Antibacterial Efficacy of Accelerated Photoactivated Chromophore for Keratitis–Corneal Collagen Cross-linking (PACK-CXL)

Olivier Richoz, MD; Sabine Kling, PhD; Florence Hoogewoud, MD; Arthur Hammer, MD; David Tabibian, MD; Patrice Francois, PhD; Jacques Schrenzel, MD; Farhad Hafezi, MD, PhD

ABSTRACT

PURPOSE: To investigate whether optimized photoactivated chromophore for keratitis–corneal collagen cross-linking (PACK-CXL) treatment settings allow accelerating treatment while maintaining antibacterial efficacy.

METHODS: *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains were irradiated with ultraviolet-A light of equal fluence but different intensity settings (18 mW/cm^2 for 5 minutes and 36 mW/cm^2 for 2.5 minutes). The killing rate was determined by comparing the number of colony-forming units between cross-linked specimens and non-irradiated controls. The potential additional effect of 0.001% benzalkonium chloride was also investigated.

RESULTS: The killing rates for *Staphylococcus aureus* were 92.5% ± 5.5% (5 minutes at 18 mW/cm^2) and 94.4% ± 2.9% (2.5 minutes at 36 mW/cm^2). For *Pseudomonas aeruginosa*, the killing rates were 93.2% ± 8.3% (5 minutes at 18 mW/cm^2) and 92.9% ± 5.0% (2.5 minutes at 36 mW/cm^2). The presence of benzalkonium chloride in the riboflavin solution did not increase the killing rate significantly.

CONCLUSIONS: The antibacterial efficacy of PACK-CXL follows the Bunsen–Roscoe law of reciprocity and can be maintained even when the irradiation intensity is considerably increased. These optimized settings may allow a shortened treatment time in the future for PACK-CXL and thus help facilitate the transition from the operating room to the slit lamp for treatment.

[*J Refract Surg*. 2014;30(12):850-854.]

Severe visual impairment due to infectious keratitis is a major cause of global blindness. In developed countries, the incidence varies between 27 and 200 in 100,000 contact lens wearers per year.[1,2] Accordingly, in the United States, where approximately 30 million Americans wear contact lenses, a central register reports 60,000 new cases per year.[3] In developing countries, infectious keratitis represents a "silent epidemic": in India alone, the estimated number of corneal ulcers is 2 million per year.[4] Minor corneal trauma is the most common underlying cause, associated with little to no access to an ophthalmologist, affordable medication, or both. This configuration leads to legal blindness in many cases.

Although the underlying pathogens in infectious keratitis may be viruses, parasites, bacteria, and fungi, the latter two are responsible for most cases.[1] Treatment of bacterial and fungal keratitis is challenging and costly,[5] and in light of emerging fluoroquinolone resistance,[5] even maximal therapy may not be enough to prevent corneal blindness.

The combination of riboflavin and ultraviolet-A has been (and is) in clinical use as an antimicrobial approach for decades (ie, transfusion medicine).[6] This combination was translated into ophthalmology in 2008, when a proof-of-concept study showed that photoactivated riboflavin is beneficial in cases of therapy-resistant infectious keratitis.[7] Now called photoactivated chromophore for keratitis–corneal collagen

From the Department of Ophthalmology (OR, FHoogewoud, AH, DT) and Genomic Research Lab, Division of Infectious Diseases (PF, JS), Geneva University Hospitals, Geneva, Switzerland; Laboratory for Ocular Cell Biology, University of Geneva, Geneva, Switzerland (SK, FHafezi); ELZA Institute, Zurich, Switzerland (FHafezi); and the Department of Ophthalmology, Keck School of Medicine, University of Southern California, Los Angeles, California (FHafezi).

Submitted: September 30, 2014; Accepted: October 20, 2014; Posted online: December 5, 2014

Supported by grants from the Swiss National Science Foundation (OR and AH).

Drs. Richoz and Hafezi are co-inventors of the PCT/CH 2012/000090 application. The remaining authors have no financial or proprietary interest in the materials presented herein.

The authors thank Myriam Girard for skilled technical assistance.

Correspondence: Farhad Hafezi, MD, PhD, University of Geneva, Rue Michel-Servet 1, 1211 Geneva 4, Switzerland. E-mail: farhad@hafezi.ch

doi:10.3928/1081597X-20141118-01

cross-linking (PACK-CXL),[8] this new treatment modality is the object of many clinical and laboratory studies.[9-17] First results indicate that the greatest benefit of PACK-CXL might be in more superficial infiltrates and early ulcers rather than in deep and advanced ulcers.[17,18] All studies on PACK-CXL performed so far have used the rather time-consuming CXL settings derived from conventional CXL for keratoconus: 3 mW/cm^2 for 30 minutes.[19] We investigated whether these settings can be optimized by accelerating the treatment while maintaining the antimicrobial efficacy, using Gram-positive and Gram-negative strains. We also tested whether the known antimicrobial effect of benzalkonium chloride would show an additive effect.

MATERIALS AND METHODS

Bacterial Strains

The methicillin-susceptible *Staphylococcus aureus* (MSSA) strain SA564 has been sequenced internally and is a strain isolated from a patient with toxic shock syndrome.[20] *Pseudomonas aeruginosa* strain PA01 is a sequenced reference isolate.[21]

Bacterial Suspension

Bacteria were treated as previously described.[22] Briefly, a suspension was prepared from fresh subcultures grown on Mueller Hinton Agar at a titer of 0.5 McFarland, corresponding to a cell density of 1 × 108 bacterial cells/mL. A 1:10 dilution in NaCl 0.9% was pre-incubated during 30 minutes with riboflavin for a final concentration of 0.1% riboflavin.

Preparation of Porcine Corneas

Freshly enucleated pig eyes were obtained from a slaughterhouse and randomly sorted into three different treatment groups (n = 3 for each group). The epithelium was removed using a hockey knife. Corneal thickness was determined by ultrasound pachymetry (SP-100; Tomey Corporation, Nagoya, Japan). Only corneas with a central thickness of 800 ± 50 µm were selected. Lamellas with a defined thickness of 150 to 200 µm were created as follows: corneas were maximally hydrated to a controlled thickness of 3,000 µm using distilled water for 24 hours. A 500-µm lamella (one-sixth of thickness) was then cut using an array of blades with a fixed distance, followed by desiccation for 24 hours. Finally, corneas were carefully rehydrated under pachymetric control to a thickness between 150 and 200 µm, as determined by ultrasound pachymetry. A 10-mm diameter disc was used for the experiments and 10 µL of the bacterial suspension were applied onto the lamella. Using corneal samples instead of Agar plates has the advantage that bacteria can penetrate higher depths and that the ultraviolet-A light is less absorbed by the tissue than the medium.

Riboflavin Solution

Riboflavin solution was prepared by mixing vitamin B2-riboflavin-5-phosphate 0.5% solution (G. Streuli & Co. AG, Uznach, Switzerland) with physiological salt solution to achieve a 0.1% isoosmolaric riboflavin solution. For the experiments involving the presence of benzalkonium chloride (Sigma Aldrich, Saint Louis, MI), the latter was added at a concentration of 0.001%.

PACK-CXL

PACK-CXL was performed at 365 µm using High Power LEDs (UV 365 µm, 1,650 mW; LED Engin, Inc., San Jose, CA). Light homogenization was achieved by surrounding the LEDs with an aluminum cylinder of 20-mm length and a diameter of 14 mm. Power was adapted using an ultraviolet-A/ultraviolet-B light meter (Model 8281E; Sper Scientific, Scottsdale, AZ) at two irradiation settings, both providing a fluence of 5.4 J/cm^2 (5 minutes @ 18 mW/cm^2 and 2.5 minutes at 36 mW/cm^2, respectively). Corneal lamellas were incubated with the bacterial suspension and irradiated. After CXL, lamellas were stored in an Eppendorff tube in 0.9% NaCl for 1 hour under aerobic conditions at 37°C, followed by counting of the number of colony-forming units.[23]

Statistical Analysis

Statistical analysis was performed using SPSS Statistics version 19 (IBM, Armonk, NY). Student's *t* test was applied for statistical comparisons. *P* values less than .05 were considered significant. All experiments were performed in triplicate.

RESULTS

The bacterial killing rate was determined for each of the four groups and is summarized in **Table 1**. **Table 2** depicts the bacterial counts for the various conditions. A graphical representation of the survival rate of *Pseudomonas aeruginosa* and *Staphylococcus aureus* is given in **Figures 1-2**. Highly significant differences ($P < .001$) were observed between control corneas and cross-linked corneas. However, no significant differences were found due to high/low irradiation. The addition of benzalkonium chloride showed the trend to increase the killing rate.

DISCUSSION

When used clinically in the cornea, photoactivation of riboflavin is called PACK-CXL[17] and might represent an attractive future adjuvant or even primary therapy in bacterial and fungal corneal infections.

TABLE 1
Mean Bacterial Killing Rate at Various Irradiance Settings in the Presence and Absence of BAC

Bacteria	Control	5 min @ 18 mW/cm²	2.5 min @ 36 mW/cm²	5 min @ 18 mW/cm² with BAC	2.5 min @ 36 mW/cm² with BAC
Pseudomonas aeurginosa (PA01)	100%	92.5% ± 5.5%	94.4% ± 2.9%	98.5% ± 1.6%	93.3% ± 6.8%
Staphylococcus aureus (SA564)	100%	93.2% ± 8.3%	92.9% ± 5.0%	98.5% ± 0.8%	97.7% ± 1.2%

BAC = benzalkonium chloride

TABLE 2
Bacterial Counts (CFU/mL) for the Different Conditions

Group	*Staphylococcus*	*Pseudomonas*
Control	14,724	13,520
18 mW/cm^2	632	1,150
36 mW/cm^2	953	821
18 mW/cm^2 plus BAC	156	170
36 mW/cm^2 plus BAC	280	740

CFU = colony-forming unit; BAC = benzalkonium chloride

Several mechanisms may be responsible for the antimicrobial effect of photoactivated riboflavin. First, riboflavin may intercalate and irreversibly bind to nucleic acids.[24] Second, photoactivated riboflavin creates reactive oxygen species,[25] which induce oxidation processes that lead to chromosomal strand breaks.[26] In clinical settings, a third mechanism comes into play in the cornea, where the combination of ultraviolet-A and riboflavin changes the tertiary structure of collagen, preventing collagenases from accessing their cleavage sites via steric hindrance.[27]

We observed a similar killing rate of approximately 93% at the 18 mW/cm^2 and 36 mW/cm^2 irradiance settings for both Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus*, respectively. We also tested whether benzalkonium chloride, a commonly used preservative in ophthalmic preparations, would enhance the killing rate. Benzalkonium chloride has known antimicrobial properties and proposed mechanisms of action include disruption of cell membranes via emulsification of membrane lipids and induction of DNA strand breaks.[28] When adding 0.001% benzalkonium chloride, the killing rate did not increase significantly.

Infectious keratitis represents a major cause of global blindness,[29,30] especially in developing countries. If PACK-CXL should be applied in these countries as a future adjuvant or even primary treatment, then the current technology needs modification. Current unmet needs include an expensive infrastructure (operating room) and a time-consuming procedure. In view of the massive reduction of pathogens on treatment with photoactivated riboflavin, it seems illogical to bring a septic patient into an aseptic operating room to apply an antiseptic procedure to the ocular surface. In consequence, the entire procedure might also be performed outside the operating room, ideally at the slit lamp, to reduce costs of the procedure. A prerequisite would be to reduce treatment time to a length that can be tolerated by a patient in the upright position.

Another important factor in the assessment of the efficacy of PACK-CXL is the depth of the ulcer. Said et al. investigated the effect of 3 mW/cm^2 for 30 minutes on advanced deep ulcers. They found no significant differences in the time to epithelial healing between the medication plus PACK-CXL group and the medication only group, but a trend in the medication plus PACK-CXL group toward less complications. They concluded that PACK-CXL might better act in rather superficial ulcers that do not implicate the deep corneal layers below 300 µm.[17] In another study involving more superficial ulcers, Price et al. concluded that PACK-CXL might be “most effective when the infection depth was limited.”[18]

All laboratory and clinical studies published to date on the treatment of infectious keratitis by CXL used the original Dresden protocol as the common setting, irradiating the cornea for 30 minutes at 3 mW/cm^2 and a wavelength of 365 µm.[7,9,11-15,19,22,31,32-34] This setting delivers a total fluence of 5.4 J/cm^2 to the corneal surface and has been adopted from the original settings used for keratoconus and postoperative ectasia.[35]

The Bunsen–Roscoe law of reciprocity states that a photochemical effect should remain the same when the same total energy (fluence) is used. This law originates from photochemistry and compares immediate photochemical reactions under different settings. The Bunsen–Roscoe law cannot easily be applied to a biological system, which generates not only immediate

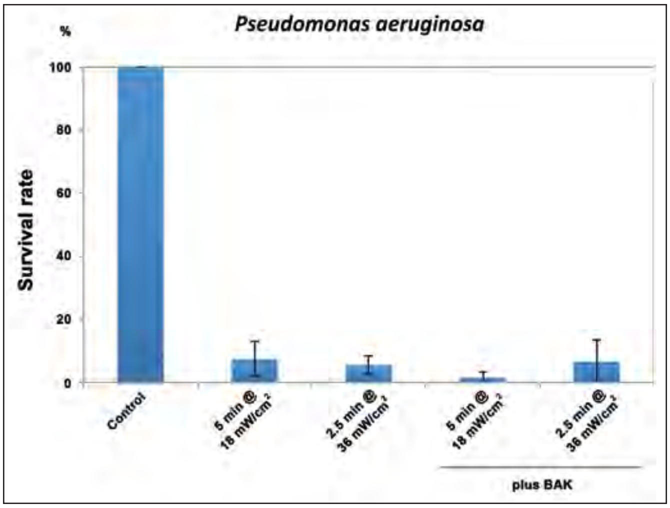

Figure 1. Survival rate of *Pseudomonas aeruginosa* P01 strain after photoactivated chromophore for keratitis–corneal cross-linking (PACK-CXL) in the absence and presence of benzalkonium chloride (BAC). Non-irradiated controls were set to 100%.

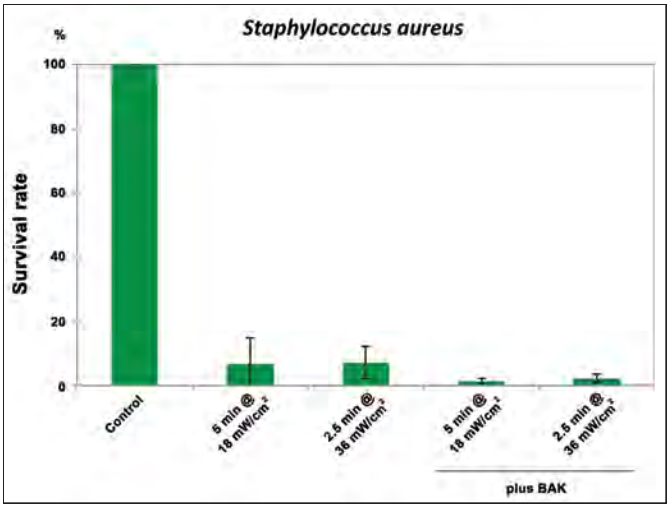

Figure 2. Survival rate of *Staphylococcus aureus* SA564 strain after photoactivated chromophore for keratitis–corneal cross-linking (PACK-CXL) in the absence and presence of benzalkonium chloride (BAC). Non-irradiated controls were set to 100%.

responses, but also additional medium- and long-term changes. Nevertheless, some commercially available CXL devices use higher intensity settings (accelerated CXL), although this modification of the technique has not yet been properly validated for use in ectasia. We have recently tested the corneal biomechanical properties at different CXL irradiances and have found that the increase in biomechanical stiffness knows limitations: when high intensities are used, the stiffness is significantly reduced.[36] In contrast, the antimicrobial efficacy of PACK-CXL seems to follow the Bunsen–Roscoe law at the irradiance levels tested in our experiments. One potential explanation might be that the killing rate of PACK-CXL depends on the oxidative stress induced by the photoactivated chromophore. The more reactive oxygen species that are created within a short period of time, the more oxidative damage is imposed to the DNA of the pathogens.[25]

We tested the antibacterial efficacy of PACK-CXL in a corneal lamella of a defined thickness of 150 to 200 µm in a proof-of-principle approach. By using this rather shallow thickness, we dissociated the effect of photoactivated riboflavin from a potential reduction in the killing rate due to insufficient activity in deeper layers. A limitation of this specific set-up was that the killing efficacy could not be tested for the standard Dresden protocol due to fast dehydration of the corneal flap during irradiation. Further studies are needed to evaluate to which stromal depth PACK-CXL will act effectively using modified fluence settings.

We showed that the antimicrobial efficacy of photoactivated riboflavin seems to follow the Bunsen–Roscoe law of reciprocity for Gram-positive and Gram-negative bacteria. When used in a PACK-CXL approach, treatment time may be substantially shortened compared to conventional CXL once our findings are clinically validated.

AUTHOR CONTRIBUTIONS

Study concept and design (PF, FHafezi, SK, OR); data collection (OR); analysis and interpretation of data (FHafezi, SK, OR, DT, FHoogewoud, JS, AH); drafting of the manuscript (FHafezi, OR, FHoogewoud); critical revision of the manuscript (PF, FHafezi, SK, OR, DT, JS, AH); administrative, technical, or material support (PF, FHafezi); supervision (FHafezi)

REFERENCES

1. Cheng KH, Leung SL, Hoekman HW, et al. Incidence of contact-lens-associated microbial keratitis and its related morbidity. *Lancet*. 1999;354:181-185.
2. Stapleton F, Keay L, Edwards K, et al. The incidence of contact lens-related microbial keratitis in Australia. *Ophthalmology*. 2008;115:1655-1662.
3. Jeng BH, Gritz DC, Kumar AB, et al. Epidemiology of ulcerative keratitis in Northern California. *Arch Ophthalmol*. 2010;128:1022-1028.
4. Whitcher JP, Srinivasan M. Corneal ulceration in the developing world: a silent epidemic. *Br J Ophthalmol*. 1997;81:622-623.
5. Goldstein MH, Kowalski RP, Gordon YJ. Emerging fluoroquinolone resistance in bacterial keratitis: a 5-year review. *Ophthalmology*. 1999;106:1313-1318.
6. Goodrich RP. The use of riboflavin for the inactivation of pathogens in blood products. *Vox Sang*. 2000;78(suppl 2):211-215.
7. Iseli HP, Thiel MA, Hafezi F, Kampmeier J, Seiler T. Ultraviolet A/riboflavin corneal cross-linking for infectious keratitis associated with corneal melts. *Cornea*. 2008;27:590-594.
8. Hafezi F, Randleman JB. PACK-CXL: defining CXL for infectious keratitis. *J Refract Surg*. 2014;30:438-439.

9. Galperin G, Berra M, Tau J, Boscaro G, Zarate J, Berra A. Treatment of fungal keratitis from fusarium infection by corneal cross-linking. *Cornea*. 2012;31:176-180.

10. Hellander-Edman A, Makdoumi K, Mortensen J, Ekesten B. Corneal cross-linking in 9 horses with ulcerative keratitis. *BMC Vet Res*. 2013;9:128.

11. Li Z, Jhanji V, Tao X, Yu H, Chen W, Mu G. Riboflavin/ultraviolet light-mediated crosslinking for fungal keratitis. *Br J Ophthalmol*. 2013;97:669-671.

12. Makdoumi K, Mortensen J, Crafoord S. Infectious keratitis treated with corneal crosslinking. *Cornea*. 2010;29:1353-1358.

13. Makdoumi K, Mortensen J, Sorkhabi O, Malmvall BE, Crafoord S. UVA-riboflavin photochemical therapy of bacterial keratitis: a pilot study. *Graefes Arch Clin Exp Ophthalmol*. 2012;250:95-102.

14. Martins SA, Combs JC, Noguera G, et al. Antimicrobial efficacy of riboflavin/UVA combination (365 nm) in vitro for bacterial and fungal isolates: a potential new treatment for infectious keratitis. *Invest Ophthalmol Vis Sci*. 2008;49:3402-3408.

15. Morén H, Malmsjö M, Mortensen J, Ohrström A. Riboflavin and ultraviolet a collagen crosslinking of the cornea for the treatment of keratitis. *Cornea*. 2010;29:102-104.

16. Pot SA, Gallhöfer NS, Matheis FL, Voelter-Ratson K, Hafezi F, Spiess BM. Corneal collagen cross-linking as treatment for infectious and noninfectious corneal melting in cats and dogs: results of a prospective, nonrandomized, controlled trial. *Vet Ophthalmol*. 2014;17:250-260.

17. Said DG, Gatzioufas Z, Hafezi F. Author reply [article published online ahead of print August 8, 2014]. *Ophthalmology*.

18. Price MO, Tenkman LR, Schrier A, Fairchild KM, Trokel SL, Price FW Jr. Photoactivated riboflavin treatment of infectious keratitis using collagen cross-linking technology. *J Refract Surg*. 2012;28:706-713.

19. Alio JL, Abbouda A, Valle DD, Del Castillo JM, Fernandez JA. Corneal cross linking and infectious keratitis: a systematic review with a meta-analysis of reported cases. *J Ophthalmic Inflamm Infect*. 2013;3:47.

20. Somerville GA, Chaussee MS, Morgan CI, et al. *Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival. *Infect Immun*. 2002;70:6373-6382.

21. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*. 2000;406:959-964.

22. Richoz O, Gatzioufas Z, Francois P, Schrenzel J, Hafezi F. Impact of fluorescein on the antimicrobial efficacy of photoactivated riboflavin in corneal collagen cross-linking. *J Refract Surg*. 2013;29:842-845.

23. Longtin Y, Schneider A, Tschopp C, et al. Contamination of stethoscopes and physicians' hands after a physical examination. *Mayo Clin Proc*. 2014;89:291-299.

24. Naseem I, Ahmad M, Hadi SM. Effect of alkylated and intercalated DNA on the generation of superoxide anion by riboflavin. *Biosci Rep*. 1988;8:485-492.

25. Kumari MV, Yoneda T, Hiramatsu M. Scavenging activity of "beta catechin" on reactive oxygen species generated by photosensitization of riboflavin. *Biochem Mol Biol Int*. 1996;38:1163-1170.

26. Kumar V, Lockerbie O, Keil SD, et al. Riboflavin and UV-light based pathogen reduction: extent and consequence of DNA damage at the molecular level. *Photochem Photobiol*. 2004;80:15-21.

27. Spoerl E, Wollensak G, Seiler T. Increased resistance of cross-linked cornea against enzymatic digestion. *Curr Eye Res*. 2004;29:35-40.

28. Ye J, Wu H, Zhang H, et al. Role of benzalkonium chloride in DNA strand breaks in human corneal epithelial cells. *Graefes Arch Clin Exp Ophthalmol*. 2011;249:1681-1687.

29. Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. *Bull World Health Organ*. 2001;79:214-221.

30. Whitcher JP, Srinivasan M, Upadhyay MP. Prevention of corneal ulceration in the developing world. *Int Ophthalmol Clin*. 2002;42:71-77.

31. Heaselgrave W, Kilvington S. Antimicrobial activity of simulated solar disinfection against bacterial, fungal, and protozoan pathogens and its enhancement by riboflavin. *Appl Environ Microbiol*. 2010;76:6010-6012.

32. Kashiwabuchi RT, Carvalho FR, Khan YA, et al. Assessing efficacy of combined riboflavin and UV-A light (365 nm) treatment of Acanthamoeba trophozoites. *Invest Ophthalmol Vis Sci*. 2011;52:9333-9338.

33. Kashiwabuchi RT, Carvalho FR, Khan YA, Hirai F, Campos MS, McDonnell PJ. Assessment of fungal viability after long-wave ultraviolet light irradiation combined with riboflavin administration. *Graefes Arch Clin Exp Ophthalmol*. 2013;251:521-527.

34. Panda A, Krishna SN, Kumar S. Photo-activated riboflavin therapy of refractory corneal ulcers. *Cornea*. 2012;31:1210-1213.

35. Hafezi F, Kanellopoulos J, Wiltfang R, Seiler T. Corneal collagen crosslinking with riboflavin and ultraviolet A to treat induced keratectasia after laser in situ keratomileusis. *J Cataract Refract Surg*. 2007;33:2035-2040.

36. Hammer A, Richoz O, Arba Mosquera S, Tabibian D, Hoogewoud F, Hafezi F. Corneal biomechanical properties at different corneal collagen cross-linking (CXL) Irradiances. *Invest Ophthalmol Vis Sci*. 2014;55:2881-2884.