Femtosecond-UVA-riboflavin (FUR) cross-linking approach to penetrating keratoplasty and anterior lamellar keratoplasty

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Abstract Purpose: To introduce femtosecond laser wound design combined with riboflavin/ultraviolet light-A (UVA) collagen cross-linking at the wound for penetrating (PKP) and anterior lamellar keratoplasty (ALK). Primary outcomes were intraocular pressure (IOP in mmHg) at burst point for the PKP group, and tensile strength (kPa) until dehiscence for the ALK group.

Methods: Human corneoscleral rims (N = 20) were mounted on artificial anterior chambers. PKP specimens underwent FUR, femtosecond laser-cut without cross-linking, or conventional corneal transplantation. PKP maximum burst IOP with progressive suture removal was assessed by a digital manometer, in triplicate and by three observers. ALK involved whole human globes (N = 10) divided into three groups using a 200-micron, 8 mm diameter donor lenticule, with or without cross-linking. Cross-linked specimens were exposed to UVA light (3 mW/cm² irradiance, 3.4 J, 370 nm wavelength) for 30 min with 0.1% riboflavin (20% Dextran) applied every 2-min. ALK tensile strength was determined using a digital tensiometer.

Keywords
Corneal collagen crosslinking; Corneal wound healing; Burst pressure; Corneal tensile strength; Penetrating keratoplasty; Anterior lamellar keratoplasty

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Results: In PKP, burst IOP was 31.32 mmHg greater for corneas that underwent the UVA-riboflavin treatment than for those that did not ($p < 0.05$). There was no significant relationship ($p = 0.719$) established between cut design (femtosecond versus conventional). On multivariate analysis, there was a mean of 15.82 mmHg higher sustainable pressure for each stabilization suture present ($p < 0.0001$). In ALK, specimens comprised of human donor and human recipient tissue combined with UVA-riboflavin therapy experienced the greatest level of adhesion strength (954.7 ± 290.4 kPa) as shown by the force required to separate the tissues, and compared to non-cross-linked specimens. Electron microscopy of ALK specimens showed non-fused and fused longitudinal cross-linked collagen fibers as well as bridges, densities, attachment plaques and primitive plasmalemmal densities.

Conclusions: Cross-linking effects of the FUR technique enable a stronger graft-recipient adhesion compared to conventional penetrating and anterior lamellar keratoplasty. Electron microscopy enabled visualization of cross-linked interface and potential bonding. The FUR approach may further lead to sutureless transplantation techniques in the future.

Setting/venue: ImagePlus Laser Eye Centre, Winnipeg, and University of Ottawa Eye Institute, Ottawa, Canada.

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1. Introduction

Femtosecond lasers have become an increasingly effective tool for corneal surgery. This is in part due to the ability to perform innovative wound design as well as the benefit of precise wound creation (Slade, 2007). Femtosecond lasers produce cavitation bubbles at a specified depth and with a specified design, and this allows for exquisite flexibility in terms of designing corneal wounds (Farid et al., 2009). Initially, this technology became popular for the creation of anterior corneal flaps in laser in situ keratomileusis (LASIK) (Binder, 2004). More recently, its use has expanded to include channel creation for intrastromal corneal ring placement (Ertan et al., 2007), penetrating keratoplasty (PKP) (Farid et al., 2009; Bahar et al., 2009) anterior lamellar keratoplasty (ALK) (Lie et al., 2010), endothelial keratoplasty (Sikder and Snyder, 2006) and astigmatic keratotomy (Bahar et al., 2008).

Collagen cross-linking has gained increasing acceptance for strengthening corneal tissue during keratoconus and corneal ectasia (Wollensak et al., 2003a,b). The combined use of ultraviolet light (UVA) with riboflavin has been reported in the literature to achieve these results (Vinciguerra et al., 2009).

The purpose of this study was to assess corneal wound tensile strength using femtosecond laser wound design combined with corneal collagen cross-linking to enhance bonding across the graft-host interface. This experimental study assessed two groups. The first group was comprised of penetrating keratoplasty (PKP) eyes using intraocular pressure (mmHg) at burst point as the primary outcome. The second group was comprised of anterior lamellar keratoplasty (ALK) specimens using tensile strength in kiloPascals (kPa) as the primary outcome measure. In both groups, femtosecond laser was used to design the wounds. Each group was comprised of non-cross-linked and collagen cross-linked tissues. Because of the combined use of Femtosecond laser, UVA light and Riboflavin, we have termed this the FUR approach, as suggested by one of the authors (MB).

2. Methods

This study was approved by the Ottawa Hospital Research Ethics Board. All authors have completed the Interagency Advisory Panel on Research Ethics’ Introductory Tutorial for the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS).

2.1. Specimens

Human cadaver eyes were obtained from the Eye Bank of Canada (Ontario Division) and the Lions Eye Bank of Manitoba and Northwest Ontario. For the PKP group, corneoscleral rims were used and mounted on a disposable Barron artificial anterior chamber (Katena Products, Inc., Denville, NJ), and according to our previous description (Bower and Rocha, 2007) (Fig. 1). Full thickness grafts were 8 mm in diameter, and created either using the femtosecond zig-zag technique or standard conventional trephination with a disposable Hess-Barron vacuum trephine (Katena Products, Inc., Denville, NJ). Only eight interrupted radial sutures were placed in all cases, using 10–0 nylon (Ethicon, Somerville NJ).

For the ALK eyes, donor and recipient whole human cadaver globes were used. Globes were pressurized to > 60 mmHg, appropriately docked under the femtosecond glass plate (Intra-
lace FS, Abbott Medical Optics, Santa Ana, CA), and 200 micron deep × 8 mm in diameter anterior lamellar grafts were dissected. All donor and recipient lenticules were cut in exactly the same fashion. Donor tissues were directly placed on the recipient bed and subdivided into either cross-linked, or non-cross-linked groups.

2.2. Corneal collagen cross-linking

In the cross-linked specimens, riboflavin 0.1% (20% Dextran) was used in the standard fashion as described clinically, one drop every two minutes for 30 min on a de-epithelialized surface, followed by one drop every 2 min in the presence of UVA light (UV-X Illumination System, IROC, Zurich, Switzerland) at 3 mW/cm², 3.4 J, 370 nm wavelength.

2.3. Primary outcome measures

The primary outcome measure for the PKP group was intraocular pressure (IOP) in mmHg at burst point, with progressive suture removal and using a digital manometer (Cole-Parmer Canada Inc., Montreal, QC, Canada). Three consecutive measurements were taken by three independent observers, using the first point of fluid expulsion as the endpoint. The maximum intraocular pressure was recorded and a mean was established.

Anterior lamellar grafts were assessed using tensile strength (stress–strain) in kPa until graft dehiscence. Determining tensile strength was performed by placing plastic pegs on the anterior surface of the anterior lamellar graft using 1–2 drops of commercially available Krazy Glue on a de-epithelialized surface and allowing it to set for 2 h. The IOP was then standardized to 15–30 mm of Hg and the mechanical force was applied (Fig. 2). Using the Instron digital tensiometer (Instron, Norwood, MA), force was applied until the graft completely separated from the stromal bed. Fig. 2 shows an example of initial resistance to the applied tearing force, followed by a sudden decline in resistance.

3. Groups

The PKP technique was comprised of 20 corneoscleral rims divided into four groups:

- **Group 1:** femtosecond-enabled keratoplasty with no collagen cross-linking.
- **Group 2:** conventional penetrating keratoplasty using a blade trephine, followed by collagen cross-linking.
- **Groups 3 and 4:** femtosecond-enabled keratoplasty. However, in Group 3 cross-linking of the tissue was performed first, followed by femtosecond laser design; in Group 4 the converse was true, with femtosecond-enabled keratoplasty first followed by collagen cross-linking.

The ALK technique included 10 whole globes divided into three groups:

- **Group 1:** ALK with human donor.
- **Group 2:** ALK with human donor followed by cross-linking.
- **Group 3:** comprised of one specimen from each group sent to the University of Ottawa Heart Institute for electron microscopy processing and analysis.

3.1. Statistical analysis

A censored regression model was employed to analyze the PKP technique. Specifically, a parametric survival model with a Gaussian distribution assumption was used to reduce the...
amount of bias that may otherwise have been present due to a small sample size. The repeated measures were taken into account for each specimen. Significance levels were set at $p < 0.05$.

The Mann–Whitney $U$ test was employed to analyze the ALK technique. This test does not assume nor require that the data follow a normal distribution, and is appropriate for analysis of small sample sizes. Since this technique has lower statistical power, significant effects generally have larger effect sizes. Significance levels were set at $p < 0.05$.

4. Results

4.1. Penetrating keratoplasty

An increased number of sutures resulted in a proportionally higher burst pressure (Fig. 3). On multivariate analysis, there was a mean of 15.82 mmHg higher sustainable pressure for each stabilization suture present ($p < 0.0001$). Even with only eight sutures present, average burst pressure was over 80 mmHg. The effect of UVA riboflavin cross-linking was assessed on IOP burst point. Comparison of non cross-linked specimens (eight sutures) versus cross-linked specimens (eight sutures) yielded a mean difference of 31.32 mmHg higher in the cross-linked group. This was statistically significant with a $p$ value equal to 0.0264 (Fig. 4).

The effect of cut design on IOP burst point was assessed, comparing conventional and femtosecond-enabled zig-zag incisions. The conventional group first underwent trephination then collagen cross-linking (Group 2), so was only compared with the group that underwent femtosecond-enabled zig-zag incisions then collagen cross-linking (Group 4). This eliminated the group that underwent collagen cross-linking first then femtosecond-enabled zig-zag incisions (Group 3), to ensure that the sequence of events would not be an extraneous variable. The conventional group (Group 2) mean burst IOP was 88.42 mmHg, and the femtosecond design followed by cross-linking (Group 4) had a mean burst IOP of 82.75 mmHg. On average, the IOP burst point was 5.67 mmHg higher in the conventional group (Group 2) compared to the femtosecond group (Group 4), but this was not statistically significant ($p = 0.719$).

Next, the sequence of the procedure was analyzed to determine whether performing the cross-linking treatment prior or subsequent to wound creation would result in significant differences. This was determined at the 8-suture level. For tissue cross-linked first, followed by femtosecond wound creation (Group 3), the mean burst IOP was 85.92 mmHg; for the reverse approach (Group 4), the mean burst IOP was 82.75 mmHg. On average, the IOP burst point was 3.167 mmHg higher when cross-linking was performed first, but this difference was not statistically significant ($p = 0.871$).

4.2. Anterior lamellar keratoplasty

Anterior lamellar tensile strength of cross-linked versus non cross-linked specimens was then compared. Median strength values were significantly higher in the cross-linked human-to-human grafts when compared to non cross-linked specimens ($954.7 ± 290.4$ kPa). The main effect of cross-linking versus non cross-linking demonstrated a definite trend toward significance; however, findings did not reach statistical significance. This is likely explained by the small sample size in addition to the use of a non-parametric analysis model, which has a higher threshold for significance.

4.3. Electron microscopy

Anterior lamellar keratoplasty specimens were sent for processing and analysis with electron microscopy at the University of Ottawa Heart Institute. Of these, only the human-to-human cross-linked specimen was viable, as the rest detached during preparation. Non-fused and fused crossed and longitudinal cross-linked collagen fibers were demonstrated. In addition, bridges, densities, attachment plaques and primitive plasmalemmal densities were observed across the graft-host junction Figs. 6–9. Finally, there was also the presence of occasional globules which appeared to be lipid in origin, within the interface.
5. Discussion

Recent technological advances have permitted alternative approaches to keratoplasty (1–14). In this study, we have shown that femtosecond wound design combined with the strengthening effects of collagen cross-linking provided by ultraviolet light/riboflavin therapy (FUR approach) results in increased burst IOP in full thickness corneal transplants, increased tensile adhesion in anterior lamellar grafts and electron microscopy evidence of corneal collagen cross-linking across the graft-host interface. Median Maximum Strength values were significantly higher in the cross-linked human-to-human condition when compared to non cross-linked specimens (Not statistically significant; see text). (Mann-Whitney U tests)

Figure 5  Tensile strength in anterior lamellar keratoplasty.

Figure 6  Electron micrograph (1000×) showing nonfused and fused (arrow) areas across the graft-host interface. Fused areas demonstrate both crossed and longitudinal cross-linked fibers.

Figure 7  Electron micrograph (4000×) showing nonfused and fused (arrow) areas across the graft-host interface. Fused areas demonstrate both crossed and longitudinal cross-linked fibers.
In the PKP technique, we demonstrated that the FUR approach enables a superior result with regard to IOP burst point. No significance was established between cut design or sequence of corneal incision and cross-linking. This has relevance, as many keratoconic corneas have now received cross-linking treatments (Vinciguerra et al., 2009). We do not anticipate a detrimental effect of cross-linking to subsequent PKP in the future, if required. Finally, as expected, a greater number of sutures enabled higher IOP burst point. However, we were surprised to learn that even the placement of eight interrupted sutures resulted in burst IOP levels higher than 80 mmHg after cross-linking, providing further evidence of the strengthening capabilities of this approach.

In the ALK technique, we established a trend toward greater adhesion in the human-to-human cross-linked group. Electron microscopy enabled visualization of the graft-host interface with evidence of crossed and longitudinal cross-linked collagen fibers, bridges, densities, attachment plaques and primitive plasmalemmal densities. These findings need to be explored further, but enhance our understanding of the potential bonding which is expected to occur across wounds in collagen cross-linking.

One weakness in this study was the fact that we were unable to assess the graft-host interface in the full thickness transplants, but plan to explore this in the future.

In conclusion, the cross-linking effects of the FUR technique enable a stronger graft-recipient adhesion compared to conventional PKP and ALK. Electron microscopy enabled visualization of the cross-linked interface. We believe that the FUR approach may open avenues to sutureless transplantation techniques in the future.

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