Pharmacologic Alternatives to Riboflavin Photochemical Corneal Cross-Linking: A Comparison Study of Cell Toxicity Thresholds

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PURPOSE. The efficacy of therapeutic cross-linking of the cornea using riboflavin photochemistry (commonly abbreviated as CXL) has caused its use to become widespread. Because there are known chemical agents that cross-link collagenous tissues, it may be possible to cross-link tissue pharmacologically. The present study was undertaken to compare the cell toxicity of such agents.

METHODS. Nine topical cross-linking agents (five nitroalcohols, glyceraldehyde [GLYC], genipin [GP], paraformaldehyde [FA], and glutaraldehyde [GLUT]) were tested with four different cell lines (immortalized human corneal epithelial cells, human skin fibroblasts, primary bovine corneal endothelial cells, and immortalized human retinal pigment epithelial cells [ARPE-19]). The cells were grown in planar culture and exposed to each agent in a range of concentrations (0.001 mM to 10 mM) for 24 hours followed by a 48-hour recovery phase. Toxicity thresholds were determined by using the trypan blue exclusion method.

RESULTS. A semiquantitative analysis using five categories of toxicity/fixation was carried out, based on plate attachment, uptake of trypan blue stain, and cellular fixation. The toxicity levels varied by a factor of 10^3 with the least toxic being mononitroalcohols and GLYC, intermediate toxicity for a nitrodiol and nitrotriol, and the most toxic being GLUT, FA, GP, and bronopol, a brominated nitrodiol. When comparing toxicity between different cell lines, the levels were generally in agreement.

CONCLUSIONS. There are significant differences in cell toxicity among potential topical cross-linking compounds. The balance between cross-linking of tissue and cell toxicity should be borne in mind as compounds and strategies to improve mechanical tissue properties through therapeutic tissue cross-linking continue to develop.

Keywords: cornea, cellular toxicity, protein cross-linking, keratoconus

Riboflavin-mediated photochemical stabilization of the keratoconic cornea is commonly known as CXL, an abbreviation for corneal (or collagen) cross-linking. This innovative therapeutic approach to strengthening corneal collagen is an exciting new treatment paradigm in ophthalmology and is revolutionizing the field of corneal therapeutics. In the short period since its inception in the late 1990s,¹ CXL has been proven effective in stabilizing patients with keratoconus (KC) and post-LASIK keratectasias and has become the standard of care in many parts of the world. The cross-linking procedure effectively halts the progression of KC and can be accompanied by an improvement in both corneal curvature (i.e., flattening or “normalization” of corneal topography) and visual acuity.

Despite these successes, the CXL therapy poses attendant risks and drawbacks related to the use of UV-A irradiation and the potential damage to the corneal endothelium and epithelial stem cells. The standard technique requires a procedure with epithelial removal (for riboflavin penetration into the corneal stroma), and exposure to a UV-A light source. The long-term effects of an acute UV light exposure such as that incurred during the CXL procedure are unclear. UV-A irradiation has been implicated in a number of deleterious effects, including lens cortical cataract and retina degeneration,^{2–4} although these

lens and retinal effects are generally associated with chronic exposures. Epithelial removal of the cornea is painful and increases the risk of incurring a corneal infection, which has been reported after CXL.^{5–8} Additionally, because the photochemical cross-linking procedure is cytotoxic to cells, caution is used in treating particularly thin corneas (<400 μ m) where the risk of corneal endothelial cell damage is increased, a complication that can lead to detrimental corneal swelling and associated opacification.⁷ In favor of its safety as a procedure, Wollensak et al.⁹ have recently reported no limbal damage following intentional riboflavin photochemical irradiation of the rabbit limbus, and Gatzoufas et al.¹⁰ report no change in endothelial cell density following high fluence CXL. Here, it should be pointed out that an improved understanding of variables involved in the CXL procedure, including the use of different approaches to light activation (i.e., high fluence irradiation), the role of oxygen, and formulation factors, could allow for safer and more effective means of carrying out the procedure in the future.

Furthermore, several research groups, including ours (Hoang QV, et al. *IOVS* 2013;54:ARVO E-Abstract 5169), are exploring the possibility of using chemical cross-linking for scleral stabilization as a means to limit pathologic axial growth

in progressive myopia.^{11,12} In this case, a chemical cross-linking approach may be favored over the photochemical method, since administration to the sclera via a sub-Tenon's injection can be performed safely and repeatedly. Previous efforts to apply the riboflavin photochemical approach to scleral cross-linking have been reported. However, several issues not applicable to corneal cross-linking arise when considering the sclera (particularly the posterior sclera) and include toxicity to the neural retina and accessibility of the UV-A light source to the posterior scleral region.¹³ In favor of this photochemical approach, a single sub-Tenon's injection can diffuse readily throughout the sub-Tenon's space, contacting a wide area of the sclera. The extent of the sub-Tenon's space, as defined by Tenon's capsule (or orbital fascia), limits the area of cross-linking to this space, favoring uniform distribution across the posterior sclera, which is the location of desired effect.¹⁴

Thus, in lieu of such concerns and potential benefits, the development of an alternative cross-linking approach that could avoid the use of UV-A light, and avoid epithelial removal (for the cornea), is less cytotoxic and could provide cross-linking to the posterior sclera without requiring a light source, and could be of significant interest to the field of ophthalmic therapeutics. This has prompted a search for candidate chemical cross-linking agents that could be used for therapeutic stabilization of either the cornea and/or sclera. In addition to the nitroalcohols that our group has been studying, several agents, including glyceraldehyde¹¹ and genipin,¹⁵ have been proposed for such uses.

In a prior study,¹⁶ we have reported in vitro toxicity levels for primary bovine corneal endothelial cells (BCECs) for three mononitroalcohols. In the current study we significantly expanded this study to include an evaluation of the in vitro cell toxicity thresholds for nine different relevant chemical cross-linking agents, using four different cell lines. The compounds evaluated included genipin, glyceraldehyde, glutaraldehyde, formaldehyde, and several nitroalcohols including a well-known brominated nitroalcohol, bronopol (Table 1). These compounds were studied by using four different cell lines representing epithelial, stromal, and endothelial cells.

MATERIALS AND METHODS

Chemicals

Nine different chemical cross-linking agents were tested in this toxicity study: 2-nitroethanol (NE; $C_2H_5NO_3$, molecular weight [MW] = 91.07), 2-nitro-1-propanol (NP; $C_3H_7NO_3$, MW = 105.09), 2-bromo-2-nitro-1,3-propanediol (bronopol [BP]; $C_3H_7BrNO_4$, MW = 199.99), methyl(1S,2R,6S)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate (genipin [GP]; $C_{11}H_{14}O_5$, MW = 226.23), and L-glyceraldehyde (GLYC; $C_3H_6O_3$, MW = 90.08) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). 2-Methyl-2-nitro-1,3-propanediol (MNPD; $C_4H_9NO_4$, MW = 135.12) and 2-hydroxy-methyl-2-nitro-1,3-propanediol (HNPD; tris hydroxymethyl nitromethane; $C_4H_9NO_5$, MW = 151.12) were from the Tokyo Chemical Industry Co. (Portland, OR, USA). Paraformaldehyde (FA; CH_2O , formula weight [FW] = 30.03) and glutaraldehyde (GLUT; $C_5H_8O_2$, FW = 100.12) were from Electron Microscopy Sciences (Hatfield, PA, USA).

Cell Culture Methods

Immortalized Human Corneal Epithelial Cell (HCEC) Culture. SV40-immortalized HCECs (a generous gift from Kaoru Araki-Sasaki, Ideta Eye Hospital, Kumamoto City, Kumamoto, Japan, via Peter Reinach) were cultured in

Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 6% fetal bovine serum (Invitrogen), 5 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 5 μ g/mL insulin (Sigma-Aldrich), and 1% penicillin and streptomycin (Invitrogen). Cells were grown in 5% CO_2 , 95% ambient air, at 37°C. When cells reached confluence, they were detached with trypsin (0.25%; Gibco, Grand Island, NY, USA) and plated into 24-well cell culture plates for toxicity testing.

Human Skin Fibroblast Culture. Normal human dermal fibroblasts from ATCC (Manassas, VA, USA) were cultured in dermal cell basal medium (ATCC) with serum-free fibroblast growth kit, which contains fibroblast growth supplements, ascorbic acid, EGF/TGF- β 1, glutamine, hydrocortisone, and insulin (ATCC). Cells were grown in 5% CO_2 , 95% ambient air, at 37°C. When cells reached confluence, they were detached and plated into 24-well cell culture plates for toxicity testing.

Primary BCEC Culture. Primary cultures of BCECs were grown by using the methods described by Grant et al.¹⁷ Ten bovine eyes were obtained from a local slaughterhouse within 5 hours post mortem. On arrival, each eye was wiped with 95% ethyl alcohol for sterilization. The cornea was excised by using sterile technique and placed in a hemispherical holder (endothelial side up). The surface was then washed with Ca^{2+} -free Dulbecco's Phosphate Buffered Saline (Invitrogen). A Ca^{2+} - Mg^{2+} -free solution containing 0.25% trypsin and 0.02% EDTA was then applied to the endothelial surface for 5 to 10 minutes after which the endothelial cells were dislodged by gentle rubbing in the presence of DMEM. The dislodged cells were then aspirated and plated into 25-cm² culture flasks (NUNC-Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5 mL DMEM plus 6% fetal bovine serum, 2 ng/mL basic fibroblast growth factor, and 2% penicillin/streptomycin. Cells were maintained in a 5% CO_2 , 95% ambient air, 37°C incubator and fed every 3 to 4 days. Cells reached confluence in 7 to 10 days, after which they were detached and subcultured. The cell suspension was divided into three 25-cm² flasks. Subsequently, the cells were plated into 24-well culture plates in preparation for toxicity studies. All studies were performed on the second or third passage.

Immortalized Retinal Pigment Epithelial Cells (ARPE) Culture. Cells from the human RPE cell line (ARPE-19 from ATCC) were cultured in DMEM with 10% fetal bovine serum. Cells were grown in 5% CO_2 , 95% ambient air, at 37°C. When cells reached confluence, they were detached and plated into 24-well cell culture plates for toxicity test.

Toxicity Setting for All Four Cell Types and Trypan Blue Staining for Cell Viability

All cells were seeded into 24-well plates at a cell density of 3×10^4 cells/well. Toxicity tests were initiated when cells were noted to reach confluence (5–7 days on average). The cells were exposed to a range of chemical concentrations (0.001 mM to 10 mM). After 24 hours, the cell culture media (including the cross-linking [XL] agent) were removed and replenished with fresh media (without XL agent) for an additional 48-hour recovery period. Following the recovery period, the cells were stained by using a modification of the trypan blue exclusion method. Briefly, the culture medium containing various chemicals was removed by aspiration and each well was rinsed with DPBS. A 0.4% trypan blue solution (Gibco) was then added for 3 to 60 minutes at room temperature (see Results for further explanation). Following this incubation period, the staining solution was replaced with fresh DPBS and washed one more time. The stained plates were then examined for cell morphology and staining characteristics under the inverted microscope (Cat No. 12-560-45; Fisher Scientific, Pittsburgh, PA, USA).

TABLE 1. The Chemical Structures of the Nine Cross-Linking Compounds Used in This Study

Name	Structure	Abbreviation
2-nitroethanol		NE
2-nitro-1-propanol		NP
2-methyl-2-nitro-1,3-propanediol		MNPD
2-hydroxy-methyl-2-nitro-1,3-propanediol		HNPD
bronopol 2-bromo-2-nitro-1,3-propanediol		BP
genipin methyl(1S,2R,6S)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate		GP
glyceraldehyde		GLYC
para-formaldehyde		FA
glutaraldehyde		GLUT

FIGURE 1. Observation criteria for toxicity are shown in control and formaldehyde-treated (1 mM and 5 mM) HCECs. We defined five main categories of cytotoxicity in ascending order as follows: alive, partially dead (<50% viable cells), dead, dead/gone, and dead/fixed (see text for further description). *Left:* Control unexposed cells are viable and able to exclude the trypan blue dye. *Middle:* An example of cells that are dead and gone (i.e., detached from the culture plate). *Right:* Cells that are dead and fixed with positive nuclear staining.

RESULTS

Trypan blue is a well-known vital dye, used to selectively color dead cells. It is a large hydrophilic, tetrasulfonated diazo dye. The mechanism of trypan blue staining is based on it being negatively charged and not interacting with cells unless the membrane is damaged.^{18,19} Thus, cells that are viable will exclude the dye, while in dead cells the dye can pass easily through the damaged cell membranes to stain the cell nucleus blue. We used the trypan blue exclusion method to test cell viability for different concentrations of cross-linking agents.

During the course of our studies we noticed that in many of the culture plates that were exposed to high concentrations of cross-linking agent, the trypan blue did not appear to stain the cell nuclei when using the standard staining protocol, although the cells were likely to be dead. We believe that this was due to fixation of the cells by the chemical cross-linking agent under study for toxicity. In other words, exposure of the cells to higher concentrations of cross-linking agent resulted in fixation of the cells and relative impermeability to the trypan blue dye. Cross-linking agents such as GLUT and FA are known as fixatives.^{20,21} Such fixative molecules have the capability to inactivate, stabilize, or immobilize proteins and form cross-linkages with their targets. Cell membrane components contain peptides, proteins, lipids, phospholipids, carbohy-

drates, carbohydrate complexes, as well as various types of RNA and DNA.²² In higher concentration (i.e., that which exceeded the toxic threshold) most of the agents studied were capable of stabilizing and fixing cell membranes and their components. By modifying the standard duration of staining from 3 minutes to 30 to 60 minutes, we were able to confirm that cells were “fixed and dead.” Figure 1 gives an example of this finding (using human corneal epithelial cells) in which cell death is shown by nuclear blue staining at 1 mM HNPd, with minimal staining at a higher concentration of 5 mM and lack of nuclear staining at 10 mM HNPd.

We defined five main categories of cytotoxicity in our experimental system: (1) alive, (2) partially dead, (3) dead, (4) dead and gone, and (5) dead and fixed. Alive cells were noted to exclude trypan blue, displayed normal morphology, and remained confluent. Dead cells were noted to remain attached to the culture dish but with nuclear trypan blue staining. Dead and gone cells were noted to be dead with contracted cell morphology and uptake of trypan blue, with areas of complete cell detachment. The dead and gone category implies greater toxicity than the dead category since all dead cells eventually detach. Dead and fixed cells were noted to have relatively normal morphology and excluded trypan blue with the standard 3-minute staining method. However, longer staining

FIGURE 2. Examples of permeability differences for trypan blue staining are shown, using three different fixative concentrations of HNPd: 1 mM (*left*), 5 mM (*middle*), and 10 mM (*right*). *Left:* A, at 1 mM HNPd cells take up the trypan blue stain. However, with increasing concentration of HNPd (5 mM and 10 mM), trypan blue can be excluded from cells. These higher-concentration cells can be stained with trypan blue by increasing the dye exposure time from 3 seconds to 60 minutes.

TABLE 2. Results of Toxicity Testing Using Immortalized HCECs

Human Corneal Epithelial Cells											
Concentration, mM	NE	NP	GLYC	MNPD	HNPD	FA, o	GLUT, o	FA, n	GLUT, n	GP	BP
10	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed
5	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Gone	Gone	Gone	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Gone
1	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Gone	Gone	Gone	Gone	Fixed	Gone			Fixed	Fixed	Gone
0.8	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
		Gone	Gone	Gone	Fixed				Fixed	Fixed	Gone
0.6	Alive	Alive	Partially dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
				Gone	Fixed				Fixed	Fixed	Gone
0.4	Alive	Alive	Alive	Partially dead	Dead	Dead	Alive	Dead	Dead	Dead	Dead
					Gone				Fixed	Fixed	Gone
0.2	Alive	Alive	Alive	Alive	Partially dead	Partially dead	Alive	Dead	Dead	Dead	Dead
									Fixed	Fixed	Gone
0.1	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Dead	Dead
										Fixed	Gone
0.01	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive
0.001								Alive	Alive	Alive	Alive
Control	Alive										

We defined five main categories of cytotoxicity in ascending order as follows: alive, partially dead (<50% viable cells), dead, dead/gone, and dead/fixed (see text for further description). Compound abbreviations are described in Table 1. o, old; n, new.

duration resulted in uptake of the blue dye into the cell nucleus. These cells were clearly dead but were fixed by the particular cross-linking agent (Fig. 2). Using these five categories, in general, we observed an all-or-none effect, using the reagent concentrations described. In a few cases, plates were observed to contain viable cells that excluded trypan blue, but also had areas in which cells had detached, indicating cell death. This category was designated as “partially dead.” In all of the plates displaying this pattern, the area covered by viable cells was less than 50%. In addition, this observation was noted primarily in experiments using the HCEC line (Table 2). In this case, the cells in these partially dead wells were considered above threshold, since we defined toxic threshold values as the highest concentration (in mM) at which all the cells were alive. It should be pointed out that this partially dead category has no relation to lethal dose, 50% (LD₅₀) values for cell toxicity. Producing LD₅₀ data, although useful and important, would have required a much smaller concentration range with finer gradations. This was not the intent of the study. Rather, this study attempted to draw comparisons between potentially useful therapeutic cross-linking agents in identically cultured cells, and therefore LD₅₀ values were not determined.

Based on which of these five criteria were observed, toxicity tables were constructed for each of four different cell lines and nine different cross-linking agents and are shown as Tables 1 through 5. In each case, cells were grown to confluence and exposed to each agent for 24 hours followed by a postexposure recovery period of 48 hours. The compounds segregated into three general groups by the toxicity thresholds observed in all four cell lines: low, intermediate, and high. Overall, the least toxic compounds were NE, NP, and GLYC with maximal toxic threshold values at 1 mM. These compounds were followed by an intermediate toxicity group that included the higher-order nitroalcohols MNPD and HNPD with maximal toxicity thresholds at 0.4 mM. The group of most toxic compounds in our cell toxicity studies included GLUT, FA, GP, and BP, which showed maximal toxicity threshold values at 0.01 to 0.02 mM (Tables 1–6). Some

variability was noted in the response of different cell types to a given agent. However, the toxic thresholds, in general, were remarkably similar across different cell types. The exception was GLYC, which showed a variable response. The toxicity threshold was very high (i.e., relatively nontoxic) with human skin fibroblasts (HSFs) and primary BCECs. For HSFs, the level was even higher than for the mononitroalcohols NE and NP. However, using ARPE-19 cells, the threshold was much lower (i.e., more toxic) than that of the mononitroalcohols, on par with the nitrodiol MNPD. Alternatively stated, ARPE-19 cells were particularly sensitive to GLYC.

Comparisons to FA and GLUT regarding cross-linking efficacy and toxicity can provide added perspective, since such agents are in widespread use and have provided decades of efficient cross-linking of a variety of tissues for purposes other than in vivo therapeutic cross-linking. In the case of FA and GLUT (using all four cell types), relatively low toxicity was shown in the first trial using reagents that had been kept under room temperature storage conditions for >6 months. Because GLUT and FA are known to form polymers during storage conditions,²³ we repeated the experiments with newly acquired reagents with guaranteed monomeric compound, using two cell lines, HCECs and HSFs. We found that significant toxicity differences existed between reagent preparations. Commercial GLUT solutions can contain a mixture of monomers and polymers. Although we used GLUT and FA solutions free of polymers and other contaminants as packaged (Cat No. 16020; Electron Microscopy Sciences), these solutions had been obtained for a previous experiment and had been stored at room temperature in the interim. When our initial results using these reagents showed relatively moderate toxicity, we considered the possibility that the reagent had degraded and repeated the experiment by using freshly obtained reagents. Using these new reagents, the GLUT toxic threshold decreased 40 to 60 times as compared with new GLUT (Tables 3, 4) and the FA toxic threshold decreased 10 to 40 times as compared to new FA (Tables 3, 4). In other words, we found that the older GLUT/FA was significantly less toxic than the fresher preparations. We suspect that the older preparations may have polymerized, diminishing the content

TABLE 3. Results of Toxicity Testing Using HSFs

Human Skin Fibroblasts											
Concentration, mM	NE	NP	GLYC	MNPD	HNPd	FA, o	GLUT, o	FA, n	GLUT, n	GP	BP
10	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Fixed	Fixed	Gone	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed
5	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Fixed	Fixed	Gone	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed
1	Dead	Dead	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Fixed	Fixed		Fixed	Fixed			Gone	Fixed	Fixed	Fixed
0.8	Dead	Dead	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Fixed	Fixed		Fixed	Fixed			Gone	Fixed	Fixed	Fixed
0.6	Alive	Dead	Alive	Dead	Dead	Dead	Alive	Dead	Dead	Dead	Dead
		Fixed		Fixed	Fixed			Gone	Fixed	Fixed	Fixed
0.4	Alive	Alive	Alive	Alive	Dead	Alive	Alive	Dead	Dead	Dead	Dead
					Fixed			Gone	Fixed	Fixed	Fixed
0.2	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Dead	Dead
								Gone	Fixed	Fixed	Fixed
0.1	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Dead	Dead
										Gone	Fixed
0.01	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive
0.001							Alive	Alive	Alive	Alive	Alive
Control	Alive									Alive	Alive

Categories and abbreviations are as listed for Table 2.

of reactive moieties, thereby resulting in lesser cytotoxicity. This view corroborates with the histologic fixation differences known to exist for polymeric and monomeric GLUT/FA. In other words, the fixative function of GLUT/FA is superior for nonpolymerized (i.e., monomeric) compound preparations.^{24,25}

Considering that CXL results in both cross-linking effect as well as complete keratocyte death in the treated area, having some idea of the balance between toxicity and cross-linking effect becomes a central issue for developing topically applied agents. It is currently unclear as to whether it is possible to cross-link extracellular matrix proteins, yet keep the adjacent cells alive. This undoubtedly will depend on the balance between toxicity and efficacy. It is understood at the outset that no conclusions may be drawn from toxicity/fixation (tox/fix) margin information using cell culture studies such as those reported here. That being said, we would like to point out that most of the agents we studied showed a gap or margin between the toxic threshold (i.e., the concentration at which all cells were alive) and concentration at which all the cells

were dead and fixed. This could provide some indication as to which compounds might be able to be effective as in vivo therapeutic tissue cross-linking agents and yet be nontoxic to adjacent cells. A note of caution is included here, as referencing the data in this way does not take into account any differences in reactivity to cross-linking agents between a cell membrane and extracellular connective tissue protein such as collagen fibrils or the proteoglycan component of the extracellular matrix. In addition, we did observe differences in tox/fix margins between cell types. For HCECs, GP showed the narrowest margin at 0.09 mM. Human skin fibroblasts were particularly susceptible to fixation by the nitroalcohols, with the narrowest tox/fix margin observed at 0.09 mM for BP, and 0.2 mM for NE, NP, MNPD, and HNPd. For primary BCECs, GP again showed the narrowest tox/fix margin at 0.9 mM. Finally, for human ARPE-19, the narrowest tox/fix margin was observed for GP/GLUT at 0.19 mM and 0.2 mM for HNPd. Interpreting the data in this way would indicate that GP and

TABLE 4. Results of Toxicity Testing Using Primary Cultured BCECs

Primary Bovine Corneal Endothelial Cells										
Concentration, mM	NE	NP	GLYC	MNPD	HNPd	FA, o	GLUT, o	GP	BP	
10	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Gone	Gone	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed
5	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
	Gone	Gone	Gone	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed	Fixed
1	Alive	Dead	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Dead
		Gone		Gone	Gone	Gone	Gone	Fixed	Fixed	Gone
0.1	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Dead
								Fixed	Fixed	Gone
0.01	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive
Control	Alive									

Categories and abbreviations are as listed for Table 2.

TABLE 5. Results of Toxicity Testing Using Human Retinal Pigment Epithelial Cells (ARPE-19)

Human Adult Retinal Pigment Epithelial Cells, ARPE-19									
Concentration, mM	NE	NP	GLYC	MNPD	HNPd	FA, o	GLUT, o	GP	BP
10	Dead Gone	Dead Gone	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed
5	Dead Gone	Dead Gone	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed	Dead Fixed	Dead Gone
1	Alive	Alive	Dead Fixed	Dead Fixed	Dead Fixed	Dead	Dead Fixed	Dead Fixed	Dead Gone
0.8	Alive	Alive	Dead	Dead Gone	Dead Fixed	Dead	Dead Fixed	Dead Fixed	Dead Gone
0.6	Alive	Alive	Dead	Dead Gone	Dead Fixed	Dead	Dead Fixed	Dead Fixed	Dead Gone
0.4	Alive	Alive	Dead	Dead Gone	Alive	Dead	Dead Fixed	Dead Fixed	Dead Gone
0.2	Alive	Alive	Dead	Partially dead	Alive	Alive	Dead Fixed	Dead Fixed	Dead Gone
0.1	Alive	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Dead Gone
0.01	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Dead Gone
0.001						Alive	Alive	Alive	Alive
Control	Alive								

Categories and abbreviations are as listed for Table 2.

possibly a nitroalcohol might have the best chance of inducing cross-link changes without killing cells.

DISCUSSION

There are hundreds of cross-linking agents that have been used for a variety of purposes including industrial as well as biomedical applications. The areas of application are vast and there is significant overlap. For example, FA²⁶ and GLUT,²⁷ two of the most widely used cross-linking agents, are used in the production of resins for manufacturing plywood, textiles, and others, as well as for leather making and rubber hardening for tires. They have also been used in important biomedical applications such as fixation of bioprostheses (i.e., heart valves, skin-grafting materials), biological scaffolds, hydrogels, and tissue fixation for histology/pathology. Some of the many commercially used cross-linking agents include other aldehyde agents such as glyceraldehydes¹¹ and methyl glyoxal²⁸; the group of carbodiimides,²⁹ GP;³⁰ imidoesters such as dimethyl adipimidate (CAS No. 14620-72-5) and dimethyl suberimidate (CAS No. 34490-86-3), Denacol-epoxys (CAS No. 930-37-0), derivatives of ethylene glycol dimethacrylate (CAS No. 2274-11-5), derivatives of methylenebisacrylamide (CAS No. 110-26-9), and divinyl benzene (CAS No. 1321-74-0). The lists are seemingly endless.

Although many of these compounds are excellent cross-linking agents for commercial, industrial, and other in vitro

cross-linking applications, the list of available agents is much more limited when considering their possible use as in vivo tissue cross-linking agents. Several aspects that are less relevant when considering in vitro use become critical when considering their clinical use. Such considerations for the cornea include efficacy under physiologic pH and temperature, permeability, coloration and effects on light transmission, and cell toxicity. The latter includes cytotoxicity, organismal toxicity, and genotoxicity (i.e., mutagenicity/carcinogenicity).

Genipin is a well-known, effective cross-linking agent derived from the *Gardenia jasminoides* plant. It has found utility in a number of applications, including fixation of cardiac valves,³¹ formation of collagen biospheres,³² fingerprinting in forensics, and tissue engineering fields, where it has been used to enhance mechanical properties.³³⁻³⁵ As well, its use as a corneoscleral cross-linking agent has been proposed.^{15,36} Its biocompatibility has been described as superior to conventional cross-linking using agents such as GLUT³⁷ with low toxicity, a low inflammatory response, and low long-term toxic release with high cross-linking activity.³⁸ In corneal cross-linking studies, GP produces a significant increase in biomechanical strength³⁶ with very low endothelial damage.¹⁵ However, in our study, comparison with other chemical cross-linking agents, including monomeric GLUT, indicated that GP is relatively toxic (albeit with strong fixation capability) by comparison to these agents.

TABLE 6. Summary of Toxic Thresholds for Nine Cross-Linking Agents With Four Different Cell Lines

	NE	NP	GLYC	MNPD	HNPd	FA	GLUT	GP	BP
Immortalized human corneal epithelial cells	0.8	0.6	0.4	0.2	0.1	0.01	0.01	0.02	0.02
Human skin fibroblasts	0.6	0.4	1	0.4	0.2	0.01	0.01	0.01	0.01
Cultured bovine corneal endothelial cells	1	0.1	1	0.1	0.1	NA	NA	0.01	0.01
Immortalized human retinal pigment epithelial cells (ARPE-19)	1	1	0.1	0.1	0.4	NA	NA	0.01	0.001

NA, not available.

TABLE 7. Some Available Representative LD₅₀ and Genotoxicity Data for the Compounds Tested in This Study

Cross-Linking Agent	LD ₅₀ Mouse Oral, mg/kg	LD ₅₀ Rat Oral, mg/kg	LD ₅₀ Mouse IP, mg/kg	LD ₅₀ Rat IP, mg/kg	Carcinogenicity/Mutagenicity Testing
NE	NA	NA	2100 ⁵⁹	NA	Neg (by NTP)
NP	NA	NA	NA	NA	Neg (by NTP)
GLYC	NA	NA	3000 ⁶⁰	2000 ⁶¹	Equivocal
MNPD	4000 ⁶²	NA	1600 ⁵⁹	NA	NA
HNPD	1900 ⁶²	NA	4000 ⁵⁹	NA	Neg
GLUT	100 ⁶³	123–650 ⁶³	13.9 ⁶³	17.9 ⁶³	NA
FA	42 ⁶⁴	84.8–100 ⁶⁵	16 ⁶⁶	NA	NA
GP	237 ⁶¹	NA	NA	NA	Equivocal ^{30,58}
BP	270–350 ⁶⁷	180–400 ⁶⁷	20–32.8 ⁶⁸	22–200 ⁶⁹	NA

IP, intraperitoneal injection; Neg, negative; NTP, national toxicology program.

Also of note, Hwang et al.³⁹ have studied optical changes induced by GP in collagen hydrogels and noted the development of strong autofluorescence with emission maxima shifts dependent on excitation wavelength. Genipin is well known to induce blue coloration effects, and similar iridoid compounds produce a variety of pigments. Thus, changes in corneal transmission and/or fluorescence could be an important consideration here. That being said, it is unclear as to whether the concentrations needed for effective GP cross-linking would simultaneously alter corneal visible light transmission, since we noted as others have, that GP is a relatively potent cross-linking agent and may require only small concentrations of reagent for inducing effects.

Glyceraldehyde is a three-carbon-sugar aldehyde (aldotriose) well known to the field of glycation research where it has been used in reactions to form products of nonenzymatic glycation products.⁴⁰ In the area of therapeutic cross-linking, GLYC has been proposed for use by Wollensak¹³ as a scleral-based treatment of progressive myopia in studies involving human and porcine sclera and in rabbit models. Glyceraldehyde has been shown to increase Young's modulus in porcine sclera in vitro by uniaxial strip testing by up to 419%.⁴¹ Glyceraldehyde cross-linking of scleral collagen has also been shown to increase biomechanical rigidity⁴² and increases scleral thermomechanical stability.⁴³ Glyceraldehyde cross-linking of cornea and sclera has also been studied by other research groups.^{44–46}

In our previous studies we have shown that aliphatic β nitroalcohol can cross-link collagenous tissue under physiologic conditions and these agents could be used as potential pharmacologic alternatives to UV-A riboflavin to stiffen corneoscleral tissues for keratoconus and related disorders.^{47,48} Nitroalcohols can function as an FA delivery system under conditions of physiologic pH and temperature.⁴⁹ In addition, their small size and water solubility favor permeability,⁵⁰ have little effect on light transmission,⁴⁷ and furthermore, although they function to deliver FA, are considerably less toxic than FA⁴⁸ and test negative in genotoxicity testing (National Toxicology Program 2012). In addition, mechanistic and catalytic studies of β -nitroalcohol cross-linking with polyamine suggest that modulation of the cross-linking reaction to speed the reaction using nitroalcohols may be possible.⁵¹

In an earlier study,¹⁶ we have reported cytotoxic thresholds for NE and NP using primary BCECs. In that study the threshold values were slightly higher than in the current study but in a similar range (1–3 mM). This slight difference is likely because of batch-to-batch variability inherent in using an unestablished cell line. Since these BCECs are not an established cell line (i.e., we prepare them fresh by using available bovine corneas), each cell batch could be slightly

different with regard to compound sensitivity. In the present study, the toxicity of each order of nitroalcohol correlated with the amount of released FA. For example, the mononitroalcohols NE and NP were the least toxic, the diol more toxic, and the triol was more toxic than the diol (except with ARPE-19 cells). The brominated nitrodiol BP turned out to be one of the most toxic agents in our experimental system. Bronopol is known to decompose into several products, including 2-bromonitroethanol, bromine, and nitrite, any of which could contribute to cytotoxicity.⁵² Bronopol has a rather lengthy history of use as a chemical preservative in personal care products such as cosmetic products⁵³ and food preservation,^{54,55} where it is used to enhance shelf life and storage. Bronopol has not generally been used as a cross-linking agent but has been known to function as an FA-donating agent⁵⁶; therefore, released FA from BP may induce cross-linking as with other aldehyde cross-linking agents.

The findings from our study are in general agreement with another form of toxicity data that can provide some level of relative toxicity between compounds, LD₅₀ values for rodents (Table 7). Such toxicity testing for FA and GLUT has been extensive, since these agents are viewed as potential occupational hazards, particularly relevant to industrial and commercial uses. Thus, an evaluation of available LD₅₀ information shows that the compounds in our high toxicity group (FA, GLUT, GP, and BP) also have the lowest LD₅₀ values for mice (i.e., most lethal), with the traditional fixatives FA and GLUT being most toxic, followed by GP and BP. The compounds in our intermediate (MNPD and HNPD) and low (NE, NP, GLYC) toxicity groups were noted to be relatively less lethal by comparison to the high toxicity group just described.

Some genotoxicity (carcinogenicity and mutagenicity) information is available for the compounds tested in this study. However, the information in this regard is incomplete but is included in Table 5. It should be noted that regarding GP, there is some disagreement in the literature as to whether GP shows relative genotoxicity or safety, particularly where it is being used in the tissue engineering field as summarized by Wang et al.⁵⁷ Such genotoxicity studies using GP were reported in 2000 (Tsai et al.³⁰) and 2002 (Ozaki et al.⁵⁸). Tsai et al.³⁰ have reported that up to a dose of 220 μ M, GP shows no genotoxicity by either micronucleus or sister chromatid exchange (SCE) assays in Chinese hamster ovary (CHO-K1) cells. However, Ozaki et al.⁵⁸ have reported that 8.8- to 44.2- μ M levels cause increases in SCE as well as the tetraploid index in V79 Chinese hamster cells. One reason for such differences are raised by Wang et al.⁵⁷ who report cell type-specific differences in alterations in gene expression whether using human fetal osteoblasts or primary porcine chondrocytes. Osteoblasts are 10 times more sensitive than chondrocytes to GP toxicity. Such cell type-specific differences, as well as

differences in exposure times, could account for the discrepancy between the genotoxicity reports of Tsai et al.³⁰ and Ozaki et al.⁵⁸ Tsai et al.³⁰ have used a Chinese hamster ovary (CHO-K1) cell line and exposed the cells to GP for 6 hours, while Ozaki et al.⁵⁸ have used a Chinese hamster lung cell line (V79) and exposed the cells to GP for 28 hours. In other words, Tsai et al.³⁰ have used a higher concentration for a shorter exposure time and Ozaki et al.⁵⁰ have used a lower concentration for a longer exposure time.

Finally, we would like to include comments regarding historical literature and comparisons that can be made with regard to the current in vivo application. A vast literature exists regarding tissue fixation using GLUT and FA, which has been in practice for many decades. That being said, gleaned information regarding the specifics of simultaneous toxicity/fixation from the literature is difficult for several reasons. First, this is a new application for tissue cross-linking, that is, inducing cross-linking in a living tissue. Second, most of these types of applications require an endpoint of fixation that is significantly greater than what we are attempting to achieve in therapeutic tissue cross-linking, be it light activated or non-light activated. Third, postmortem tissue is often used and so the tissue is already dead. Furthermore, the conditions for tissue cross-linking vary between studies in many potentially important ways. This includes differences in pH, temperature, concentration, duration of cross-linking, and substrate/tissue types. All of these factors can affect the cross-linking effect and can be modulated in vitro. As well, as we noted in this study, different preparations of FA and GLUT can have variable effects based on differences in the polymerization of cross-linking reagent. Variability in penetration of cross-linking reagent into tissues can also alter the induced cross-linking effect. Thus, without “head-to-head” comparison studies, it is difficult to compare studies owing to these variables. Regarding BP, there is very little literature available that we are aware of regarding it as a tissue cross-linking agent since its applications have largely been focused on its antibacterial preservative function, albeit via FA liberation.

In conclusion, there are significant differences of cell toxicity among topical cross-linking compounds, with mono-nitroalcohols and GLYC being the least toxic and GP and BP being the most toxic, for the same concentration. The question as to whether toxicity and fixation go hand in hand has yet to be determined. In other words, is cytotoxicity (or some degree thereof) necessary to induce therapeutic tissue cross-linking? The experience with riboflavin photochemical cross-linking suggests that cell death (keratocytes) may be necessary to induce a mechanical tissue effect, since keratocyte death is routinely encountered after CXL. Some degree of cell death may be acceptable, particularly if the cross-linking effect is significant. Are there pharmacologic cross-linking agents that may have properties that favor cross-linking efficacy over cell toxicity? Thus, the balance between tissue fixation (or cross-linking) and cell toxicity should be borne in mind as compounds and strategies to improve mechanical tissue properties through therapeutic tissue cross-linking continue to develop.

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