First Proposed Efficacy Study of High Versus Standard Irradiance and Fractionated Riboflavin/Ultraviolet A Cross-Linking With Equivalent Energy Exposure

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Purpose: To document the first presented report in December 2008 of high irradiance riboflavin/ultraviolet A (UVA) corneal cross-linking in comparison with that of standard irradiance and of fractionated exposure to increase the time for oxygen diffusion into the cornea.

Methods: After in vitro studies of oxygen depletion and cross-linking density using type 1 human collagen gels, 36 ex vivo porcine globes were deepithelialized and exposed to 0.1% riboflavin drops in carboxymethylcellulose solution every 5 min for 3 initial doses and then throughout irradiation afterward. Six eyes each were irradiated with 370-nm UVA light at 2, 3, 9, and 15 mW/cm^2 continuously and 15 mW/cm^2 fractionated (with alternate cycles of 30 s "ON" and 30 s "OFF" exposure) using an equivalent radiant exposure of 5.4 mJ/cm. The final six eyes received no UVA exposure as a control. The exposed corneas were then dissected and subjected to extensiometry. Analysis of variance with Bonferroni post hoc test was performed between groups.

Results: The stress required to induce a 10% strain for the control eyes (no UVA) was $100.6\pm20.9\times10^3$ N/m^2 in comparison with the stress of 3 mW/cm^2 (standard irradiation) at $146.7\pm17.6\times10^3$ N/m^2 (P=0.009). The stress at the other equidose irradiances of 2, 9, 15 continuously, and 15 mW/cm^2 fractionated were 140 ± 21.9, 162.8 ± 70, 154.1 ± 70, and $163.0\pm64\times10^3$ N/m^2, respectively. When comparing the irradiances of 15 mW/cm^2 continuously and fractionated to the standard irradiation, the stress was not statistically different (P=0.799 and 0.643), respectively.

Conclusion: High irradiance riboflavin/UVA cross-linking with equivalent energy exposure demonstrates comparable efficacy in stiffening corneal collagen with standard irradiance, but with considerably less exposure time. Over the past 6 years, since this report was first presented, the use of high irradiance cross-linking has been gaining popularity.

Key Words: Cross-linking—Riboflavin—High irradiance—Oxygen.

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When riboflavin/ultraviolet A (UVA) collagen cross-linking was first introduced and compared with other cross-linking methods, it was defined as most efficacious at a UVA irradiance of 3 mW/cm^2 for an exposure of 30 or 45 min.[1] The first clinical publication of riboflavin/UVA cross-linking for progressive keratoconus used this same UVA dose, 3 mW/cm^2 for 30 min, and successfully altered the course of keratometric progression to that of keratometric regression with continued improvement over time.[2] As a result, every early clinical study of riboflavin/UVA corneal cross-linking (CXL) used this UVA exposure.[3–7]

The standard irradiance of 3 mW/cm^2 for 30 min is not only efficacious in stiffening the cornea in cases of keratoectasia and keratolysis[8,9] but also has been demonstrated as efficacious in sterilizing microbial keratitis[10] because of the cytotoxic effect of the reactive oxygen species (ROS) generated during the cross-linking process.[11] The cytotoxic effect also leads to keratocyte apoptosis within the cornea,[12] and when deep enough could lead to endothelial cell toxicity.[13] As a result, the established safety criteria of 400-μm thickness and total energy exposure of 5.4 J/cm^2 has become the accepted norm for clinical practice.[14] According to safety studies of collagen cross-linking, the maximum recommended UVA exposure seems to be more dependent on the total energy (5.4 J/cm^2) rather than the UVA irradiance (3 mW/cm^2).[14] As the creation of ROS necessary for cross-linking is directly proportional to the irradiance of UVA light, cross-linking with the higher irradiance should require less exposure time for similar efficacy, while still maintaining the safety criteria. Effective cross-linking requires the presence of oxygen, in addition to sufficient penetration of riboflavin and UVA exposure.[15] As oxygen is transported into the cornea at a fixed rate of 45 nL/cm^2 per second[16] and is more rapidly consumed with higher irradiances beyond the first 30 s of exposure, fractionated UVA exposure (30 s "ON" and 30 s "OFF") should aid in maintaining effective cross-linking at the higher irradiances. In 2008, we first proposed that the efficacy of high irradiance riboflavin/UVA CXL was similar to that of standard irradiance and that fractionated exposure may enhance the efficacy and/or safety by increasing the time for oxygen diffusion into the cornea (Fourth International Congress of Corneal Cross-linking, Dresden, Germany, December 5–6, 2008). Since that time, rapid high irradiance cross-linking has been adopted clinically. This article documents our early unpublished studies on accelerated and fractionated cross-linking and discusses its clinical adoption and relevance today.

METHODS

Preliminary testing of cross-linked collagen gels[17] was performed before the extensiometry studies of cross-linked porcine

corneas to characterize the linear relationship of oxygen consumption and cross-linking density with increasing UVA irradiance.

Oxygen Depletion

Placing a microneedle sensor (PreSens GmbH, Regensburg, Germany) into a cuvette filled with type 1 human collagen gel (9 mg/mL) (Innomed, Purepol, Freemont, CA) and riboflavin 0.1% solution (Sigma Aldrich, Saint Louis, MO) allows for the measurement of pO2 with increasing UVA irradiance. Before UVA irradiation, pure oxygen was bubbled through the sample for 30 min to create an oxygen-rich environment. The pO2 was then measured, and then on starting UVA exposure, the time for 50% decline of pO2 was recorded for both the standard and high irradiance dosing. The exposure was continued until the pO2 reached a 5% value.

Cross-Linking Density

The temperature for collagen denaturation depends on the cross-link density, with higher temperatures signifying higher density. Differential scanning calorimetry (DSC) (DSC 1; Mettler-Toledo International Inc., Columbus, OH) was used to measure both the shrinkage and denaturation temperatures of riboflavin-saturated collagen gels cross-linked with both standard and high irradiance dosing.

Porcine Eye Extensiometry

For extensiometry testing, 36 porcine globes were each deep-ithelialized and exposed to 0.1% riboflavin drops in carboxymethylcellulose solution every 5 min for 3 initial doses. Thirty of these eyes were then irradiated, while maintaining the every 5-min dosing of riboflavin 0.1%, with 370-nm UVA light each for an equivalent radiant exposure of 5.4 mJ/cm^2 but with irradiances of 2 mW/cm^2 for 45 min (6 eyes), 3 mW/cm^2 for 30 min (6 eyes), 9 mW/cm^2 for 10 min (6 eyes), 15 mW/cm^2 for 6 min (6 eyes), and 15 mW/cm^2 for 12 min with fractionation of UVA delivery in cycles of 30 s ON and 30 s OFF (6 eyes). The final six eyes received no UVA exposure as a control. The exposed corneas were then dissected into 5×8 mm strips of collagen and subjected to stress/strain testing. The extensometer used a load cell (Althen DMS KA 31E series, Kelkheim, Germany) that would record the amount of force (stress) required to achieve a 10% strain. Analysis of variance with Bonferroni post hoc test was performed within groups to access the statistical significance of stiffness variation.

RESULTS

Oxygen Depletion

The pO2 before UVA irradiance was recorded at 100% and declined to 50% pO2 values within 245 s (4.08 min) (Fig. 1A) using 3 mW/cm^2 and 46 s (0.77 min) (Fig. 1B) with the high irradiance dosing of 16 mW/cm^2. The O_2 consumption rate (and hence the generation of ROS) was linearly associated with the UVA irradiance (R^2=0.997), being greater than 5 times faster with the 16 mW/cm^2 dosing.

Cross-Linking Density

Using DSC, the temperature for denaturation of cross-linked collagen gels was recorded as 52°C for 3 mW/cm^2 and 56°C for 15 mW/cm^2. Shrinkage temperature for these gels was also measured at 47°C for 3 mW/cm^2 and 53°C for 15 mW/cm^2. Although the total radiant exposure was equivalent at 5.4 J/cm^2, the higher irradiance dosing revealed greater cross-linking density in these cross-linked collagen gels.

Porcine Eye Extensiometry

The standard irradiance of 3 mW/cm^2 for 30 min required a stress of $146.7\pm17.6\times10^3$ N/m^2 to induce a 10% strain in comparison with the control (no UVA), which stretched 10% with $100.6\pm20.9\times10^3$ N/m^2 (P=0.009). With a lower than standard UVA irradiance of 2 mW/cm^2 for 45 min, a slightly lower stress of $140\pm21.9\times10^3$ N/m^2 was required, whereas for a 3× higher UVA irradiance of 9 mW/cm^2 for 10 min, a slightly higher stress of $162.8\pm70\times10^3$ N/m^2 was recorded, but neither were statistically significantly different from the standard dose (P>0.05). Statistical significance was also not observed with the 5× higher than standard UVA irradiance of 15 mW/cm^2, with continuous exposure for 6 min at $154.1\pm70\times10^3$ N/m^2 (P=0.799) nor for the fractionated exposure of 12 min at $163\pm64\times10^3$ N/m^2 (P=0.643). Figure 2 displays the mean±SD of stress to induce a 10% strain in collagen strips subject to the above UVA irradiance exposures.

DISCUSSION

Extensiometry is the gold standard for determining the stiffness and tensile strength of biologic tissue and is a practical measure of the effectiveness of cross-linking techniques.[18] It does so by measuring the stress required to achieve a determined strain (elongation), and in this study, we used 10% strain, as this is the most commonly tested strain value. As the viscosity of the riboflavin 0.1% solution impacts the rapidity of reoxygenation and may impact the uniformity of UVA delivery with shorter higher intensity exposures, we have chosen to use riboflavin 0.1% solution in a vehicle of carboxymethylcellulose, rather than dextran, as is more commonly used in clinical studies.[2] The dextran vehicle shows no difference from carboxymethylcellulose in effectiveness of cross-linking and is generally preferred clinically to prevent the corneal swelling found with deepithelialization.

Our testing shows that riboflavin/UVA cross-linking is statistically significantly more effective in stiffening porcine corneas than riboflavin 0.1% solution in carboxymethylcellulose alone (without UVA). More specifically, riboflavin with UVA exposures at 2, 3, 9, and 15 mW/cm^2 (using an equivalent total energy exposure of 5.4 J/cm^2) are each equally effective and each statistically more effective than riboflavin without UVA. When fractionating the UVA exposure with multiple cycles of 30 s ON and 30 s OFF, the extensiometry is again equally as effective as without fractionation. In theory, fractionation of the UVA delivery should improve the degree of cross-linking, especially with the faster higher irradiance exposures where oxygen is consumed more quickly. However, our study does not reveal significant improvement but rather shows statistically similar efficacy.

The reason we might expect higher stress/strain with fractionation is actually the same reason we might expect lesser stress/strain with high irradiance alone that of oxygen depletion.[19] Our preliminary testing of oxygen depletion using riboflavin-saturated type 1 collagen gels reveals a 50% pO2 within 245 s for an irradiance of 3 mW/cm^2, whereas half the oxygen is consumed in 46 s for the higher irradiance of 16 mW/cm^2, a nearly linear relationship. In (Figure 1A and B), the decline in oxygenation of the

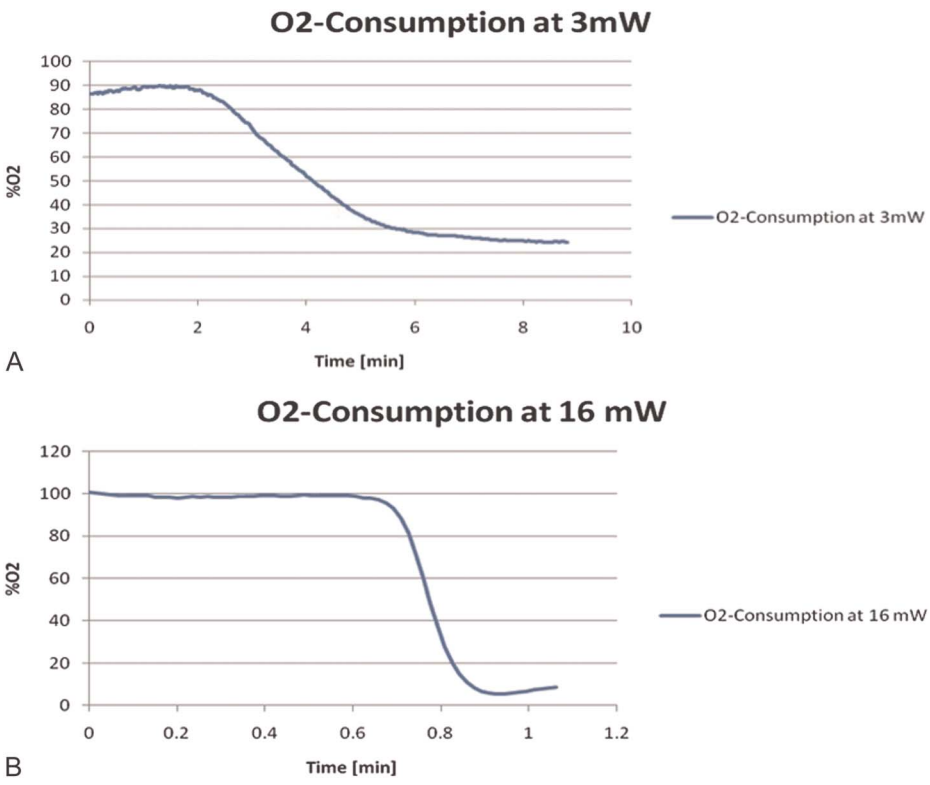

FIG. 1. Depletion time of tissue oxygen concentration during riboflavin/UVA cross-linking being inversely proportional to the UVA irradiance. A 50% depletion of oxygen is noted in 245 s when irradiated with 3 mW/cm^2 (A) and 46 s (5.3× less time) when irradiated with 16 mW/cm^2 (5.3× more energy) (B). full color online

collagen gel is much more rapid with high irradiance CXL, although the decline does not begin until at least 30 s of exposure has elapsed. Restorative diffusion of oxygen into a normal epithelialized cornea (without riboflavin) has been reported to achieve a steady state pO_2 at 300+ μm depth within 30 to 45 s,[16] and reoxygenation is even faster with deepithelialized cornea. As a result, we have selected a fractionation cycle of 30 s ON and 30 s OFF to avoid any decline in efficacy when using the high irradiance exposure. In our study, however, the decline without fractionation was not observed nor was there a significant improvement in cross-linking efficiency with fractionation. This may be explained, at least in part, by the relatively small sample size, which curiously may also have impacted the higher standard deviation in stress values with the higher irradiances. Perhaps with greater numbers, the small increases in stress with high irradiance and even fractionation might be better defined with tighter standard deviations and better display statistical significance. Despite the statistics, we believe that higher irradiance with fractionation should be further investigated to maximize the theoretical benefit of tissue oxygenation.[19]

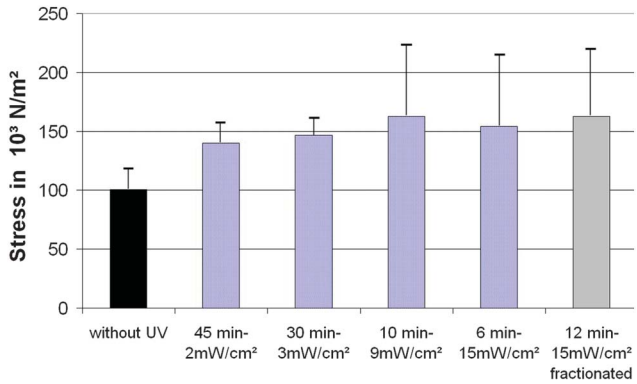

FIG. 2. Extensiometry stress values for 10% strain of riboflavin-soaked porcine corneal strips treated without UV and with UV cross-linking at a total dose of 5.4 J/cm^2 using low irradiance (2 mW/cm^2), standard irradiance (3 mW/cm^2), high irradiance (9 mW/cm^2, 15 mW/cm^2) and fractionated (30 s ON and 30 s OFF sequence) high irradiance (15 mW/cm^2) exposures. The strips with standard irradiance UV cross-linking are significantly stiffer than those without UV exposure (P=0.009) but not significantly different from those with high irradiance (P=0.799) or fractionated high irradiance exposure (P=0.643).

The greater curiosity in our results is not as much the lack of decline in stress/strain with higher irradiances but rather a non-significant increase in comparison with standard dosing. Perhaps, additional cross-linking effectiveness is achieved with high irradiances that are not appreciated with the standard dosing. This assertion is supported by DSC studies of riboflavin-saturated collagen gels that are cross-linked with UVA light at 3 mW/cm^2 for 30 min, where the temperature for denaturation is 47°C, and 15 mW/cm^2 for 6 min, where the temperature for denaturation is higher at 54°C. Here, the higher DSC temperature is indicative of greater tissue strength in the high irradiance cross-linked collagen gels.[20]

As a practical consideration, the rapidity of high irradiance cross-linking offers great advantages in clinical applications, where 30 min is considered too long for most clinicians and their patients. In a previous publication of intrastromal injection of riboflavin for cross-linking of deep regions in the cornea, as with bullous keratopathy, the shorter time of high irradiance UVA exposure (6 min) allows a single dose of riboflavin to diffuse into the cornea

both proximal and distal to the interface and then fully cross-link the deep tissue before the riboflavin clears from the cornea.[21] Without the short exposure of high irradiance cross-linking, intrastromal injection techniques would not be possible unless multiple injections were performed during the cross-linking process.

Although our study reveals a similar efficacy of high irradiance and standard irradiance cross-linking, it does not say anything about the safety of this dosing. The equivalent total energy of exposure infers that high irradiance meets the established safety requirements, but confirmatory safety studies of corneal haze, reepithelialization, endothelial viability, and immunohistochemistry with standard cross-linking[22,23] need to be repeated with high irradiances. The fact that other ophthalmic technologies are safely using much higher levels of UVA irradiance, such as the light adjustable lens (IOL) with transcorneal exposures of 250 mW/cm^2 for 3 min (without the use of riboflavin),[24] leads us to believe that high irradiance short-exposure cross-linking would be safe clinically.

At the time of our presentation of these data in 2008, high irradiance cross-linking had only been performed in the single reported case of bullous keratopathy with no adverse effects.[21] Since that time, over the past 6 years, both the safety and efficacy of high irradiance cross-linking has been clinically studied with progressive keratoconus[25–28] and also prophylactically in conjunction with laser in situ keratomileusis (LASIK) (i.e., LASIK Xtra).[29–31] In each of these studies, the impact of the high irradiance was to shorten the time exposure for cross-linking without compromising the effect or outcome. With advancing keratoconus, high irradiances up to 30 mW/cm^2 were comparable with the standard 3 mW/cm^2 in both halting and reducing the K_{max} values without safety concern.[27] With the thinner corneas undergoing high myopic LASIK, prophylactic high irradiance cross-linking of up to 30 mW/cm^2 showed no change in LASIK outcomes, except for the presence of a demarcation line, which signified the relative effectiveness of the extra step of cross-linking.[31] With topographic-guided hyperopic LASIK, however, statistically significant less regression over a 2-year period was seen in eyes receiving high irradiance cross-linking (10 mW/cm^2) under the flap compared with those without cross-linking.[32] This latter study reveals a direct clinical benefit from LASIK in conjunction with high irradiance cross-linking (i.e., LASIK Xtra), which would be nearly impossible to perform with standard irradiance cross-linking.

Despite these positive clinical findings, conflicting reports of efficacy have been cited in experimental extensiometry studies by Hafezi et al. because of the rapid depletion of oxygen with high UV irradiances.[33,34] This difference in standard versus rapid cross-linking was not found in similar studies by Mrochen et al., except when exceeding irradiances of 50 mW/cm^2 or when testing second harmonic reflection imaging in whole globes with a more elevated intraocular pressure.[35–37] This brings us back to the idea of fractionated UV exposures, whereby the depletion of oxygen when the UV light is turned ON has the opportunity to undergo restoration while the UV light is turned OFF. In the past 5+ years, this concept has not been further addressed in the literature, although unpublished reports of its widespread use by a group of U.S. investigators from CXL-USA (B. Trattler, M.D., personal communication, 2014) suggest that it will have a prominent place in enhancing the efficacy of high irradiance cross-linking in the future.

In summary, the concept of high irradiance cross-linking with equivalent energy exposure follows the established safety criteria and demonstrates equivalent efficacy in comparison with the standard cross-linking parameters. Although fractionation of delivery (30 s ON and 30 s OFF) theoretically enhances the efficacy of high irradiance cross-linking, our study in porcine eyes shows no statistical improvement. Nevertheless, we still believe that fractionation will likely be beneficial in reducing the amount of UVA exposure necessary for successful cross-linking in the future. The benefit of high irradiance dosing capitalizes on the rapidity of completion and has ergonomic advantages and strategic implications for future cross-linking techniques.

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