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Enzymatic Digestion of Porcine Corneas Cross-linked by Hypo- and Hyperosmolar Formulations of Riboflavin/ultraviolet A or WST11/Near-Infrared Light

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Purpose: To assess enzymatic digestion rate after Riboflavin (RF) and Water-Soluble-Taurine (WST11) based corneal cross-linking (CXL), with or without the addition of high molecular weight dextran (RF-D and WST-D).

Methods: Eighty-eight paired porcine corneas were cross-linked by either RF (n = 11) or RF-D (n = 11) and ultraviolet light (UVA), or WST11 (n = 11) or WST-D (n = 11) and near-infrared (NIR) light, or used as paired control (n = 44). Corneal buttons of treated and paired control eyes were placed in a 0.3% collagenase solution. Time to full digestion and remaining dry sample weight after six hours were compared.

Results: A strong treatment effect was seen with all four formulations, as all controls had been fully digested whilst all treated samples were still visible at the experiment's endpoint. After irradiation, central corneal thickness was significantly higher in samples treated with hypo-osmolar formulations, compared to dextran enriched formulations ($P < 0.001$). Dry sample weight after digestion was nonsignificantly different between corneas treated by the four different formulations ($P = 0.102$). Average dry sample weight was 1.68 ± 0.6 (n = 10), 2.19 ± 0.50 (n = 8), 1.48 ± 0.76 (n = 11), and 1.54 ± 0.60 (n = 9) mg, for RF, RF-D, WST11, and WST-D treated samples, respectively. Enzymatic resistance was similar for RF and WST based CXL ($P = 0.61$) and was not affected by the addition of dextran ($P = 0.221$).

Conclusions: Both RF and WST11 based CXL significantly increases resistance to enzymatic digestion, with similar effect for hypo-osmolar and hyperosmolar (dextran enriched) formulations.

Translational Relevance: Our findings indicate these formulations are interchangeable, paving the way for the development of novel PACK-CXL protocols for thin corneas and deep-seated infections.

Introduction

After its introduction in 2003 by Wollensak et al.,¹ Riboflavin/ultraviolet A (RF/UVA) cross-linking (CXL) has become a widely accepted treatment for

keratoconus (KC), and multiple long-term clinical studies have shown its safety and efficacy.²⁻⁴ The mechanism of action of RF/UVA CXL is thought to involve the formation of new bonds between the collagen bundles and surrounding proteoglycans within the corneal stroma.⁵ Besides increasing

Table. Studies Reporting on Enzymatic Digestion After Corneal Cross-Linking

Model	Essay	Treatment	Results	References
Porcine	Pepsin, trypsin, collagenase	RF/UVA	Slower digestion with higher irradiance	Spoerl et al. ²⁷
Porcine	Collagenase	RF/UVA	Increased resistance for both superficial and deeper crosslinked flaps	Schilde et al. ²⁸
Ox (fragmented) Porcine (acellular)	Collagenase Collagenase	SM+NM Genipin	Lower protein residue in controls Highly reduced digestion after Genipin treatment	Naderi et al. ²⁹ Liu et al. ³⁰
Porcine	Pepsin	RF/UVA	Highly reduced digestion after RF/UVA treatment	Hayes et al. ⁵
Bovine	MMPs	RF/UVA	Resistance to cleavage by MMPs 1, 2, 9, and 13	Zhang et al. ³¹
Human	Collagenase	RF/UVA	Increased resistance with longer irradiation up to 30 minutes	Arafat et al. ²²
Porcine	Collagenase	RB/GL	Reduced digestion after RB/GL treatment	Wang et al. ³²
Porcine	Pepsin	RF/UVA	Dresden protocol parameters most effective	Aldahlawi et al. ³³
Human (FS-LASIK)	Collagenase	RF/UVA	Increased resistance of deeper stroma after treatment	Kanellopoulos et al. ³⁴
Human	Collagenase	RF/UVA	Longer RF impregnation increases resistance	Laggner et al. ³⁵
Porcine	Pepsin	RF/UVA	Greater resistance with higher irradiation dose	Aldahlawi et al. ³⁶
Porcine (incl. epi-on) Rabbit	Pepsin Collagenase	RF/UVA RB/GL	Hypo-osmolar RF less effective RB/GL at high irradiance comparable to RF/UVA Dresden protocol	Aldahlawi et al. ³⁷ Fadlallah et al. ²¹
Human	Collagenase	RF/UVA	Dresden achieves greatest resistance, compared to accelerated protocols	Kanellopoulos et al. ³⁸
Human	Collagenase	VP/RL	VP/RL comparable to RF/UVA	Alageel et al. ¹²
Human (incl. trans-epi)	Collagenase	RF/UVA	Epi-on RF/UVA comparable to untreated controls	Cruzat et al. ³⁹
Mouse	Pepsin	RF/UVA	Digestion speed inversely correlated to biomechanical stiffness	Kling et al. ⁴⁰
Rat	Collagenase	RF/UVA	Threshold dose of 0.54 J/cm ² to increase resistance	Zhu et al. ⁴¹
Porcine	Pepsin	RF/UVA	Significant increase in resistance with higher RF concentrations	O'Brart et al. ¹⁸

SM, sulfur mustard; NM, nitrogen mustard; MMP, matrix metalloproteinases; RB/GL, rose Bengal/green light; FS-LASIK, femtosecond laser assisted in situ keratomileusis; epi-on, epithelium in situ; VP/RL, Verteporfin/red laser; trans-epi, transepithelial.

corneal biomechanical properties, corneal CXL was also shown to increase resistance against enzymatic digestion (Table). Increased proteinase activity and reduced expression of proteinase inhibitors in keratoconic corneas play an important role in corneal thinning, a hallmark of progressive disease.⁶ Increased

resistance to keratolysis is therefore an important mechanism of action in arresting keratoconus progression. Keratolysis is also a major complication of corneal infections, resulting in significant thinning and even corneal melting. RF/UVA CXL has therefore been suggested as a novel treatment modality for

infectious keratitis (IK), with promising preliminary results.⁷

Despite the revolution introduced by RF/UVA CXL in the treatment of KC, there are still drawbacks to overcome, mainly related to the toxic nature of UVA irradiation.⁸ To prevent irreversible endothelial damage and allow safe CXL treatment, corneal thickness has to be at least 400 μm .⁹ In corneal ectatic disorders such as KC and IK, this is often a problem because stromal thinning frequently progresses beyond this safety threshold. In the treatment of KC, several solutions have been suggested to overcome this problem, the most established of which is application of hypo-osmolar RF where dextran or other high molecular weight molecules are omitted from the formulation to induce corneal swelling above the 400 μm safety threshold.¹⁰

In IK, progressive corneal melting with subsequent stromal thinning often occurs rapidly after onset of the disease, *a priori* rendering these patients unsuitable for currently available RF/UVA CXL treatment. Thus patients suffering from IK may greatly benefit from a CXL modality that would allow treatment of thin corneas, postponing or preventing the need for invasive corneal transplantation.

To provide a safe alternative for patients with thin corneas, novel chromophores that can be excited at safer wavelengths have been investigated.^{11–13} In 2012, our group established the stiffening capabilities of a water-soluble bacteriochlorophyll derivative, water-soluble-*taurine* (WST11), which is excited by near-infrared (NIR) light at 755 nm.¹³ Because NIR light by itself in the applied intensities is nontoxic to the eye, corneal thickness may be reduced below the current threshold of 400 μm without endangering the corneal endothelium or deeper ocular structures.¹⁴ Although we have shown safe, efficient, and long-term stiffening in rabbits, enzymatic resistance of WST11/NIR CXL had not yet been determined.^{15,16} Moreover, in this study we compare for the first time corneal resistance to enzymatic digestion following application of hypo-osmolar and hyperosmolar (dextran enriched) RF, currently used in clinical practice.

Methods

Chromophore Formulations

Four different chromophore formulations were prepared: (1) hypo-osmolar riboflavin (RF), (2) hyperosmolar riboflavin (RF-D), (3) hypo-osmolar WST11 (WST11), and (4) hyperosmolar WST11 (WST-D). Riboflavin formulations were prepared

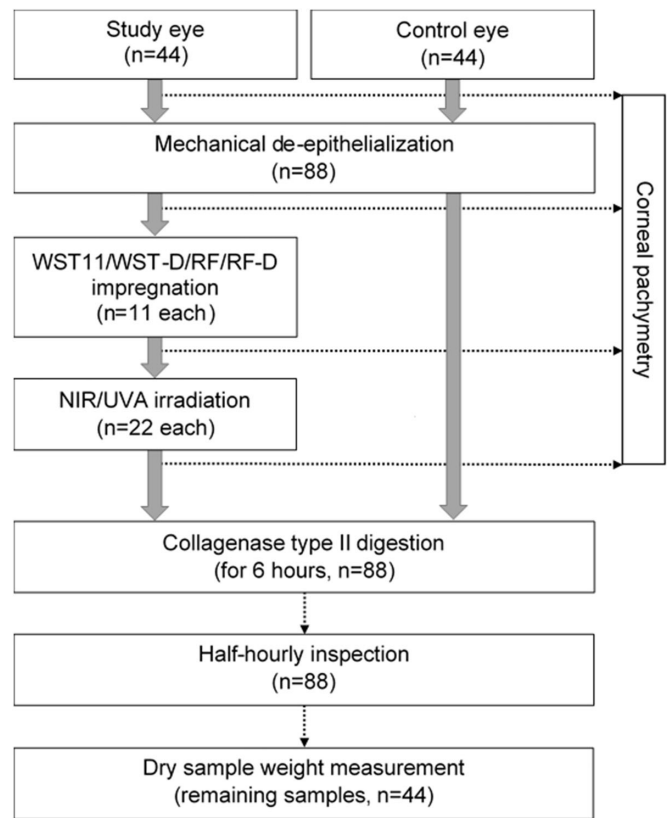


Figure 1. Study flowchart. Eighty-eight paired porcine eyes were mechanically de-epithelialized. One eye of each pair served as control ($n = 44$), whereas the contralateral eye was treated by one of the following: WST11 ($n = 11$) or WST-D (dextran enriched, $n = 11$) impregnation with consecutive NIR irradiation, or RF ($n = 11$) or RF-D ($n = 11$) impregnation with UVA irradiation. Between steps, if applicable, central corneal thickness was measured by ultrasound pachymetry. After treatment, samples were subjected to a collagenase type II digestion assay, during which samples were inspected half-hourly. After six hours dry sample weight of remaining samples ($n = 44$) was measured to quantify the degree of digestion.

from 0.1% Riboflavin-5'-phosphate (F6750; Sigma-Aldrich, St. Louis, MO, USA) in 0.9% saline solution and used as is (RF) or enriched by 20% dextran 500 kD (RF-D; Leuconostoc spp. Mr 450,000-650,000, Sigma-Aldrich). Similarly, WST11 formulations were prepared from 0.25% WST11 (Steba Laboratories, Rehovot, Israel) in 0.9% saline solution, without (WST11) or with addition of 20% dextran 500 kD (WST-D). All solutions were corrected to a pH 7.2 to 7.3.

Treatment Procedure

Figure 1 shows a flow chart of the applied procedures. Eighty-eight porcine corneas were obtained in pairs from a local abattoir within two hours of enucleation, in accordance to the Association for

Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.¹⁷ Per pair, one eye received full CXL treatment by either RF/UVA, RF-D/UVA, WST11/NIR, or WST-D/NIR ($n = 11$ per group), the contralateral eye ($n = 11$ per group) serving as paired control. Central corneal thickness (CCT) was determined for all corneas as an average of five consecutive measurements using an ultrasound pachymeter (Humphrey ultrasonic pachymeters; Humphrey Instruments, San Leandro, CA, USA) at four different time points: (1) before and (2) after de-epithelialization, (3) after chromophore impregnation, and (4) irradiation, if applicable. The corneal epithelium of all corneas, both treated and control, was removed mechanically up to the limbus using a blunt hockey knife. After de-epithelialization, a corneoscleral button was cut from the control eyes, tightly wrapped in clingfilm and aluminum foil, and frozen at -80°C until further processing. Eyes in the treatment group were treated according to established protocols.^{1,13} In short, eyes were impregnated through one of the aforementioned chromophore formulations by placing a chromophore filled plastic cup on top of the cornea for 30 (RF/RF-D) or 20 (WST11/WST-D) minutes. The corneas were then placed under UVA LED (RF/RF-D) or NIR laser light source (WST11/WST-D), calibrated to deliver $3\text{ mW}/\text{cm}^2$ or $10\text{ mW}/\text{cm}^2$, respectively, to the corneal surface for 30 minutes. During irradiation the corneas were topically rehydrated in five-minute intervals to avoid dehydration. Corneoscleral buttons were then cut and frozen, similarly to the control group, until further testing.

Enzymatic Assay

After defrosting, the corneoscleral buttons were unwrapped and a central 8mm button was punched and transferred to 12-well plates. Each well contained 2 mL of freshly prepared and cooled 0.3% collagenase of the same batch (420 u/mg; Type 2, Worthington Biochemical Corporation, Lakewood, USA) and Dulbecco's phosphate buffered saline solution (Biological Industries, Kibbutz Beit-Haemek, Israel) solution. The solution was measured to ensure a stable pH of 7.38, which allows for collagenase's optimal activity. Samples were covered in aluminum foil to prevent light exposure and transferred to a shaker rotating at 170 rpm in an incubator set at 37°C . A collagenase digestion assay that is most commonly used in studies on corneal enzymatic digestion was applied (Table). Testing parameters were calibrated to achieve full digestion of native corneal samples in approximately five hours. Pilot studies showed this timeframe

allowed for optimal differentiation between treatment groups, without the need to renew the collagenase solution as it loses its activity over time, deemed undesirable because fluid exchange may disrupt the remaining corneal tissue.

Quantification of Digestion

Pilot studies showed initial swelling in anterior-posterior direction, after which the posterior stroma detached and completely dissolved, leaving a thin lamella of anterior stroma behind. The dimensions of this remaining lamella appeared to remain quite stable over time in the CXL treated groups, thus measuring the samples' surface dimensions provided incomplete data. Therefore we adopted the approach established by O'Brart et al.¹⁸ to measure the samples' dry weight. During a six hours digestion phase in the collagenase solution, sample appearance ($n = 88$) was assessed every 30 minutes by a blinded observer (J.B.) and time to full digestion was noted if no tissue was seen anymore. After six hours, the visibly remaining samples ($n = 44$) were transferred to Eppendorf containers and placed in a lyophilizer (Gamma 2-16 LSCplus; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) set at 0.2 mbar for 72 hours to fully dry the remaining corneal samples. Consecutively, the dry sample weight was determined. All measurements were done blinded from the received treatment.

Statistical Analyses

Baseline CCT characteristic were analyzed for control and treated groups, and for hypo-osmolar and hyperosmolar formulations using a one-way analysis of variance (ANOVA). Similarly, the sample's dry weight of treated corneas amongst chromophore groups and dextran addition was analyzed using a one-way ANOVA. The level of statistical significance was set at 0.05 for all analyses. Statistical calculations were done with SPSS software (version 25; IBM Corp., Armonk, NY, USA).

Results

Baseline Characteristics

Figure 2 shows the CCT per chromophore and treatment group before de-epithelialization, after de-epithelialization, and for the treated eyes after chromophore impregnation and irradiation. CCT at baseline was comparable between chromophore groups for both control ($P = 0.148$) and treated

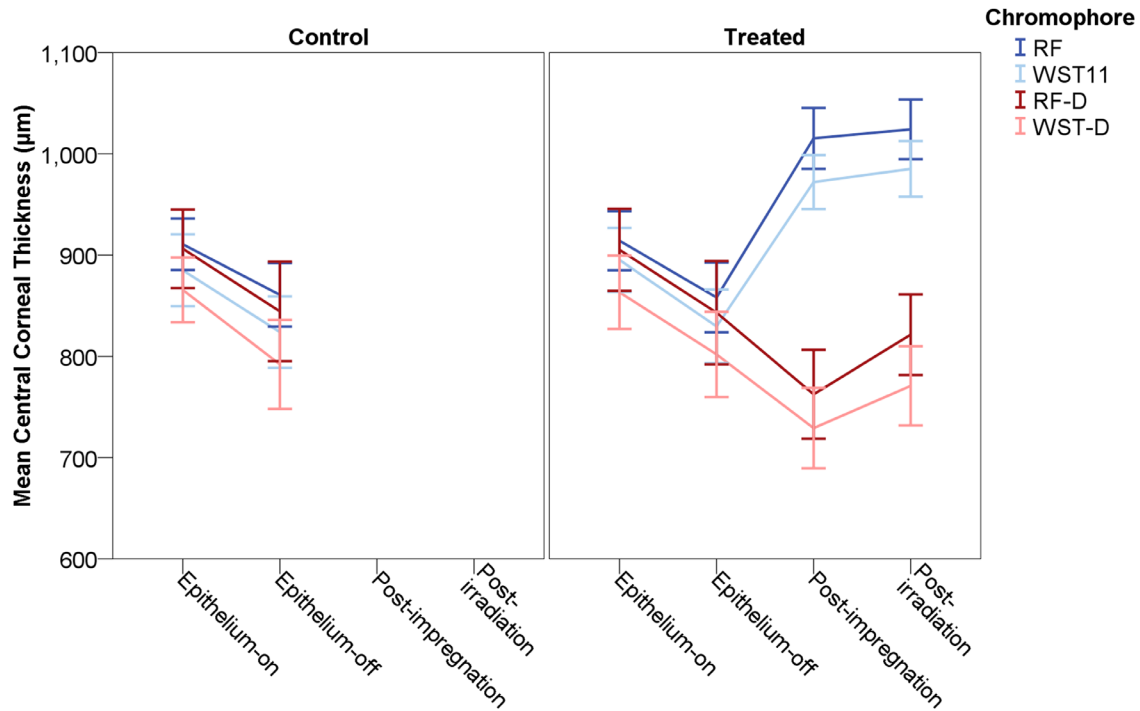


Figure 2. Mean central corneal thickness (CCT) for control ($n = 44$) and treated ($n = 44$, $n = 11$ per chromophore) corneas. CCT was determined for each sample as the average of five consecutive measurements, and was repeated at different timepoints: (1) epithelium-on, (2) epithelium off, (3) post-chromophore impregnation (treated group only), and (4) postirradiation (treated group only). At baseline, no significant difference in CCT was seen between chromophore groups for both control ($P = 0.148$) or treated ($P = 0.124$) corneas. Hypo-osmolar chromophore impregnation induced significant swelling compared to impregnation by hyperosmolar formulations ($P < 0.001$). CCT in micrometer, error bars indicate 95% confidence interval.

($P = 0.124$) eyes. As expected, samples were significantly ($P < 0.001$) thicker after impregnation by hypo-osmolar chromophore formulations, compared to impregnation by hyper-osmolar formulations.

Residual Sample Weight

All control samples ($n = 44$) had been completely digested. In the treated group ($n=44$), average dry sample weight measurements read 1.68 ± 0.6 ($n = 10$), 2.19 ± 0.50 ($n = 8$), 1.48 ± 0.76 ($n = 11$), and 1.54 ± 0.60 ($n = 9$) mg, for RF, RF-D, WST11, and WST-D treated samples, respectively. Six samples were excluded from analysis, as after 72 hours in the lyophilizer the Eppendorfs containers of these samples still contained clearly visible liquid due to blockage of the venting holes. No significant difference in dry weight was seen between the four chromophore formulations (between-subject effect, $P = 0.102$). Similarly, no significant differences were seen between RF-based (RF and RF-D) and WST11-based (WST11 and WST-D) formulations (between-subject effect, $P = 0.061$), between hypo-osmolar (RF and WST11) and hyper-osmolar

(RF-D and WST-D) solutions (between-subject effect, $P = 0.221$).

Time to Full Digestion

All treated samples remained after six hours of digestion, while all control samples had been completely digested. For the control samples, average time to digestion was 5.4 ± 0.2 , 5.4 ± 0.4 , 5.5 ± 0.3 , and 5.5 ± 0.4 hours in the RF, RF-D, WST11, and WST-D subgroups ($n = 11$ each) respectively. No difference in time to full digestion was seen between the four chromophore groups ($P = 0.864$).

Discussion

RF/UVA corneal CXL has been shown to effectively arrest KC progression, revolutionizing the treatment of KC.²⁻⁴ The induced CXL by RF/UVA application relies on the formation of new bonds within the collagenous stroma, shown to increase corneal

stiffness and resistance against enzymatic digestion.⁵ Its success and limitations have led to the investigation of new drug formulations or chromophores and light combinations like WST-D/NIR for preventing potential toxicity induced by the RF/UVA application.^{11,13} The bacteriochlorophyll derivative-WST11 is a novel drug, developed as photogenerator of hydroxyl and superoxide radicals. Although approved as first-line treatment for localized prostate cancer in the setting of intravenous infusion, we have looked at the possible utilization of WST11 in the setting of topical application for the treatment of KC. We showed significant and persistent stiffening of the cornea *in vivo*, with X-ray diffraction and electron microscopy studies showing unaltered corneal microstructure and transparency.^{13,19} Increased proteolytic activity is believed to be a major driver of disease progression in KC and corneal melting in IK.²⁰ Thus, after establishing biomechanical and structural effects of WST11/NIR treatment, in this study we assess its effect on resistance against enzymatic digestion.

This study shows highly increased resistance against enzymatic digestion by both RF and WST11 based corneal collagen CXL in both treatment types. Several studies evaluated the keratolytic resistance of different CXL protocols (Table), mainly focusing on RF/UVA. Although the time lag for full digestion of untreated control samples in our study is in line with previous publications, direct comparison should consider differences in treatment protocols and available information regarding study parameters—in particular the enzymatic activity of the applied assay.^{12,21,22} Therefore we chose to directly compare WST/NIR and RF/UVA CXL. All four formulations showed a very strong reduced rate of enzymatic digestion after treatment, indicating the keratolytic resistance of both RF/UVA and WST/NIR derived corneal stiffening. This may be in part the mechanism of action in arresting keratoconic progression and makes CXL techniques interesting in the treatment of IK.

To maintain physiological corneal thickness, clinically applied RF contains dextran, which can be omitted or reduced in thin corneas to induce corneal swelling. If corneal swelling can increase corneal thickness above the threshold of 400 μm CXL safety can be guaranteed. If this threshold cannot be achieved, patients may require transplantation surgery. This safety mechanism relies on achieving a minimal stromal RF concentration, such that sufficient UVA is attenuated before it reaches deeper ocular structures, so as not to induce a photochemical response near the fragile endothelium. In contrast, WST-D is activated by NIR light at an intensity that it is safe to ocular

structures.²³ Thus WST-D/NIR safety profile can rely on the diffusion profile of WST11 within the cornea, rather than light attenuation, as no endothelial damage will occur without WST11 present at the endothelial level. WST11 penetration depth can be controlled by the addition of high molecular weight dextran at different concentrations, as dextran concentration is inversely related to WST11 penetration.¹³ It is this control on penetration depth, combined with the application of safe NIR light, which may give WST-D/NIR CXL clinical relevance because it may allow for safe corneal CXL on thin corneas when RF/UVA CXL cannot be applied.

Besides the treatment effect of both chromophores, our results indicate no statistically significant difference in resistance to enzymatic digestion between the four formulations tested or between dextran-free and dextran-enriched formulations. Although not statistically significant, a notable higher residual dry weight is seen in favor of RF-D. Considering the dry weight of a nontreated porcine cornea is 9.1 ± 0.5 mg, 18.5%, 24.1%, 16.3%, and 16.9% stromal tissue remained in this study for RF, RF-D, WST11, and WST-D treated corneas, respectively.²⁴ Although RF-D may result in more residual stromal tissue (5.6%–7.8% compared to the other three formulations), the other formulations provide greater safety due to induced swelling and utilization of nontoxic irradiation, as described above. This is of particular importance in the treatment of IK, as IK is often associated with corneal thinning rendering corneal thickness below the safety threshold of 400 μm for RF-based CXL. This balance between efficacy and safety may present a clinical dilemma.

As with previous studies investigating the enzymatic resistance of CXL methods, the *in vitro* model has limitations in mimicking the *in vivo* situation. Although it provides a good comparison between techniques, the clinical relevance remains to be determined and residual dry sample weight may not correlate directly to the clinical effect or relevance. *In vivo* studies are needed to further assess treatment efficacy in IK. Most *in vivo* studies have proven to be challenging, as the degree of infection is hard to control and the time point for performing CXL is debated. Current available studies on PACK-CXL were mainly conducted in a relatively advanced stage, with deep infectious penetration, often as a last resort.^{25,26} Early CXL treatment may result in better effect as the treatment is predominantly effective in the anterior stroma. For deeper infections, potential benefits of WST-D/NIR CXL should be investigated, given its earlier described safety profile allowing to treat thinner corneas, and deep tissue penetration of NIR.

In conclusion, this study shows that both WST11 and RF based CXL have a strong anti-keratolytic effect, which may underlie the mechanism of action in both arresting keratoconus progression and preventing stromal melting in IK. Reducing dextran concentration is used clinically to allow for CXL in severely affected corneas, but its effect on the antikeratolytic effect of CXL had not been investigated previously. Our results show that the addition of 20% high molecular weight dextran does not affect keratolytic resistance. This finding is important in CXL of thin corneas, as often seen in progressed KC and IK. WST-D/NIR CXL may provide a safe alternative in thin corneas due to the differences in safety profile. Although RF/UVA CXL has shown great results in arresting KC, studies investigating other CXL techniques should be encouraged to better personalize and broaden the application of CXL treatment.

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