Supplement A:

**Details and background of the PACK-CXL protocol modifications evaluated in this study**

1) Acceleration of the PACK-CXL treatment using higher irradiation intensities is desirable since it facilitates the delivery of (effect boosting) higher energy PACK-CXL doses and decreases treatment time and costs. Recent reports have demonstrated accelerated CXL to be safe in patients [1-4]. The oxygen dependence of the cross-linking effect of CXL could be one reason for the decrease in biomechanical stiffening as a result of CXL acceleration [5-8]. Currently, there is no consensus on how acceleration influences the tissue stabilizing and antimicrobial effects of CXL [7, 9-13].

2) Increasing the total dose of irradiation energy delivered to the treatment area (fluence) increases treatment effect [13-15]. Moderate fluence increases (7.2 J/cm$^2$) led to significantly longer enzymatic digestion times in porcine corneas *ex vivo* compared to corneas cross-linked with routine fluences (5.4J/cm$^2$) [14]. Both Backman et al. and Kling et al. demonstrated large increases in antibacterial efficacy with large fluence increases from the routine 5.4 J/cm$^2$ up to 27 J/cm$^2$ [13, 15]. *In vitro* bacterial killing rates increased from 50% to >90% with double fluences of 10.8 J/cm$^2$ and to 100% with triple fluences of 16.2 J/cm$^2$ and higher [15-18]. Significant side effects are unlikely to occur with triple fluence protocols since topography-guided CXL protocols with regional fluences ranging from 7.2 to 15 J/cm$^2$ have been used for human keratoconus patients without detrimental effects [19]. Also, Nicklin et al. reported the use of 15-20 J/cm$^2$ fluences in preclinical studies on nonhuman primates and 10-15 J/cm$^2$ fluences in a clinical safety study on blind human eyes without negative effects on the corneal endothelium or retina [20].

3) An increased riboflavin concentration theoretically focuses the PACK-CXL effect in the superficial stroma and could therefore decrease effect depth [21]. At the same time, tripling the

concentration of riboflavin from the standard 0.1% to 0.3% increased resistance of corneal tissue to enzymatic digestion after CXL *ex vivo* [21-25].

4) The supplementation of $D_2O$ enhances the half-life of singlet oxygen and thus theoretically increases the number of created cross-links during PACK-CXL, increasing corneal stiffness [26]. Supplementation with $D_2O$ can potentially overcome oxygen depletion during accelerated protocols and might therefore increase the PACK-CXL-induced corneal resistance to enzymatic digestion, without affecting treatment depth. $D_2O$ concentrations under 30% are generally considered safe for systemic treatment [27].

5) Use of hydroxypropyl methylcellulose (HPMC) as a carrier drives riboflavin deep into the corneal tissue, potentially providing a deeper treatment effect, and improving tissue resistance to enzymatic digestion [28]. However, infectious keratitis is often accompanied by severe edematous tissue swelling of the corneal stroma, which can be reduced by using hypertonic concentrations of dextran. Due to the hydration-dependent stiffening effect of CXL, significant corneal edema may reduce tissue stabilization. [29-31]. Additionally, recent studies in keratoconus patients demonstrated worse visual outcomes with the use of HPMC compared to dextran [32].

6) A lack of riboflavin replenishment during the irradiation phase may increase treatment depth, by allowing riboflavin concentrations in the superficial stroma to decrease, and UVA to reach deeper layers with high riboflavin concentrations [23].

Supplement B:

**Materials and Methods:**

*PACK-CXL procedure*

Porcine eyes were obtained from the local abattoir and processed within 10 hours of slaughter. Only eyes with normal anterior segment, based upon focal light source examination, were included. Similar to previously reported methods [29-31], corneas were first de-epithelialized using a surgical blade and then saturated with riboflavin solution for a period of 20-30 minutes at room temperature. For the fluorescent imaging assay, the treatment border was first marked with a skin biopsy punch to create an eight mm diameter circle in the center of the cornea, within which the cornea was deepithelialized. Depending on the treatment group, corneas were saturated with 0.1% riboflavin and 20% dextran (Riboflavin solution for Corneal Cross-Linking (CXL), Peschke® D, PESCHKE Trade GmbH, Huenenberg, Switzerland); 0.1% riboflavin and HPMC (Riboflavin solution for Corneal Cross-Linking (CXL), Peschke® M, PESCHKE Trade GmbH, Huenenberg, Switzerland) or pharmaceutically prepared solutions of 0.4% riboflavin and 20% Dextran, or 0.1% riboflavin, 30% $D_2O$ and 20% Dextran, or 0.4% riboflavin, 30% $D_2O$ and 20% Dextran. See Tables 1a and 1b for details of the chromophores used in the various experiments. Excess riboflavin was removed prior to UVA irradiation.

Corneas were irradiated with an 11 mm (pepsin digestion assay) or 8 mm (fluorescent imaging assay) diameter beam of 365-nm UVA light (PXL Velvet 345, Peschke Trade GmbH, Switzerland). Riboflavin was replenished every 3 to 5 min during UVA irradiation and between additional UVA cycles (except for the no riboflavin replenishment group). Eyes in the control group were deepithelialized but did not receive riboflavin or UVA irradiation. 'Riboflavin treated/no UVA' and 'dextran treated/no UVA' control groups were not included since previous studies have demonstrated that corneas in such control groups have identical

resistance to enzymatic digestion to 'no riboflavin/no UVA' control eyes [7, 14]. The PACK-CXL protocol details are presented in Tables 1a and 1b, and Figure 2 illustrates the experimental protocols.

Central corneal thickness (CCT) was measured by ultrasound pachymeter (PachPen; Accutome) at two timepoints: after de-epithelialization and after saturation with riboflavin. In accordance with previously published protocols [29-31], and to reduce the variance in corneal hydration prior to UVA irradiation, the duration of riboflavin saturation was adjusted to the CCT measurements after de-epithelialization. Corneas with a CCT under 750μm, between 751 and 800μm, and exceeding 800μm, were saturated with riboflavin for 20 minutes, 25 minutes, or 30 minutes, respectively. Analysis of variance (ANOVA) testing was performed to detect differences in CCT between treatment groups (measured after riboflavin saturation). To detect differences between specific treatment groups, Tukey's 'Honest Significant Difference' method was used. Each experiment was analyzed separately. Forty missing values were recorded, which were considered to be "missing at random", since the missing values were due to equipment failure, which was not associated with the CCT values. Missing values were imputed with the function "missForest" in R. A paired T-test was used to check whether dextran saturation led to a significant reduction in CCT.

*Pepsin digestion assay*

After PACK-CXL treatment, full-thickness corneal buttons with an eight mm diameter were removed with a skin biopsy punch from the center of the irradiated area and placed in 5 ml tubes containing 4ml of a 5 % pepsin solution (Pepsin from porcine gastric mucosa, powder, ≥ 500 U/mg; SIGMA, catalog no. 77160) at a pH of 1.1.

Samples were incubated in the oven (Hybridization Oven/Shaker, Amersham Life Science) at 25$^{o}$C, shaken every other day and monitored for signs of digestion until measurement of the

dry weight at day 8 (DW8) or 16 (DW16) after incubation. Dry weight measurements were performed according to previously described methods [14, 22, 33, 34]. Briefly, the corneal button residues were removed from the pepsin solution and placed in the oven for 3 hours at 60°C. After this period, the samples were weighed on an analytical laboratory scale (Laboratory scale, OHAUS).

Prior to the main experiments, preliminary experiments were performed to validate the methods. The appropriate pepsin concentration, the corneal dry weight measurement timepoints, and the heating time necessary to arrive at a constant sample dry weight, were defined based on the results of these preliminary experiments.

Time to complete digestion and changes in corneal button diameter have been used as endpoints to quantify corneal resistance to enzymatic digestion in previous publications [7, 14, 33]. Neither of these endpoints were used in this study, since tissue fragmentation and turbidity of the pepsin solution increased with incubation time. As a result, tissue presence could not be quantified objectively after 20 days of incubation, significantly biasing the parameters "time to complete digestion" and "changes in corneal button diameter".

*Fluorescent imaging assay*

- **Sample preparation**

After PACK-CXL treatment, eyes were incubated in cell culture medium (ThermoFisher, MEM alpha, no nucleosides, catalog no. 22561021) for 24 hours at 20°C, with the cornea raised above the culture medium surface. Following incubation, the corneas were dissected, bisected, and placed in a 20% dextran solution for 45 minutes to reduce tissue swelling. The corneas were subsequently washed and cryopreserved. Six μm thick cryosections of each cornea were placed onto positively charged glass slides and further processed for fluorescent biomarker staining with DAPI (ProLongTM Diamond Antifade Mountant with DAPI, ThermoFisher

Scientific) and phalloidin (Alexa Fluor 488® phalloidin, ThermoFisher Scientific), according to product guidelines (full fluorescent biomarker staining protocol available as Supplement C).

- **Fluorescent imaging and image analysis**

Samples were imaged using a slide scanner (Digital Slide Scanner 3D Histech Pannoramic 250). All images were acquired using a 20 and 220 ms exposure time, and a 61% and 100% excitation, in the DAPI and Alexa 488 channels, respectively. Focus set up during imaging was automatically performed based on the DAPI signal.

Three cutouts of the treated central cornea were prepared per image using QuPath-0.2.3 [35]. All cutouts were oriented with the anterior side of the cornea on the left side of the image in Image J 1.53a (Figure 2c and 2d). The boundary between slide surface and deepithelialized cornea was manually marked to define the region of interest used for image analysis. An additional cutout from the untreated, peripheral, epithelialized cornea was prepared from each image as negative control. A dedicated CellProfiler [36] pipeline, available at GitHub repository under https://github.com/sstoma/ia-project-ems, was created to further process images for data analysis. Collagen compaction, and changes to the nuclear morphology and actin cytoskeleton of keratocytes in the anterior corneal stroma have previously been described as a result of epithelium-off CXL [37-39]. Cytoskeletal actin was identified in the Alexa 488 channels, by segmenting objects using the IdentifyPrimaryObjects module (Figure 2d.). In addition, evenly sized and spaced sampling regions were identified to measure the overall Alexa 488 fluorescence signal, including both background and cytoskeletal actin fluorescence, as a measure of stromal collagen compaction (Figure 2c). The final segmentation and its parameters were confirmed via visual inspection of the output. Images in which automatic segmentation resulted in too many artifacts were excluded from the final analyses (details of the selection available at GitHub repository under https://github.com/sstoma/ia-project-ems). As a result, 52 of 423 images were excluded from the cytoskeletal actin identification dataset. Different

morphometric and intensity-based features were computed for objects identified in the channels of interest and correlated to their distance from the anterior tissue edge. We created an additional computational channel, in which Alexa 488 was cleaned using background subtraction implemented in ImageJ [40].

- **Data analysis of CellProfiler output**

Analysis of CellProfiler output was performed with Python using Pandas [41], and plots were created using Seaborn [42] library. The script is provided at GitHub repository under https://github.com/sstoma/ia-project-ems. First, various annotation sources were merged, and an initial exploratory data analysis was performed for Quality Control. We pooled data based on the experimental groups. The error envelopes visible on plots for different experimental conditions mark variability between images (Figure 4). Data analysis was performed on cytoskeletal actin identification, and background and cytoskeletal actin fluorescence measurement data originating from the corneal tissue between 50 and 800μm from the corneal surface. The first 50μm of tissue was excluded from the data analyses to avoid fluorescent staining artifacts. Measurements originating from deeper than 800μm from the corneal surface were excluded from the data analyses, since the measurements typically reached a plateau at a maximum of 600μm from the corneal surface. In addition, absolute measurements were normalized and displayed as ratios, by calculating average baseline corneal tissue measurement values for objects in the 900-1000μm range from each image, and then dividing each absolute measurement by these average baseline corneal tissue values.

Stromal collagen compaction was analyzed based on the intensity of the non-specific background phalloidin-Alexa 488 fluorescence signal. The mean object counts were also analyzed in the phalloidin-Alexa 488 channel. The cytotoxic post-CXL effect was evaluated based on cytoskeletal fragmentation by counting the number of objects labeled with mean gray value intensities above the Alexa 488 channel threshold.

- **Automatic computation of the estimated treatment depth**

Assessment of the PACK-CXL treatment depth was based on the estimated collagen compaction depth. Treatment depth was estimated by computing a derivative of smoothed phalloidine background intensity values, aggregated by averaging different images of the same experimental condition, as a function of distance from the tissue edge. Computed derivative values were compared to an empirically established threshold. The first distance measurement from the endothelial side of the tissue sections for which the derivative reached a smaller value than the threshold was assumed to represent the PACK-CXL penetration depth. We assume this algorithm to identify the tissue depth at which structural collagen changes cease to be identified and collagen compaction stops. The details of this procedure are available in the GitHub repository under https://github.com/sstoma/ia-project-ems. To ensure the robustness of this analysis, we checked different thresholds and observed that the choice of threshold (within a reasonable range) does not change the qualitative results when comparing between conditions (code allowing for reproduction is available; tested derivate_thr = [-0.05, -0.01] every 0.005).

*Temperature assay*

Four porcine eyes were de-epithelialized and the corneas saturated for 20 minutes with 0.1% riboflavin (Riboflavin solution for Corneal Cross-Linking (CXL), Peschke® D, PESCHKE Trade GmbH, Huenenberg, Switzerland). Excessive riboflavin was removed, and the corneas were treated with an accelerated PACK-CXL protocol (5.4 J/cm$^2$, 2 minutes at 45mW/cm$^2$). PACK-CXL was repeated five times, without riboflavin solution replenishment between irradiation cycles, such that the corneas received a fluence of 32.4 J/cm$^2$. The temperature of the corneal surface was measured eight times: before riboflavin saturation, after saturation and after each irradiation cycle (Supplementary Figure 1). An infrared thermometer, which can obtain exact results from a distance, was used to measure the temperature of the corneal surface

(TIF 260, Standard infrared thermometer ebro ®, Xylem Analytics). To check whether increased doses of energy have an effect on the corneal surface temperature, we used a mixed model. To adjust for clustering, the eye was considered as a random effect in the model.

*Experimental layout*

Six hundred and eighty-eight corneas were used in the pepsin digestion assay, divided in five experiments. These experiments were performed in batches of 20 to 30 eyes, which were processed during the same day. In each batch, the corneas were randomly allocated to the control group or one of the various treatment groups representing the PACK-CXL protocol modifications investigated. In experiment 1 **(Acceleration)**, 10 minute 9 mW, and 2 minute 45 mW protocols were compared to 30 minute 3 mW, 5.4 J/cm$^2$ protocols (135 corneas). In experiment 2 **(Fluence)**, 10.8, 16.2, 21.6, 26 and 32.4 J/cm$^2$ protocols were compared to a 10 minute 9 mW, 5.4 J/cm$^2$ protocol (263 corneas). In experiment 3 **(Riboflavin concentration & $D_2O$ supplementation)**, 0.1 and 0.4% riboflavin concentrations were compared, and the supplementation of $D_2O$ was evaluated, using 10 minute 9mW, 5.4 J/cm$^2$ protocols (125 corneas). In experiment 4 **(Riboflavin carrier)**, HPMC was compared to Dextran as chromophore carrier using a 10 minute 9mW, 5.4 J/cm$^2$ protocol (122 corneas). In experiment 5 **(Riboflavin replenishment)**, two 10 minute 9mW + 2x 2 minute 45mW, 16.2 J/cm$^2$ protocols were compared, during which riboflavin was either replenished during and between irradiation cycles, or not (41 corneas). See Tables 1a and 1b for the detailed experimental layout.

Information gathered during the pepsin digestion assay allowed us to select promising PACK-CXL parameters for the fluorescent imaging assay. Based on the results we selected the 10 minute 9mW, 5.4J /cm$^2$ PACK-CXL protocol as the reference protocol. The following

treatment groups were evaluated in the fluorescent imaging assay: **(Control epi-on)** epithelium untouched (86 corneas); **(Control epi-off)** epithelium removed (25 corneas); **(Fluence 5.4 J/cm$^2$**) 10 minute 9 mW, 5.4 J/cm$^2$ fluence protocol (33 corneas); **(Fluence 5.4 J/cm$^2$, 0.4% Riboflavin**) 0.4% riboflavin, 10 minutes 9mW, 5.4 J/cm$^2$ fluence protocol (14 corneas)**; (Fluence 5.4 J/cm$^2$, HPMC**) HPMC as a carrier, 10 minutes 9mW, 5.4 J/cm$^2$ fluence protocol (12 corneas)**; (Fluence 16.2 J/cm$^2$**) 10 minute 9mW + 2x 2 minute 45mW, 16.2 J/cm$^2$ protocol (14 corneas); **(Fluence 16.2 J/cm$^2$, no Riboflavin replenishment**) riboflavin was not replenished during and between irradiation cycles (12 corneas)

*Data Analysis*

Analysis of variance (ANOVA) testing was performed to detect differences in CCT between treatment groups (measured after riboflavin saturation). To detect differences between specific treatment groups, Tukey's 'Honest Significant Difference' method was used. Each experiment was analyzed separately. Forty missing values were recorded, which were considered to be "missing at random", since the missing values were due to equipment failure, which was not associated with the CCT values. Missing values were imputed with the function "missForest" in R. A paired T-test was used to check whether dextran saturation led to a significant reduction in CCT.

Linear models were built to assess whether treatment type has an effect on corneal dry weight at day 8 (DW8) or day 16 (DW16). The model was adjusted for two variables: 1) CCT measurements after riboflavin saturation, 2) date at which corneas were processed. The outcomes - DW8 and DW16 - were analyzed separately for each experiment. Differences between specific treatment groups were assessed via Tukey's 'Honest Significant Difference' method. The sample sizes needed to detect differences in mean corneal weight between treatment groups were calculated based on reported standard deviations (SD of 7 [34], SD of 1

[14] and SD of 0.24 [22]), and a power of 80% and type one error of 5% were assumed. The minimum sample group size was 6 corneas per treatment group.

To check whether increased doses of energy have an effect on the corneal surface temperature, we used a mixed model. To adjust for clustering, the eye was considered as a random effect in the model.

The statistics program R 3.1.2., with packages multcomp and nlme were used for all statistical calculations. Data sets and R scripts are available at the GitHub (https://github.com/sstoma/ia-project-ems).

Supplement C:

**Fluorescent biomarker staining protocol.**

1. Wash tissue sections twice with PBS saline, pH 7.4.
2. Fix the sample in 3.7% formaldehyde solution in PBS for 10 minutes at room temperature.
   Note: Methanol can disrupt actin during the fixation process. Therefore, it is best to avoid any methanol containing fixatives. The preferred fixative is methanol-free formaldehyde.
3. Wash three times with PBS.
4. Place each coverslip in a glass petri dish and extract with 0.1% Triton X-100 in PBS for 5 minutes.
5. Wash three times with PBS.
6. Pre-incubate tissue with PBS containing 1% bovine serum albumin (BSA) for 20 minutes prior to adding the phallotoxin staining solution.
7. Dilute 5 µL stock solution into 200 µL PBS for each coverslip to be stained. To reduce nonspecific background staining with these conjugates, add 1% BSA to the staining solution. When staining more than one coverslip, adjust volumes accordingly. For a stronger signal, use 2 or 3 units per coverslip.
8. Place the staining solution on the coverslip for 30 minutes at room temperature (generally, any temperature between 4°C and 37°C is suitable). To avoid evaporation, keep the coverslips inside a covered container during the incubation.
9. Wash three or more times with PBS.
10. For long-term storage, the tissue should be air dried and then mounted in a permanent mounting medium. Use Diamond Antifade medium containing DAPI. Specimens prepared in this manner retain actin staining for at least six months when stored in the dark at 2–6°C.

*Required reagents*

- Phalloidin (Alexa Fluor 488® phalloidin, ThermoFisher Scientific) – prepare following manufacturers guidelines, including 1% BSA to minimize the amount of phalloidin that binds to the tube.
- PBS
- Methanol-free formaldehyde
- Triton X-100
- Mounting media – ProLongTM Diamond Antifade Mountant with DAPI, ThermoFisher Scientific
- Optional: BSA

Phalloidin is pH sensitive: at elevated pH, a key thioether bridge is cleaved and phalloidin loses its affinity for actin. Coverslips are to be kept in a covered container to minimize solution evaporation during incubation.

**Supplementary Table 1. Corneal central thickness before and after saturation with riboflavin.**

| | Pepsin digestion assay | | Fluorescent imaging assay | |
|---|---|---|---|---|
| | Central Corneal Thickness | | | |
| **Experiments** | **Before [µm]** | **After [µm]** | **Before [µm]** | **After [µm]** |
| *1: Acceleration* | | | | |
| Control | 720 ± 58 (554 – 826) | NA | - | - |
| 30 min | 762 ± 53 (606 – 853) | 659 ± 55 (438 – 740) | - | - |
| 10 min | 756 ± 43 (654 – 850) | 647 ± 44 (527 – 716) | - | - |
| 2 min | 764 ± 49 (666 – 872) | 659 ± 43 (438 – 740) | - | - |
| *2: Fluence* | | | | |
| Control | 741 ± 50 (642 – 898) | NA | 730 ± 43 (643 - 816) | NA |
| 5.4 J/cm$^2$ | 774 ± 54 (647 – 869) | 659 ± 39 (561 – 721) | 738 ± 46 (645 - 805) | 665 ± 45 (603 - 757) |
| 10.8 J/cm$^2$ | 765 ± 53 (673 – 890) | 665 ± 48 (568 – 758) | - | - |
| 16.2 J/cm$^2$ | 758 ± 43 (687 – 857) | 661 ± 41 (584 – 759) | 744 ± 36 (674 - 799) | 680 ± 39 (624 - 749) |
| 21.6 J/cm$^2$ | 762 ± 69 (628 – 990) | 663 ± 56 (562 – 858) | - | - |
| 27 J/cm$^2$ | 788 ± 44 (707 – 887) | 676 ± 30 (634 – 778) | - | - |
| 32.4 J/cm$^2$ | 768 ± 73 (649 – 971) | 664 ± 64 (554 – 860) | - | - |
| *3: Riboflavin concentration & $D_2O$ supplementation* | | | | |
| Control | 736 ± 56 (651 – 865) | NA | - | - |
| 0.1 $B_2$ | 718 ± 35 (672 – 800) | 592 ± 33 (515 – 656) | 744 ± 40 (681 - 819) | 628 ± 51 (472 - 677) |
| 0.1 $B_2$ + $D_2O$ | 738 ± 54 (627 – 823) | 584 ± 46 (456 – 654) | - | - |
| 0.4 $B_2$ | 756 ± 37 (634 – 810) | 626 ± 51 (507 – 707) | 743 ± 42 (671 - 837) | 622 ± 63 (466 - 736) |
| 0.4 $B_2$ + $D_2O$ | 744 ± 47 (667 – 846) | 605 ± 62 (495 – 731) | - | - |
| *4: Riboflavin carrier* | | | | |
| Control | 759 ± 58 (628 – 855) | NA | - | - |
| HPMC | 762 ± 36 (675 – 836) | 768 ± 39 (671 – 868) | 735 ± 45 (656 - 816) | 764 ± 53 (684 - 873) |
| Dextran | 760 ± 36 (703 – 849) | 673 ± 37 (603 – 759) | - | - |
| *5: Riboflavin replenishment* | | | | |
| Control | 710 ± 34 (632 – 764) | NA | - | - |
| Yes | 731 ± 35 (639 – 791) | 643 ± 47 (563 – 715) | - | - |

| | | | | |
|---|---|---|---|---|
| No | 730 ± 46 (659 – 807) | 640 ± 47 (571 – 732) | 738 ± 43 (674 - 810) | 706 ± 43 (634 - 781) |

Data presented as mean ± standard deviation (minimum – maximum); NA = not applicable

**Supplementary Table 2. Statistical analysis of corneal dry weight measurements at day 8 or 16.**

| | Day 8 | | | Day 16 | | |
|---|---|---|---|---|---|---|
| ***Experiments*** | **Sample size** | **Point estimate [weight in µg]** | **95% CI [weight in µg]** | **Sample size** | **Point estimate [weight in µg]** | **95% CI [weight in µg]** |
| *1: Acceleration* | | | | | | |
| Control | 14 | - 27.7 | from -54 to -1.35 | 14 | -41.4 | from -69 to -13.7 |
| 30 min | 18 | 15.2 | from 9.6 to 20.9 | 18 | 16.6 | from 11.2 to 22 |
| 10 min | 18 | 16.9 | from 11 to 22.7 | 18 | 16.5 | from 10.9 to 22.1 |
| 2 min | 18 | 20.4 | from 14.6 to 26.2 | 17 | 14 | from 8.7 to 19.3 |
| *2: Fluence* | | | | | | |
| Control | 31 | -14.1 | from -44.5 to 16.3 | 28 | -21.5 | from -36.8 to -6.3 |
| 5.4 J/cm$^2$ | 16 | 17.8 | from 10 to 25.5 | 14 | 13.7 | from 10.2 to 17.3 |
| 10.8 J/cm$^2$ | 16 | 24.2 | from 16.6 to 31.9 | 14 | 15.8 | from 12.2 to 19.4 |
| 16.2 J/cm$^2$ | 16 | 27.7 | from20 to 35.4 | 14 | 18.6 | from 15 to 22.1 |
| 21.6 J/cm$^2$ | 29 | 26.2 | from 19.4 to 32.9 | 27 | 22.6 | from 19.7 to 25.4 |
| 27 J/cm$^2$ | 15 | 29.2 | from 20.7 to 37.8 | 14 | 25 | from 21.6 to 28.5 |
| 32.4 J/cm$^2$ | 15 | 27 | from 18.5 to 35.6 | 14 | 23.8 | from 20 to 27.5 |
| *3: Riboflavin concentration & $D_2O$ supplementation* | | | | | | |
| Control | 12 | -61.1 | from -151.1 to -29 | 13 | -19.6 | from -54.4 to 15.2 |
| 0.1 $B_2$ | 12 | 35.5 | from 15.5 to 55.6 | 13 | 18 | from 9.2 to 26.7 |
| 0.1 $B_2$ + $D_2O$ | 12 | 25.2 | from 5.1 to 45.2 | 13 | 14.2 | from 4.8 to 23.6 |
| 0.4 $B_2$ | 12 | 34.3 | from 16.5 to 52 | 13 | 18.1 | from 10.7 to 25.5 |
| 0.4 $B_2$ + $D_2O$ | 12 | 29 | from 9.1 to 49 | 13 | 15.5 | from 7.5 to 23.5 |
| *4: Riboflavin carrier* | | | | | | |
| Control | 19 | -109.7 | from -203 to -16.4 | 19 | 14.5 | from -19 to 48 |
| HPMC | 21 | 20.4 | from 8.8 to 32 | 21 | 20 | from 15.2 to 24.9 |
| Dextran | 21 | 37.3 | from 21.7 to 52.8 | 21 | 16.5 | from 10.4 to 22.5 |
| 5: *Riboflavin replenishment* | | | | | | |
| Control | 6 | -56 | from -145 to 33 | 7 | -47.6 | from 105 to 10 |
| Yes | 7 | 33.6 | from 21 to 46 | 7 | 26.5 | from 16.4 to 36.6 |
| No | 7 | 35.7 | from 24 to 47.5 | 7 | 21 | fom 10 to 31.6 |

**Supplementary Table 3. Differences in sample dry weight measurements at day 16 between treatment groups, expressed in estimated differences to control group and related p value.**

| *1: Acceleration* | Control | | 10 min | | 2 min | |
|---|---|---|---|---|---|---|
| | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* |
| 30 min | 16.6 | <0.001 | 0.1 | 1 | 2.6 | 0.64 |
| 10 min | 16.5 | <0.001 | - | - | | |
| 2 min | 14 | <0.001 | -2.6 | 0.67 | - | - |

| *2: Fluence* | Control | | 5.4 J/cm$^2$ | | 10.8 J/cm$^2$ | | 16.2 J/cm$^2$ | | 21.6 J/cm$^2$ | | 27 J/cm$^2$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* |
| 5.4 J/cm$^2$ | 13.7 | <0.001 | - | - | | | | | | | | |
| 10.8 J/cm$^2$ | 15.8 | <0.001 | 2.1 | 0.89 | - | - | | | | | | |
| 16.2 J/cm$^2$ | 18.6 | <0.001 | 4.9 | 0.07 | 2.8 | 0.65 | - | - | | | | |
| 21.6 J/cm$^2$ | 22.6 | <0.001 | 8.8 | <0.001 | 6.76 | 0.001 | 4 | 0.17 | - | - | | |
| 27 J/cm$^2$ | 25.1 | <0.001 | 11.2 | <0.001 | 9.3 | <0.001 | 6.5 | 0.04 | 2.5 | 0.71 | - | - |
| 32.4 J/cm$^2$ | 23.7 | <0.001 | 10 | <0.001 | 7.9 | 0.004 | 5.1 | 0.19 | 1.2 | 0.99 | -1.3 | 0.98 |

| *3: Riboflavin concentration & $D_2O$ supplementation* | Control | | 0.1% riboflavin | | 0.1% riboflavin + $D_2O$ | | 0.4% riboflavin | |
|---|---|---|---|---|---|---|---|---|
| | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* | *Estimate* | *P value* |
| 0.1 $B_2$ | 17.9 | 0.001 | - | - | | | | |
| 0.1 $B_2$ + $D_2O$ | 14.2 | 0.027 | -3.7 | 0.59 | - | - | | |
| 0.4 $B_2$ | 18.1 | <0.001 | 0.14 | 1 | 3.87 | 0.65 | - | - |
| 0.4 $B_2$ + $D_2O$ | 15.5 | 0.002 | -2.45 | 0.87 | 1.3 | 0.98 | -2.6 | 0.84 |

| *4: Riboflavin carrier* | Control | | HPMS | |
|---|---|---|---|---|
| | *Estimate* | *P value* | *Estimate* | *P value* |
| HMPC | 20 | <0.001 | - | - |
| Dextran | 16.5 | <0.001 | - 3.6 | 0.5 |

| *5: Riboflavin replenishment* | Control | | No | |
|---|---|---|---|---|
| | *Estimate* | *P value* | *Estimate* | *P value* |
| Yes | 26.5 | 0.0016 | 5.5 | 0.21 |
| No | 21 | <0.001 | - | - |

1. Knyazer, B., et al., *Accelerated Corneal Cross-linking as an Adjunct Therapy in the Management of Presumed Bacterial Keratitis: A Cohort Study.* J Refract Surg, 2020. **36**(4): p. 258-264.
2. Famose, F., *Evaluation of accelerated collagen cross-linking for the treatment of melting keratitis in ten cats.* Vet Ophthalmol, 2013.
3. Famose, F., *Evaluation of accelerated collagen cross-linking for the treatment of melting keratitis in eight dogs.* Vet Ophthalmol, 2014. **17**(5): p. 358-67.
4. Knyazer, B., et al., *Accelerated Corneal Cross-Linking With Photoactivated Chromophore for Moderate Therapy-Resistant Infectious Keratitis.* Cornea, 2018. **37**(4): p. 528-531.
5. Hammer, A., et al., *Corneal biomechanical properties at different corneal cross-linking (CXL) irradiances.* Invest Ophthalmol Vis Sci, 2014. **55**(5): p. 2881-4.
6. Richoz, O., et al., *The Biomechanical Effect of Corneal Collagen Cross-Linking (CXL) With Riboflavin and UV-A is Oxygen Dependent.* Transl Vis Sci Technol, 2013. **2**(7): p. 6.
7. Aldahlawi, N.H., et al., *Standard versus accelerated riboflavin-ultraviolet corneal collagen crosslinking: Resistance against enzymatic digestion.* J Cataract Refract Surg, 2015. **41**(9): p. 1989-96.
8. Wernli, J., et al., *The efficacy of corneal cross-linking shows a sudden decrease with very high intensity UV light and short treatment time.* Invest Ophthalmol Vis Sci, 2013. **54**(2): p. 1176-80.
9. Arafat, S.N., et al., *UV cross-linking of donor corneas confers resistance to keratolysis.* Cornea, 2014. **33**(9): p. 955-9.
10. Kanellopoulos, A.J., Y.L. Loukas, and G. Asimellis, *Cross-Linking Biomechanical Effect in Human Corneas by Same Energy, Different UV-A Fluence: An Enzymatic Digestion Comparative Evaluation.* Cornea, 2016. **35**(4): p. 557-61.
11. Suter A, H.S., Kling S, and et al., *The bactericidal effect of standard and accelerated CXL protocols on bacterial isolates associated with infectious keratitis in veterinary patients*, in *ACVO 49th Annual Conference and Trade Show*. 2018: Minneapolis.
12. Gilardoni, F., *In-vitro efficacy of accelerated high-fluence PACK-CXL with riboflavin for bacterial keratitis.* 2019: International CXL Experts Meeting. p. 65.
13. Backman, A., et al., *The efficiency of cross-linking methods in eradication of bacteria is influenced by the riboflavin concentration and the irradiation time of ultraviolet light.* Acta Ophthalmol, 2014. **92**(7): p. 656-61.
14. Aldahlawi, N.H., et al., *Enzymatic Resistance of Corneas Crosslinked Using Riboflavin in Conjunction With Low Energy, High Energy, and Pulsed UVA Irradiation Modes.* Invest Ophthalmol Vis Sci, 2016. **57**(4): p. 1547-52.
15. Kling, S., et al., *High Fluence Increases the Antibacterial Efficacy of PACK Cross-Linking.* Cornea, 2020. **39**(8): p. 1020-1026.
16. Makdoumi, K. and A. Backman, *Photodynamic UVA-riboflavin bacterial elimination in antibiotic-resistant bacteria.* Clin Exp Ophthalmol, 2016. **44**(7): p. 582-586.
17. Makdoumi, K., et al., *Evaluation of antibacterial efficacy of photo-activated riboflavin using ultraviolet light (UVA).* Graefes Arch Clin Exp Ophthalmol, 2010. **248**(2): p. 207-12.
18. Shen, J., et al., *Effect of Ultraviolet Light Irradiation Combined with Riboflavin on Different Bacterial Pathogens from Ocular Surface Infection.* J Biophys, 2017. **2017**: p. 3057329.
19. Nordstrom, M., et al., *Refractive improvements and safety with topography-guided corneal crosslinking for keratoconus: 1-year results.* Br J Ophthalmol, 2017. **101**(7): p. 920-925.
20. Nicklin, A., et al. *Safety of high-dose, transepithelial, accelerated CXL with supplemental oxygen on human and monkey eyes*. in *International CXL Experts' Meeting*. 2018. Zurich.
21. Lin, J.T., *The Role of Riboflavin Concentration and Oxygen in the Efficacy and Depth of Corneal Crosslinking.* Invest Ophthalmol Vis Sci, 2018. **59**(11): p. 4449-4450.

22. O'Brart, N.A.L., et al., *An Investigation of the Effects of Riboflavin Concentration on the Efficacy of Corneal Cross-Linking Using an Enzymatic Resistance Model in Porcine Corneas.* Invest Ophthalmol Vis Sci, 2018. **59**(2): p. 1058-1065.
23. O'Brart, D.P.S., et al., *Author Response: The Role of Riboflavin Concentration and Oxygen in the Efficacy and Depth of Corneal Crosslinking.* Invest Ophthalmol Vis Sci, 2018. **59**(11): p. 4451-4452.
24. Lin, J.T., *Influencing Factors Relating the Demarcation Line Depth and Efficacy of Corneal Crosslinking.* Invest Ophthalmol Vis Sci, 2018. **59**(12): p. 5125-5126.
25. Schumacher, S., M. Mrochen, and E. Spoerl, *Absorption of UV-light by riboflavin solutions with different concentration.* J Refract Surg, 2012. **28**(2): p. 91-2.
26. McCall, A.S., et al., *Mechanisms of corneal tissue cross-linking in response to treatment with topical riboflavin and long-wavelength ultraviolet radiation (UVA).* Invest Ophthalmol Vis Sci, 2010. **51**(1): p. 129-38.
27. Hatta, J., et al., *Heavy water inhibiting the expression of transforming growth factor-beta1 and the development of kaolin-induced hydrocephalus in mice.* J Neurosurg, 2006. **104**(4 Suppl): p. 251-8.
28. Ehmke, T., et al., *Comparison of Corneal Riboflavin Gradients Using Dextran and HPMC Solutions.* J Refract Surg, 2016. **32**(12): p. 798-802.
29. Hatami-Marbini, H. and S.M. Jayaram, *Relationship between initial corneal hydration and stiffening effects of corneal crosslinking treatment.* J Cataract Refract Surg, 2018. **44**(6): p. 756-764.
30. Hatami-Marbini, H. and A. Rahimi, *Stiffening effects of riboflavin/UVA corneal collagen cross-linking is hydration dependent.* J Biomech, 2015. **48**(6): p. 1052-7.
31. Hatami-Marbini, H. and S.M. Jayaram, *Effect of UVA/Riboflavin Collagen Crosslinking on Biomechanics of Artificially Swollen Corneas.* Invest Ophthalmol Vis Sci, 2018. **59**(2): p. 764-770.
32. Rapuano, P.B., et al., *Corneal collagen crosslinking in patients treated with dextran versus isotonic hydroxypropyl methylcellulose (HPMC) riboflavin solution: a retrospective analysis.* Eye Vis (Lond), 2018. **5**: p. 23.
33. Aldahlawi, N.H., et al., *An investigation into corneal enzymatic resistance following epithelium-off and epithelium-on corneal cross-linking protocols.* Exp Eye Res, 2016. **153**: p. 141-151.
34. Aldahlawi, N.H., et al., *Standard versus accelerated riboflavin–ultraviolet corneal collagen crosslinking: Resistance against enzymatic digestion.* Journal of Cataract & Refractive Surgery, 2015. **41**(9): p. 1989-1996.
35. Bankhead, P., et al., *QuPath: Open source software for digital pathology image analysis.* Sci Rep, 2017. **7**(1): p. 16878.
36. Stirling, D.R., et al., *CellProfiler 4: improvements in speed, utility and usability.* BMC Bioinformatics, 2021. **22**(1): p. 433.
37. Bottos, K.M., et al., *Immunofluorescence confocal microscopy of porcine corneas following collagen cross-linking treatment with riboflavin and ultraviolet A.* J Refract Surg, 2008. **24**(7): p. S715-9.
38. Esquenazi, S., et al., *Immunofluorescence of rabbit corneas after collagen cross-linking treatment with riboflavin and ultraviolet A.* Cornea, 2010. **29**(4): p. 412-7.
39. Gallhoefer, N.S., et al., *Penetration depth of corneal cross-linking with riboflavin and UV-A (CXL) in horses and rabbits.* Vet Ophthalmol, 2016. **19**(4): p. 275-84.
40. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis.* Nature Methods, 2012. **9**(7): p. 671-675.
41. Jeff Reback, et al., *pandas-dev/pandas: Pandas 1.0.3*. 2020, Zenodo.
42. Waskom, M.L., *seaborn: statistical data visualization*. 2021: Journal of Open Source Software.