic activity or optical quality. This result contrasts in part with the same previous study, where BAK 0.001% significantly reduced lens metabolic activity, measured from 72h post-exposure. The difference can be attributed to the exposure duration, which was 50% longer than in the current study.

For UV exposure, Youn et al.[22] exposed lenses to a UVB radiation source, as compared to the combined UVA and UVB spectrum in this study. However, both studies report significantly increased BVD variability compared to control at 14 days post-exposure. Our study also confirmed the previous finding that changes in metabolic activity of the lens are detected earlier than changes in optical quality, independent of the treatment conditions.[22]

This research performed by Xu et al. suggested that BAK and UV interact to produce additive and synergistic effects on human corneal epithelial cell lines.[11] The study found that BAK 0.005% and BAK 0.001% combined with UV produced additive and synergistic effects on metabolic activity, respectively. The findings were consistent irrespective of the treatment order.[11] Conversely, our study demonstrated that the effect of combined BAK and UV exposure on lens metabolic activity was not significantly different than the effect of BAK alone. This was demonstrated when the lens was treated first with BAK followed by UV. It is notable that the synergistic and additive effects on HCEC were observed at relatively low doses of BAK. BAK 0.001% did not produce significant toxicity to the bovine lens in the

current study. Neither an additive nor synergistic reaction would be expected from combined exposure if the dose of BAK was not sufficient to produce toxicity. UV also did not have a potentiation effect on low dose BAK in these experiments. Withrow et al. also reported additive toxicity for BAK and UVA on mouse lymphoma cell survival and mutation frequency.[41] A key difference between the two formerly mentioned studies evaluating combined toxicity and the current study was the use of mouse lymphoma and immortalized human corneal epithelial cell lines, as compared to whole primary tissue. A mouse lymphoma cell line represents a marked divergence from the nature of lens cells of bovine origin. For immortalized cell lines, the genotype must be transformed from primary cells in order to produce the occurrence of infinite generations of cells. A number of alternate functions are reported in immortalized cell lines as compared to primary cells, including altered production and responses to cell mediator molecules, growth kinetics, and respiration decoupling.[42] An additional concern with the growth of immortalized cells is the possibility that through the multitude of generations of cells, further drift from the primary cell genotype will occur. Possible reasons for the difference in the effects of BAK and UV on HCEC as compared to whole lenses can be attributed to the structural organization of lenses as compared to suspended cells, as well as the differences that exist between whole lenses and immortalized cells. These differences affect the mechanism of toxicity, as described below. The findings from this study emphasize the importance of reproducing experiments in primary cell culture.

5.2 Mechanisms of toxicity

Benzalkonium chloride is a quaternary ammonium salt, with a long hydrophilic hydrocarbon chain and cationic head group. It is classified as a surfactant compound, and behaves as a detergent in an aqueous solution.[22] Its unique structure imparts superior antimicrobial properties, stability in solution, and allergic tolerance as compared to other preservatives.[20-23, 25, 43] However, BAK-preserved solutions are also reported to induce greater toxicity.[21, 44] The adverse effects of BAK to structures of the anterior segment have been thoroughly reported. BAK is known to disrupt the tear film, the corneal epithelium, stroma and endothelium, and conjunctival cells. [24, 45-47] Though the potential side effects are well known, the exact biological mechanism by which BAK induces toxicity has not been defined. Several *in vitro* studies however have demonstrated the participation of certain biological pathways during BAK toxicity. A recent study by Datta and colleagues found that BAK lowered mitochondrial oxygen consumption and adenosine triphosphate (ATP) activity at the level of mitochondrial complex I in corneal epithelial cells.[48] Another study performed using Chang conjunctival cells found that BAK was associated with H_2O_2 and superoxide anion production.[47] This study suggested that the processes of free radical production, inflammation, and apoptosis in ocular tissue are interconnected.[47] Dutot and colleagues examined the effects of BAK on the activation of the P2X7 death receptor found in corneal, conjunctival, lens, and retinal epithelial cells. The P2X7 receptor can be activated through interaction with reactive oxygen species, and is thought to prompt production of inflammatory cytokines, particularly interleukin-1 (IL-1) and interleukin-6 (IL-6). The results indicated that P2X7 receptor activity increased in response to BAK for ocular surface

epithelia.[49] Another study using cultured lens epithelial cells treated with BAK induced production of inflammatory markers, particularly PGE_2, IL-1, and IL-6.[50] BAK exposure also reportedly activates the canonical Wnt signaling pathway, as determined by Zhou et al. (2011) from experiments on corneal epithelial cells lines and rat corneas.[51] Other reports of BAK toxicity have commented that the long BAK hydrocarbon tail which improves the penetrative properties of preserved solutions additionally permits interaction with the lipid layer of the tear film and cellular membranes. BAK disperses membrane lipids which consequently triggers cell component leakage and lysis.[52] The results of the current study, in which lenses do not recover in from decreases in metabolic activity or optical quality after exposure to BAK 0.01%, are consistent with this account, since cell lysis would reduce the overall cell number and thus impede both metabolic activity and cell recovery mechanisms.

UV radiation induces photochemical damage to the eye through both direct and indirect mechanisms.[14] The direct mechanism involves the absorption of UV light by cellular chromophores, including nucleic acids and proteins. The indirect mechanism of UV damage to the lens involves the generation of reactive oxygen species and free radicals, causing peroxidation of cellular lipids, and modification of DNA and proteins.[13] UVB reportedly produces damage primarily through the direct pathway, while UVA produces damage through the indirect pathway.[14] The consequences of UV damage to the lens often indicate oxidative stress.

The anterior lens epithelium is the site of initiation for several events of UV-induced toxicity and cataract. The anterior location consequently involves receiving the greatest exposure level of UV within the lens. Reactive oxygen species and free radicals are known to

target the phospholipids and proteins of cellular membranes. These events result in dysregulation of the ionic balance of the lens, particularly the balance of Ca^{2+} ions.[18] This could also lead to the aggregation of lens crystallin proteins.[18] The lens epithelium additionally contains most of the organelles contained within the lens, including nuclei and mitochondria. Consequently, the epithelium is a major site of enzyme production and metabolic activity. Damage to lens nuclei may be produced from the formation of pyrimidine dimers. A study by Bantseev on lens, retinal, and corneal epithelial cell lines demonstrated that UVB produced shrunken nuclei. As UV produces cellular dysfunction, apoptosis may be induced and the extracellular spaces between lens fibre cells may be altered, producing changes in refractive index and loss of transparency.[53] Another study reported lack of mitochondrial movement and fragmentation of mitochondria after irradiation of bovine lenses and retinal pigment epithelial cells with UVB.[54] Disturbance of cell mitochondria would then inhibit the production of cell energy as ATP.

The results from this study suggest that BAK and UV light in the range of 280 to 400 nm do not interact to enhance the independent toxicities of one another on whole lenses. This finding is consistent with information published by Withrow and colleagues and the Japanese pharmacopeia, which report that BAK does not absorb wavelengths of light greater than 290 nm.[41, 55] Xu et al. also performed a photoreactivity test between BAK and UV (280 – 400 nm), wherein cells were exposed both to UV-treated and untreated BAK. The results indicated that the toxicity of UV-treated BAK was similar or slightly lower than untreated BAK. It is unlikely then that a photosensitization interaction between BAK and UV would occur.

The present study met the threshold of toxicity for both BAK and UV, at a level of approximately 20% reduced activity as compared to control, to permit the observation of additive or synergistic effects. If the reduced metabolic activity of the lens reflected having reduced the overall number of live cells, the expected result for two damages would be additive. It is likely then that the cells were damaged and rendered less functional, but not necessarily necrotic after each exposure. Moreover, an additive effect would not be expected if the cells vulnerable to BAK were the same vulnerable to the effects of UV. The current study evaluated the metabolic activity of cells, which for the lens is carried out by the mitochondria found in the epithelium and superficial cortical fiber cells. Studies of bovine and rat lenses demonstrated that the mitochondria in lens epithelial cells and superficial cortical fiber cells are distinct.[56, 57] Lens epithelial cell mitochondria are densely concentrated surrounding the cell nucleus. In contrast, the mitochondria found in superficial cortical fiber cells are longer, are less densely concentrated, and not associated with the cell nucleus. [56, 57] A study led by Bantseev and colleagues evaluated the effect of sodium dodecyl sulfate (SDS) on the bovine lens.[58] Similar to BAK, SDS possesses a long hydrocarbon tail with an ionic head, which in slight contrast to BAK is negatively charged. The analysis found that SDS reduced mitochondrial number and length in the epithelium and superficial cortical fiber cells. Superficial cortical fiber cells were particularly vulnerable to the effects of SDS. Another study which evaluated the mechanism of damage of BAK on mitochondria found that cells with a mitochondrial DNA point mutation were particularly susceptible to BAK.[48] Further research is needed to confirm that the superficial cortical cells are particularly vulnerable to the effects of BAK and UV. This hypothesis is potentially consistent with the

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results of previous work by our group using HCEC, which found synergistic and additive effects of BAK and UV. Unlike whole lenses which possess cellular and mitochondrial heterogeneity, the cell culture of the previous study was likely more uniform. The combined effects of BAK and UV on lens epithelial cell lines were not examined as part of this study; this is a potential avenue for further investigation how BAK and UV interact with the lens, as well as in different cell cultures. Further research would also be required to confirm the nature of combined toxicity of BAK and UV *in vivo*. Unlike the conditions of the current *in vitro* test, the eye *in vivo* would significantly dilute the concentrations of BAK tested through blink action and transportation through circulation.

5.3 *In vitro* assessment of ocular toxicity with the bovine lens

The Draize test is the current *in vivo* standard for assessing the ocular irritancy and toxicity. The test is performed by administering a 0.5 mg or mL of a test substance to the skin or lower conjunctival sac of an alert animal, typically an albino rabbit. The conjunctiva, cornea, and iris are monitored over time for symptoms of damage, and are ranked by the observer. However, this test has been criticized for several reasons in the scientific literature. A review by Wilhelmus reports that rater judgments are subjective, consequently producing significant intra-rater and inter-rater variability.[38] The test is also weakly sensitive and results are not consistently replicable.[38] Moreover, the associated animal discomfort and pain raises ethical concerns. Considerable effort has been put forth in developing *in vitro* models to reduce reliance on the Draize test for toxicity testing. Yet, there is no single or battery of tests which are validated and universally accepted to supersede the Draize test as the standard for assessing ocular toxicity. Further, despite the significant role that the lens performs with in

focusing light to the retina, there is no component of the Draize test which evaluates the toxicity of the test substance to the lens.

The bovine lens, cornea, and conjunctiva are embryologically-derived from surface ectoderm; similar to human embryological development.[59] The bovine lens also possesses similar physiology and morphology to the human cornea.[22] The current model uses a serum supplement for the lens culture medium which is of the same animal origin as the culture tissue. The bovine lens has been previously reported as a suitable model for assessing the toxicity for surfactants, alcohols, UV exposure, and other xenobiotic substances.[22, 58-60] The bovine lens performs consistently between samples in the alamarBlue and ScanTox assay, as shown in Tables IV-1 and IV-4. Unlike excised corneas in culture, the lens is capable of retaining its refractive function. The results of this study suggest that the bovine lens model is suitable for assessing the effects of combined toxic exposures.

A valuable *in vitro* alternative to the Draize test would perform a sensitive, objective, valid, reliable, low-cost, robust, and specific assessment of cell viability.[61] The alamarBlue assay is a non-toxic indicator of cell metabolic activity. The indicator is metabolized through reduction and oxidation mechanisms concentrated within cell mitochondria for its reduction from resazurin to resorufin.[61] This chemical reaction yields a product with measurable fluorescence and absorbance, and colour change from blue (resazurin) to pink (resorufin). The alamarBlue assay is reported to have greater sensitivity to cell activity than similar assays such as MTT.[62] Additionally, the MTT protocol involves a solubilization step to precipitate the dye from cells which would induce lysis and defeat the objective of long-term culture.

An ideal method for the evaluation of cytotoxicity *in vitro* would thus involve a battery of assays. Previous work has demonstrated that metabolic activity is well-correlated with induction of lens optical disturbance.[63] The ScanTox assay is a non-intrusive evaluation, performed without the need to directly handle lenses or remove them from their culture medium. This method is capable of long-term lens culture, while maintaining optical integrity and refractive function. This may be ideal for the evaluation of toxicity of benzalkonium chloride and similar compounds, whose partially lipophilic nature may bind to ocular tissues and produce delayed effects. This also lends itself well to the study of UV toxicity, which was observed in this and previous studies by our group to exhibit a latency period.[22] The system is additionally capable of monitoring recovery from the effects of UV radiation and cold cataract.[64, 65] Long-term tissue culture is useful for measuring toxicity as repair mechanisms are effected.[60] This study used this method for a period of 20 days, though it has been reported to be sustainable up to 1000 hours.[60] The control lenses in this study were cultured successfully with consistent optical performance, and with low variability between samples. The results of the current study reproduced the assessments of toxicity for BAK and UV from previous studies well, and the control data between the studies are comparable.[22] As an alternative to the use of live animals, which require additional maintenance, the use of abattoir-supplied lenses provides an accessible and low-cost test for ocular irritancy. The consistently of results can be attributed in part to the objective measurements produced by an automated laser-scanner, in contrast to judgments made by multiple observers.

In summary, the results of this study suggest that combined exposure to BAK and UV does not produce a significant interactive toxicity on the lens. The study confirmed previous work which has examined the concentration-dependent nature of BAK damage, and the latent effects of UV on the lens.[11, 22] This work also suggests that the ideal method for assessing toxicology *in vitro* for individual and combined exposures is multifaceted, including work with cell lines and primary tissue, and using multiple assay types. Metabolic activity and optical quality together provide an effective assessment of lenticular toxicity from preservatives and ultraviolet radiation.

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