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INVESTIGATING THE EFFECT OF DISSOLVED OXYGEN-ASSISTED CORNEAL CROSS-LINKING (CXL) ON PORCINE CORNEAS

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By

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ABSTRACT

INVESTIGATING THE EFFECT OF DISSOLVED OXYGEN-ASSISTED CORNEAL CROSS-LINKING (CXL) ON PORCINE CORNEAS

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Thesis supervised by Bin Yang, PhD

Corneal cross-linking is a clinical procedure that is known to stop the progression of keratoconus, an eye disease that affects the cornea's structure, ultimately leading to vision loss in its advanced stages. The typical treatment plan includes riboflavin and UV-A exposure in the hope to increase the mechanical properties of the cornea. There are two types of CXL pathways, with Type-II CXL requiring oxygen. Naturally, the dissolved oxygen is limited in the cornea; therefore, limiting the effect of Type-II CXL. This study proposes to improve the Type-II CXL contribution by integrating dissolved oxygen during the standard CXL treatment used in today's practice. The enhancement of the cornea's mechanical properties was evaluated for oxygen-assisted CXL (O2CXL). Overall, the O2CXL showed a significant increase in biomechanical

enhancement as compared to the standard CXL. Such enhancement could be attributed to the supplied oxygen, which prolonged the Type-II CXL and improved its stiffening effect.

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LIST OF ABBREVIATIONS

CXL – Corneal Cross-linking

STCXL – Standard Corneal Cross-linking

O2CXL – Oxygen-Assisted Corneal Cross-linking

RF – Riboflavin

KCN – Keratoconus

INTRODUCTION

Our sense of sight is one of the important ways we absorb information. Visual perception is made possible in part by the cornea, which represents the outer structure of the eye [1]. Specifically, the cornea's transparency enables light to pass into the retina, making the cornea play a significant role in ocular refraction; therefore, damage to the cornea and its visual structure can cause detrimental effects such as vision loss [2].

Typically, minor corneal injuries that damage the cornea's surface can heal independently; however, treatment could lead to a more invasive approach in severe cases, such as corneal ectasia. Corneal ectasia disorders represent a group of disorders characterized by the ocular structure changing due to weakness or thinning of the cornea [3]. Such deformation of the ocular structure leads to protrusion, irregular astigmatism, and a profound impact on the visual acuity [3]. Fortunately, there are a variety of treatment approaches being studied to manage various forms of ectasia as researchers are trying to find alternatives to avoid invasive corneal surgeries—such as corneal cross-linking (CXL), a minimally invasive procedure.

With corneal ectasia disorders, the biomechanical strength of the cornea is reduced [3]. In turn, corneal cross-linking was introduced to halt the progression of ectasia disorders, particularly to treat keratoconus, and ultimately enhance the corneal rigidity [4]. Further research is needed to optimize the CXL treatment protocol to accelerate the time while maintaining efficacy. This study aims to adopt the standard Dresden CXL protocol, which is currently used in medical practice, while also introducing dissolved oxygen into the procedure to understand if dissolved oxygen would show a significant improvement in the cornea's rigidity as compared to the standard CXL procedure. This study hopes to recognize further the importance of

incorporating additional oxygen and its role in the CXL treatment, potentially accelerating the standard Dresden treatment plan in future practice.

CHAPTER 1. The Human Cornea

The cornea comprises of five layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium [Figure 1] [5]. The epithelium is the cornea's outermost layer, which serves as a protection field layer [6]. The endothelium is the cornea's innermost layer, which maintains the cornea's transparency by regulating the stromal hydration [6]. The function of the endothelium is essential because excess fluid could result in making a person's vision hazy [6]. The Bowman's layer, which lies beneath the epithelium, and the Descemet's membrane, which lies above the endothelium, serve as protection against injury and infection and maintain the cornea's transparency [6].

The stroma constitutes around 90% of the corneal thickness and comprises of collagen fibrils coated by different proteoglycans. The biomechanical characteristics of the cornea depend on the anatomical and biomechanical properties of the stroma [2]. The major component of the corneal stroma is collagen fibrils organized into bundles known as lamellae, which account for the transparency and mechanical strength of the normal cornea [5]. Keratocytes are distributed throughout the lamellae, essential for mechanical strength and the cornea's transparency [5, 7]. Ultimately, the keratocytes synthesize new collagens and proteoglycans while also secreting collagenases and other enzymes to degrade the old stroma matrix [5, 7]. This is crucial to maintaining the stroma's collagen scaffold and extracellular matrix—primarily upon injury as keratocytes become active during wound healing [7]. Ultimately, any abnormality in the cornea's structure or composition will disrupt the mechanical stability of the cornea.

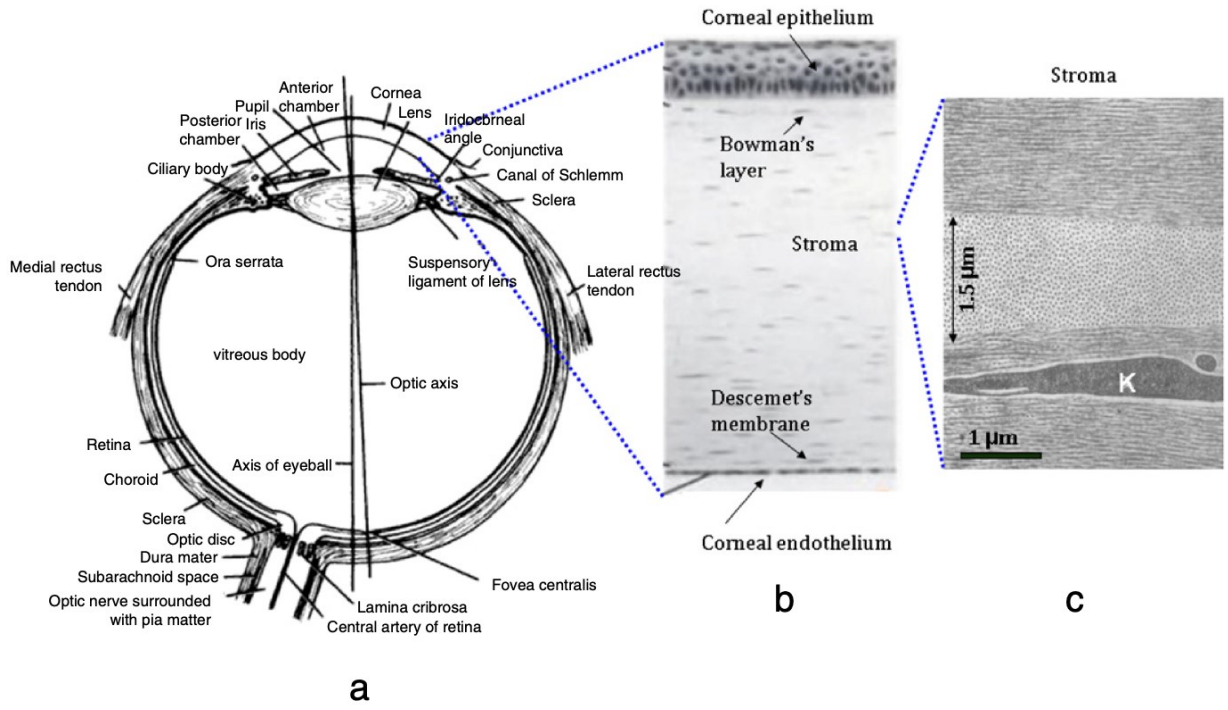

Figure 1. A cross-section of the human eye, with an expanded view of the cornea's five layers [6].

CHAPTER 2. Keratoconus

Keratoconus (KCN) is the most common form of corneal ectasia [8]. KCN is often manifested as a bilateral condition as it affects both eyes; however, there are rare instances that it presents itself as unilateral or asymmetric in severity. Generally, KCN begins to affect people at a young age, but most cases become clinically apparent in the late teens to early twenties [8]. The global prevalence of KCN is 1.38 cases per 1000 populations [9]. Though widely researched, the etiology of KCN is unknown; however, the condition is multifactorial and could vary depending on the severity of the disease. Genetic abnormalities, environmental factors, and post-refractive procedures could be fundamental reasons for its predisposition [9].

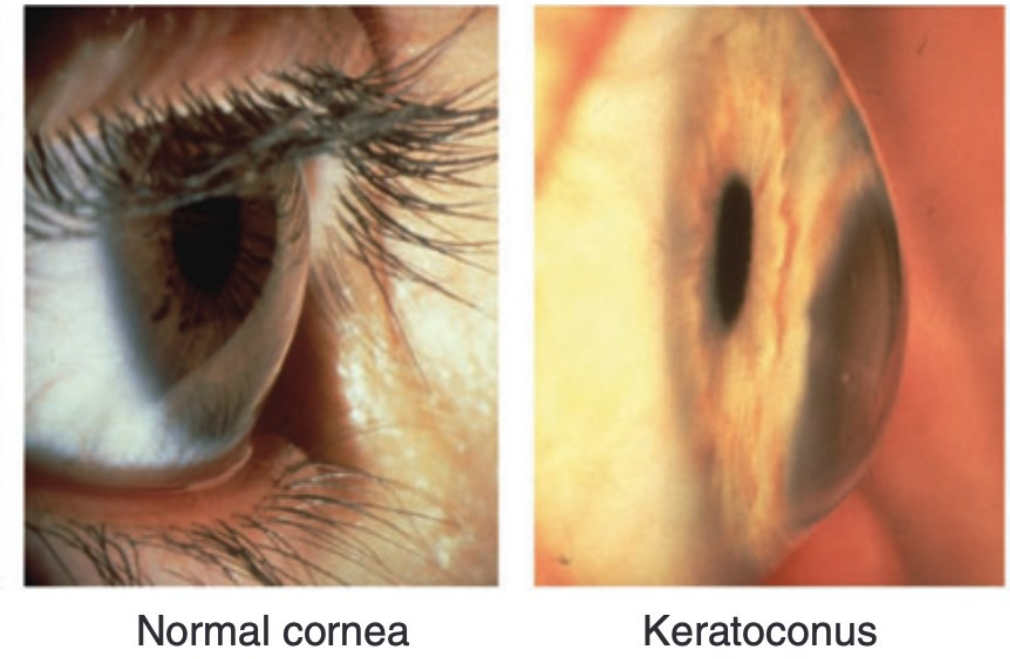

Figure 2. Normal cornea compared to an individual with keratoconus [10]. The corneal degeneration leads to a protrusion of the thinned cornea. The cone shape of the cornea is why it

has been named Keratoconus, derived from the Greek words *kera*, meaning cornea, and *konus,* meaning cone.

2.1 Signs and Symptoms

One of the main challenges for the prognosis of this disease is that KCN typically does not produce any signs or symptoms in its incipient stages; therefore, the condition goes unnoticed unless specific tests, such as corneal topography, are conducted. In general, the organization of collagen fibers, ground substance, and cells all determine the mechanical properties of the cornea's viscoelastic tissue. These factors are known to be affected in individuals who have KCN [11]. Three common signs characterize KCN: stromal corneal thinning, discontinuity or defects of the Bowman's layer, and iron deposits within the corneal epithelium layer (Fleischer's Ring) [8].

The etiology of Fleischer's Ring is relatively unknown; however, Hiratsuka et al. suggest two different explanations related to the cornea's thinning and iron deposition. Hiratsuka et al. indicate that the disturbance to the corneal epithelium could lead to iron accumulation, ultimately changing the interaction between the epithelium and the stroma and changing the metabolism of collagen fibers and thins the stroma [12]. The alternative explanation could be due to the changes in iron metabolism that can occur in the epithelium, which directly causes the thinning of the stroma [12]. Such evidence for this explanation is seen in iron-requiring proteins in the collagen synthesis [13]. Iron is known to be a required co-factor in the formation of hydroxylysine, which plays a crucial role in stabilizing intra- and intermolecular crosslinks that affect the diameter of collagen fibers in the cornea [12, 14]. A study has displayed that hydroxylysine levels are lower

in patients with keratoconus, which implies an abnormality in iron metabolism could affect the development of these iron lines and potentially cause the stromal thinning [15]. Clinically, Fleischer's ring presents itself as a yellow-brown circle line in moderate to advanced stages of KCN. Ultimately, the line implies an accumulation of iron deposits from the tear film onto the cornea, resulting in severe corneal curvature changes induced by the disease [Figure 3] [8].

Other deficiencies in individuals who have KCN include: the corneal epithelium's basal cells degenerate and grow towards Bowman's layer. The basal cell density is also decreased in comparison to a normal cornea. Also, in the stroma, studies have shown a decrease in the number of lamellae and keratocytes, degradation of fibroblasts, changes in the gross organization of the lamellae, and uneven distribution of collagen fibrillar mass and inter-and intra- lamellae when compared to a normal cornea [Figure 4] [16, 17]. Another characteristic sign is the presence of Vogt's striae produced due to the compression of the Descemet's membrane [Figure 5] [8]. In severe KCN cases, breakage in the Descemet's membrane has caused corneal hydrops, which is the sudden opaqueness of the cornea due to edema [Figure 3] [8].

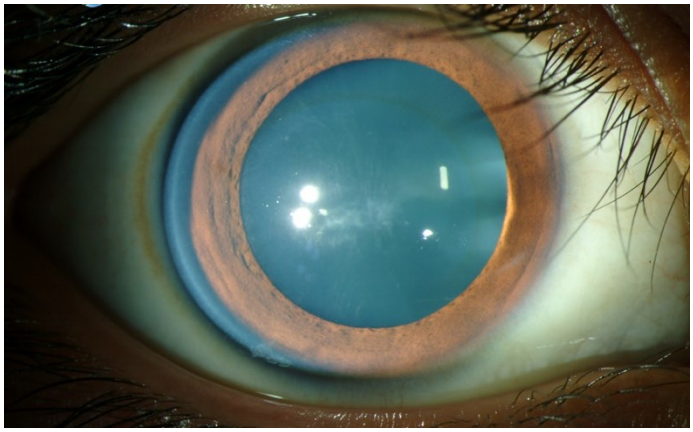

Figure 3. Fleischer's Ring is present on an individual with KCN [18]. The characteristic presents itself as a yellow-brown ring around the base of the cornea.

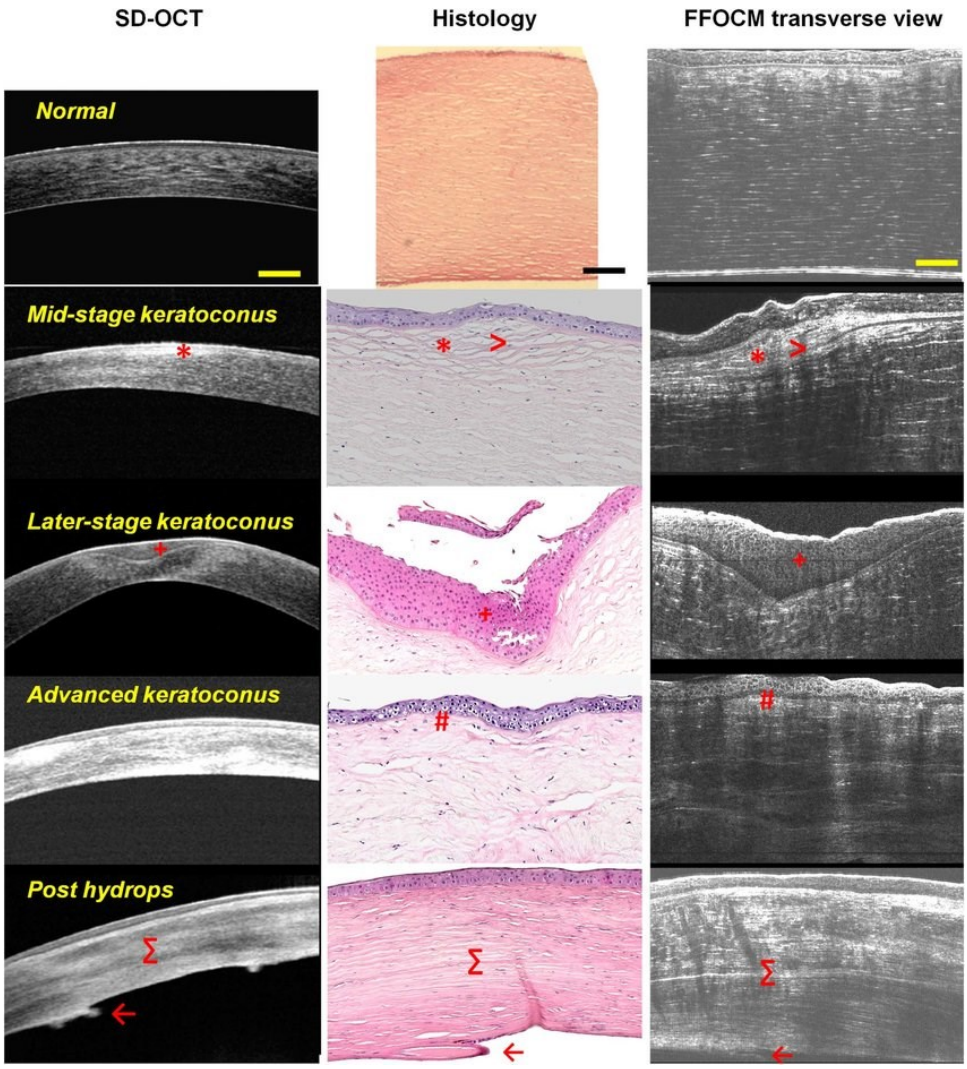

Figure 4. Cross-sectional views of a normal cornea and progressive keratoconus from early to advanced stages [17]. Imaged through Spectral-domain optical coherence tomography (SD-OCT), Full-field optical coherence microscopy (FFOCM), and standard histology. Indicators include: > Bowman's layer interruption; * Fibrotic tissue; + thickened epithelium; # Bowman's layer absent; Σ stromal scarring.

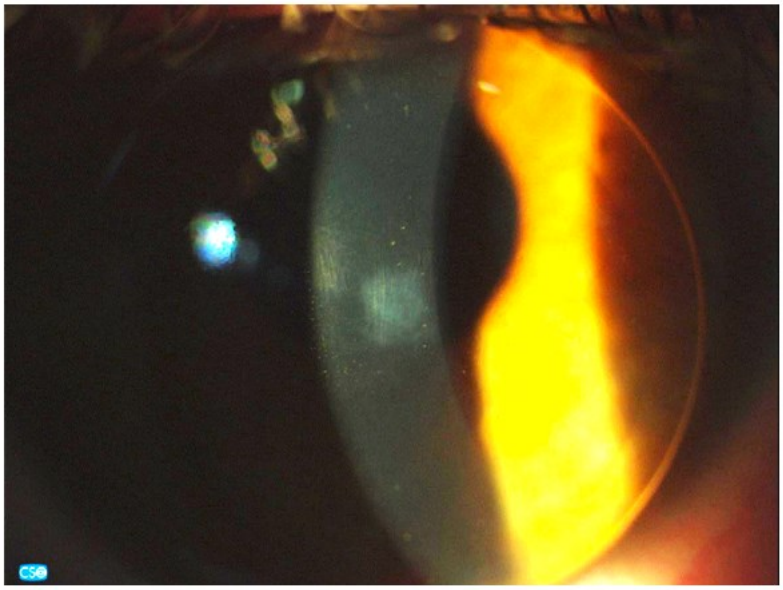

Figure 5. The clinical characteristic of Vogt's striae on an individual with KCN. Shown are vertical lines in Descemet's membrane. This image is taken from Romero-Jiménez et al. review paper on Keratoconus [8].

2.2 Treatment methods

Eyeglasses are usually prescribed to manage the disorder if detected in its early stages. However, suppose the disease progresses to a more moderate or severe case, in which the cornea severely weakens, and there is an increase in irregular astigmatism. In that case, its progression manifests in a significant loss of visual acuity that cannot be compensated with eyeglasses. Other treatment methods include contact lenses. As modern technology improves, the advances in contact lens technology and specialty lenses for irregular corneas have made visual rehabilitation for KCN patients more available. When a severe disorder case arises, surgical approaches

include penetrating keratoplasty, deep anterior lamellar keratoplasty, or intracorneal ring segments could be used as a treatment method [8]. These methods have been considered to improve an individual's vision, but it does not slow the progression of the disease, and the plans are typically costly. Countless studies have suggested that CXL impacts keratoconus progression by strengthening and stabilizing the collagen lamellae, which results in mechanical stiffening of the cornea. The CXL treatment method improves the patient's refractive error by reducing irregular astigmatism caused by the biochemical instability of the cornea.

CHAPTER 3. Corneal Cross-linking (CXL)

The CXL medical treatment is a minimally invasive intervention that utilizes ultraviolet A (UV-A) and riboflavin (vitamin B2) to halt the progression of corneal ectasias like Keratoconus [4]. The introduction of cross-links in the stroma of cornea tissue was first recognized in the late 1990s when a study done by Seiler et al. investigated the possibility of cross-links in corneal tissue to increase stiffness [19]. Seiler et al. compared untreated porcine corneas to treated porcine corneas with riboflavin and UV- irradiation, which showed nearly a 70% enhancement in the corneal stiffness [19]. This initial study was the first to introduce the epithelium- off approach, also known as the Dresden protocol or standard protocol, which is widely used in current studies and eventually became a Food and Drug Administration-approved treatment used in current practice. Several studies have demonstrated that the promising CXL treatment delays or halts the progression of keratoconus by improving the patient's corneal shape and visual acuity.

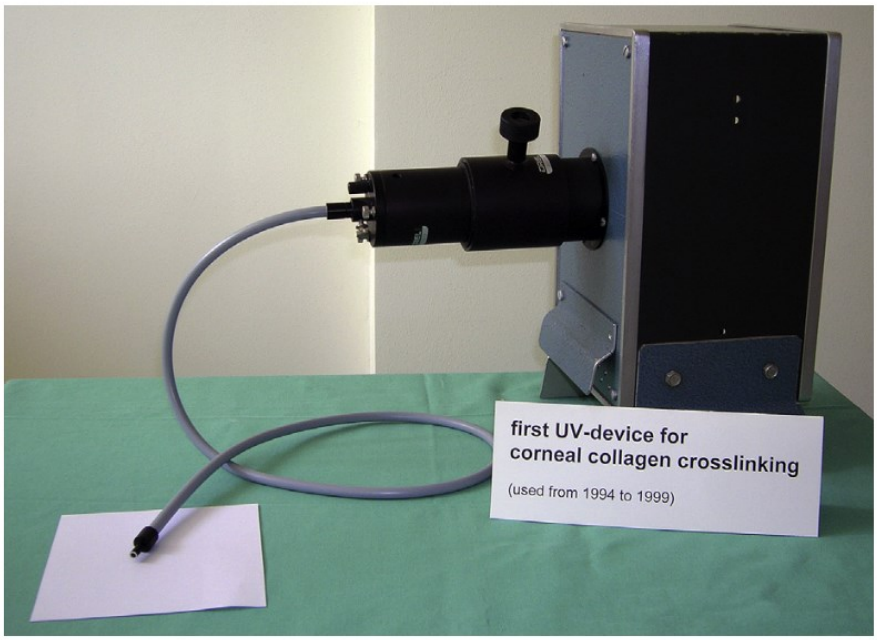

Figure 6. The first UV light-emitting device was used in the Seiler et al. research study [4].

3.1 The role of riboflavin (RF) and UVA irradiation in CXL

RF plays a vital role as the primary inducer of the standard photochemical reaction in CXL. Its alkyl isoalloxazine structure allows for absorption over a wide range of the light spectrum, particularly an absorption peak at the UVA light range [4]. Additionally, the photosensitization reaction that RF catalyzes involves the production of singlet oxygen that reacts with collagen fibers and proteoglycans [20]. An epithelium-off approach is implemented in the standard protocol to maximize the CXL treatment and efficacy of stromal CXL treatment since it allows for enough RF concentration to penetrate the cornea [20]. UVA light interacts with RF to activate oxidative and glycosylation pathways that lead to collagen cross-links formation [20]. The prime absorption peak of RF at 370 nm is ideal for the practical CXL effect.

Generally, the standard protocol found that 3mW/cm^2 of energy for 30 minutes provided maximum efficacy of tissue stiffening.

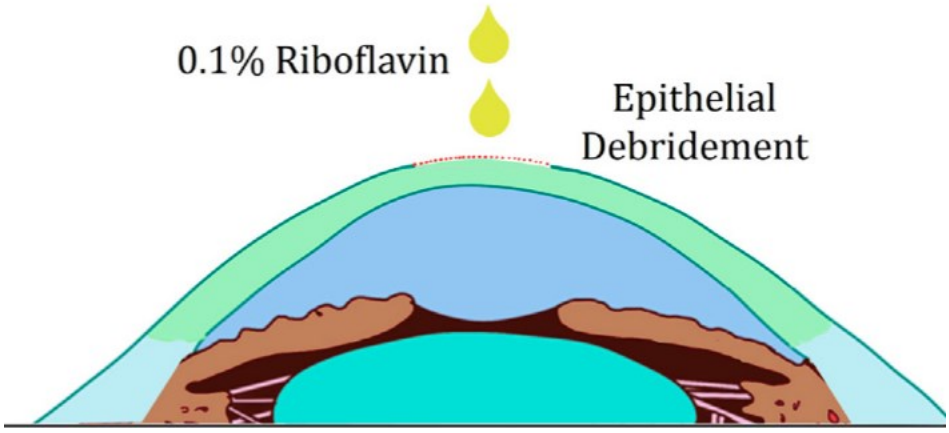

Figure 7. Illustration of the standard CXL protocol [2]. The standard CXL treatment in practice uses 30 minutes of 0.1% Rf drops after removing the epithelium layer. Following this procedure, the cornea is exposed to 30 minutes of 370 nm UV-A illumination at 3mW/cm^2.

3.2 CXL pathways

Once RF absorbs UVA light energy, it excites into a triple-state that undergoes two types of oxidative reactions, anaerobic (Type-I) and aerobic (Type-II) [Figure 8]. Since the oxygen concentration in the cornea is modified by UV-A irradiance and temperature, the oxygen concentration quickly decreases at the beginning of the UV-A exposure [20, 21]. The time-dependence of both type-I and type-II photochemical mechanisms in corneal cross-linking with riboflavin is considered. Both types of oxidative reactions aid in the initiation of covalent bonds; however, further research has proved that oxygen is fundamental in increasing the biomechanical rigidity of the cornea [22]. More specifically, the study by Richoz et al. performed epithelium-off CXL on ex-vivo porcine corneas in a low-oxygen environment. Their results displayed that CXL in a low-oxygen setting could cause a decrease in biomechanical rigidity as compared to the regular oxygen leveled environment; therefore, oxygen is critical in increasing corneal stiffness [Figure 9] [22].

As for the human eye, most of the corneal oxygen supply is provided by the atmosphere, accessible through diffusion from tears. Consequently, the limited oxygen supply within the cornea restricts the overall CXL effect of the Type-II pathway. As the study above displays that oxygen is fundamental in CXL treatment, further research needs to be conducted on incorporating oxygen into the standard CXL protocol and comparing the biomechanical impact of oxygen combined CXL to the typical standard CXL protocol in practice. Such research would continue to validate the need for oxygen in CXL to aid in the increase of biomechanical properties.

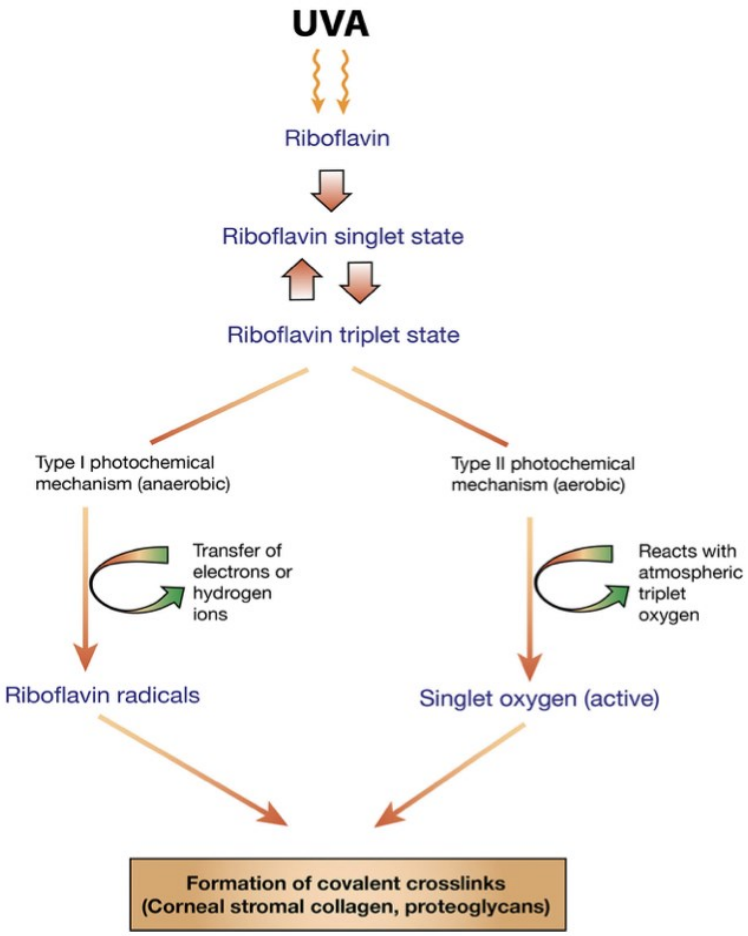

Figure 8. Mechanism of CXL with riboflavin and UV-A [21]. Anaerobic (Type-I) and aerobic (Type-II) pathways lead to crosslinks forming within collagen fibers after CXL treatment.

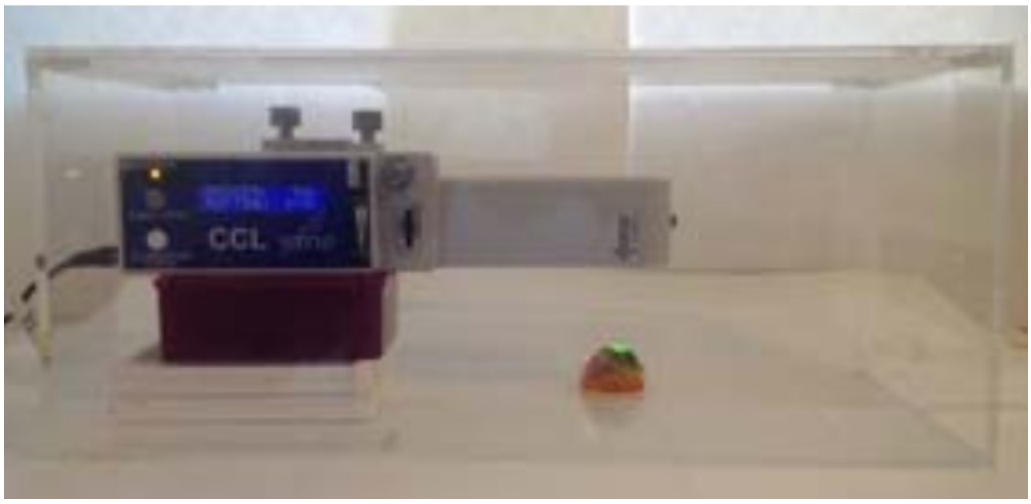

Figure 9. The experimental setup of Richoz et al.'s research [22]. The experimental set-up consists of a sealed oxygen-low/helium environment during CXL treatment. The results suggest that low oxygen does not show a high corneal stiffness effect, proving that oxygen is needed during treatment.

CHAPTER 4. Methods

4.1 CXL Preparation

Porcine eye preparation:

24 Porcine eyes were obtained from a slaughterhouse 6-hour postmortem. A razor blade and surgical scissors removed the excess soft muscular tissues surrounding the porcine eyeball. Once finished removing the excess tissue, the porcine eyes were wrapped in parafilm and placed in the freezer at -78℃. The 24 porcine eyes were randomly divided into two groups: 12 standard CXL groups and 12 O2CXL groups. The standard CXL group will follow the Dresden protocol without added dissolved oxygen. The O2CXL group will serve as the group which is exposed to dissolved oxygen before and during RF solution staining.

Riboflavin (RF) solution staining:

Before putting the whole ocular globe into the chamber filled with RF, 10% ethyl acetate with PBS solution was applied dropwise to remove the epithelial tissue of the eye using a razor blade. Riboflavin solution at a concentration of 0.146% was used for both standard CXL and O2CXL groups. The whole globe of the eye was submerged in the riboflavin cornea facing down in a 3D-printed chamber for 30 minutes before CXL treatment.

Specifically, for O2CXL, the RF solution was saturated with oxygen for 20 minutes before cornea staining. The RF solution was maintained with the oxygen supply during the 30 minutes RF staining process of the corneal portion of the whole globe.

CXL treatment:

Before each treatment for CXL, two identical cornea strips were dissected along the short axis. Given the sizeable intra-sample variation of mechanical properties, one strip was chosen as a reference with no CXL treatment. The other cornea strip underwent CXL treatment [Figure 10]. All CXL-treated corneal strips experienced 365 nm UV light at 3 mW/cm^2 for 30 minutes.

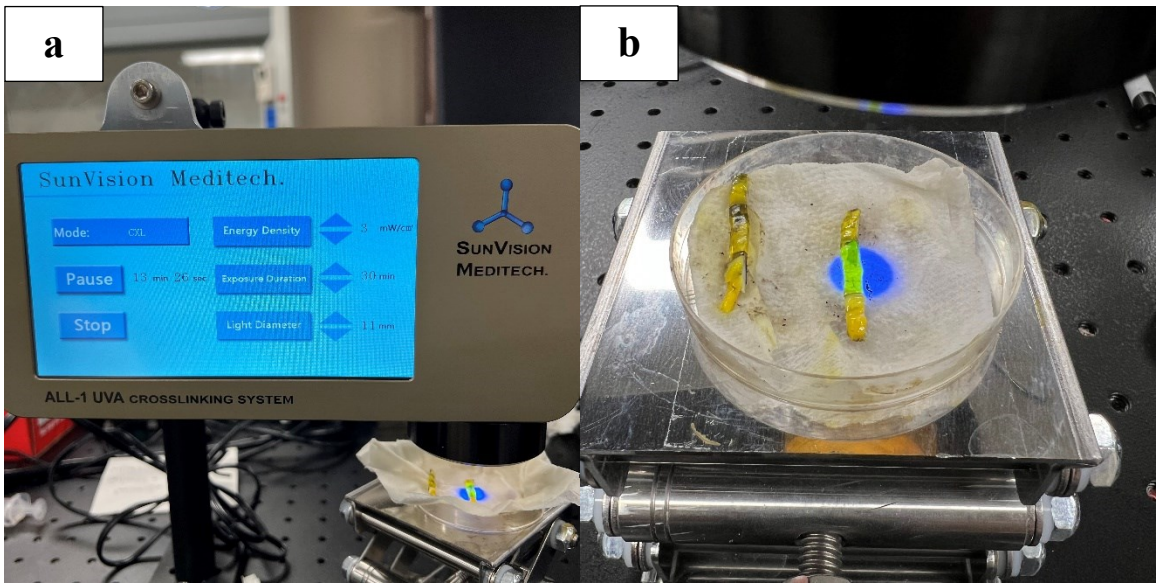

Figure 10. Cross-linking device and setup. Figure 10a is the UV-A crosslinking system used in many CXL treatment practices. Figure 10b shows the cornea strip assigned for CXL treatment under the UV-A light and the reference strip is placed to the side. Both cornea strips were hydrated with water accordingly.

4.2 Mechanical Testing

3D Printing:

Fusion 360 was used to design and fabricate clamps for the uniaxial tests, and the chamber was used to submerge the cornea into the RF solution [Figure 10]. 3D printed razor spacers were fabricated to ensure the width of the cornea strips was controlled.

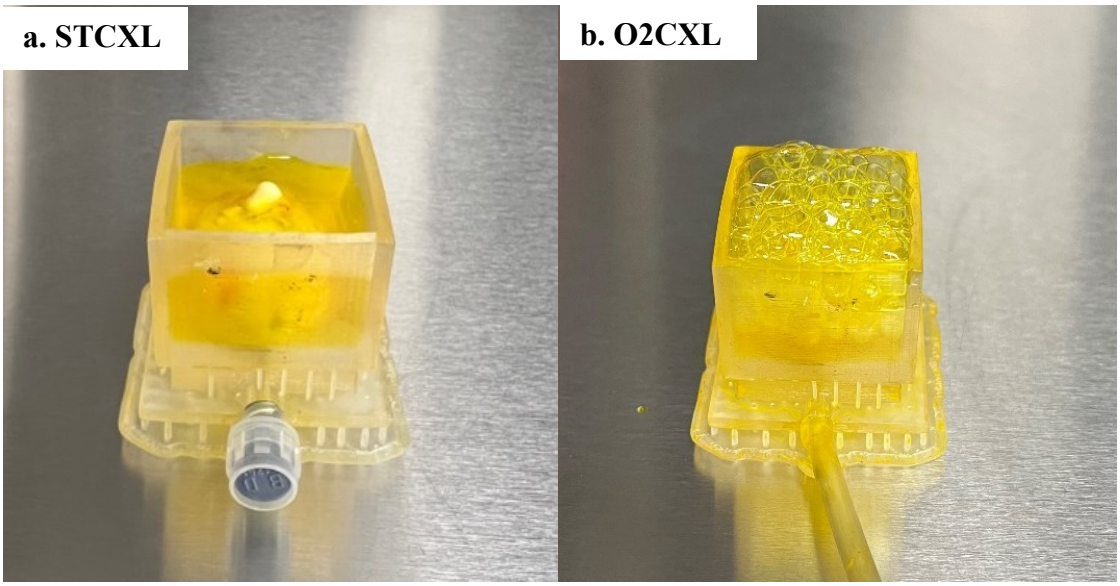

Figure 11. 3D printed chamber that holds the whole globe of the porcine eye—which is submerged in 0.146% RF solution. Figure 11a. is the STCXL setup. Figure 11b. is the O2CXL setup with 0.25 psi of oxygen released from the attached tube and into the chamber with 0.146% RF solution.

Uniaxial tensile tests and Analysis.

The stiffness of the cornea was measured and determined by performing a uniaxial tensile test. A force gauge of 20 Newtons was used for the uniaxial tests. Before testing, both cornea strips were measured for length, width, and thickness. Uniaxial tensile testing was performed for reference and treated corneal strips [Figure 12]. The tangential modulus at 10% strain was determined, and the enhancement for each group was calculated using MATLAB.

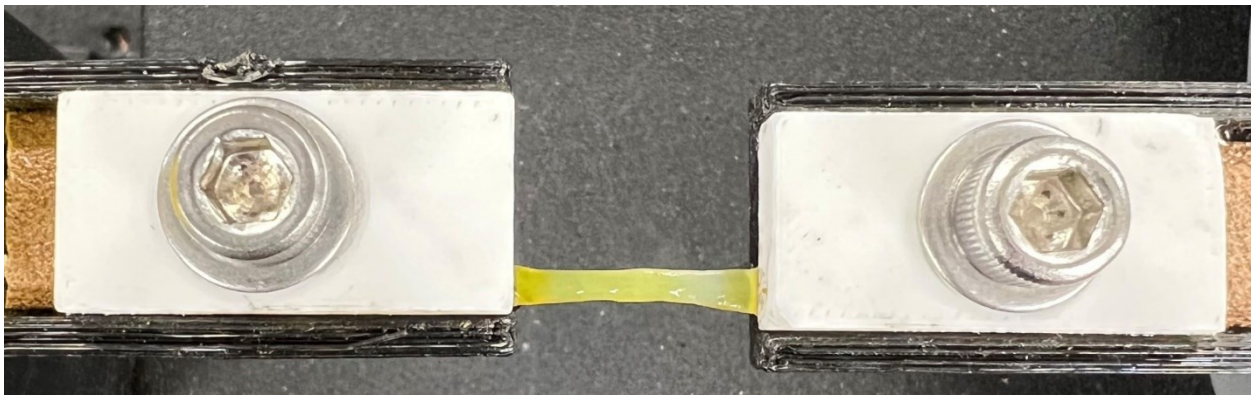

Figure 12. Uniaxial tensile test clamps with cornea strip. A 20N force gauge was used to measure pull force and determine the young's modulus.

Repeatability study.

A baseline group of porcine eyes without RF solution and CXL treatment was tested to validate the improvement of standard CXL and O2CXL tests once conducted.

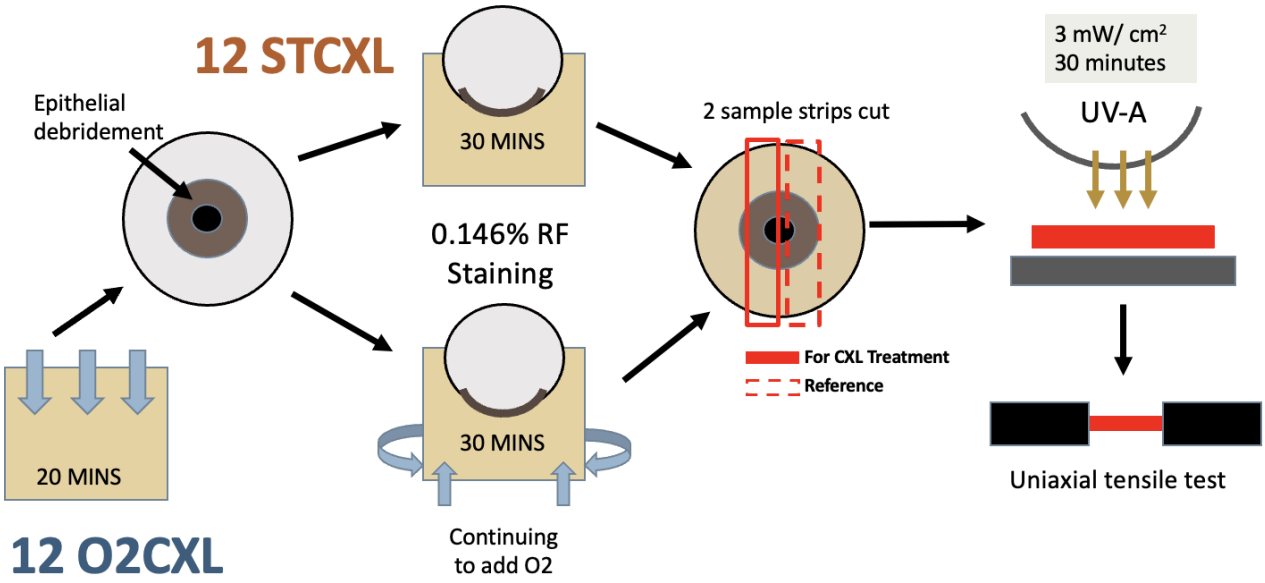

Figure 13. A schematic of the complete experimental methods conducted for this research. For O2CXL, additional 20 minutes of oxygen integration for the RF solution was conducted prior to adding the whole globe into the solution. After the 20 minutes, the epithelium was removed and the whole globe was added to the solution. Oxygen was continually added to the 30-minute RF staining process. For STCXL, the whole globe was added into the RF solution after the removal of the epithelium.

CHAPTER 5. Results

As for the repeatability study, the results indicate that each porcine eye shows a slight variation in mechanical properties. Based on these results, two cornea strips are dissected from 1 eye to test for STCXL and O2CXL tests. One cornea strip will serve as a reference with no CXL treatment, while the other cornea strip will undergo CXL treatment. Overall, the difference in mechanical properties showed eye 1 having a 2.2% difference and eye 2 having a 7.9% [Table 1].

At 10% strain, the treated corneal strip with O2CXL resulted in an average of 131.9% ± 34.78 increase in tangential modulus compared to the non-treated corneal strip samples [Figure 16]. The treated samples with STCXL showed an average improvement of 33.35% ± 2.96 [Figure 16]. O2CXL models are 295.5% more effective than the standard CXL.

Table 1. Repeatability study shows large baseline inter-sample variation.

	Eye 1 at 10% strain	Eye 2 at 10% strain
1 Cornea Strip: Sample 1	9.1	5.9
1 Cornea Strip: Sample 2	9.3	5.47
Difference	2.2%	7.9%

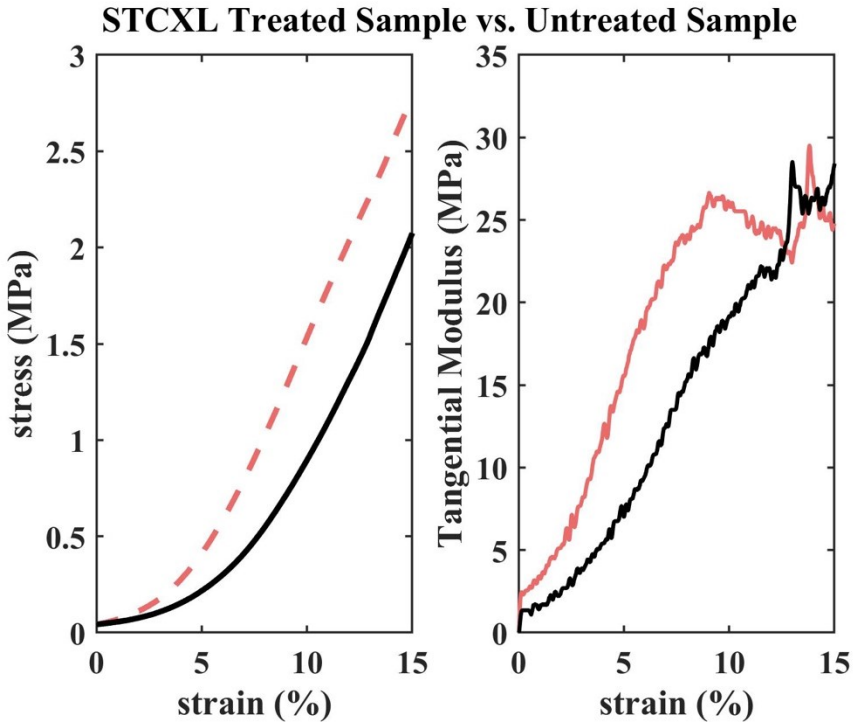

Figure 14. Porcine eye #2, Raw data of STCXL Treated Sample strip vs. Untreated Sample strip. There was an improvement of 35.45% when the STCXL treated cornea strip was compared to the untreated sample (reference strip with no CXL treatment).

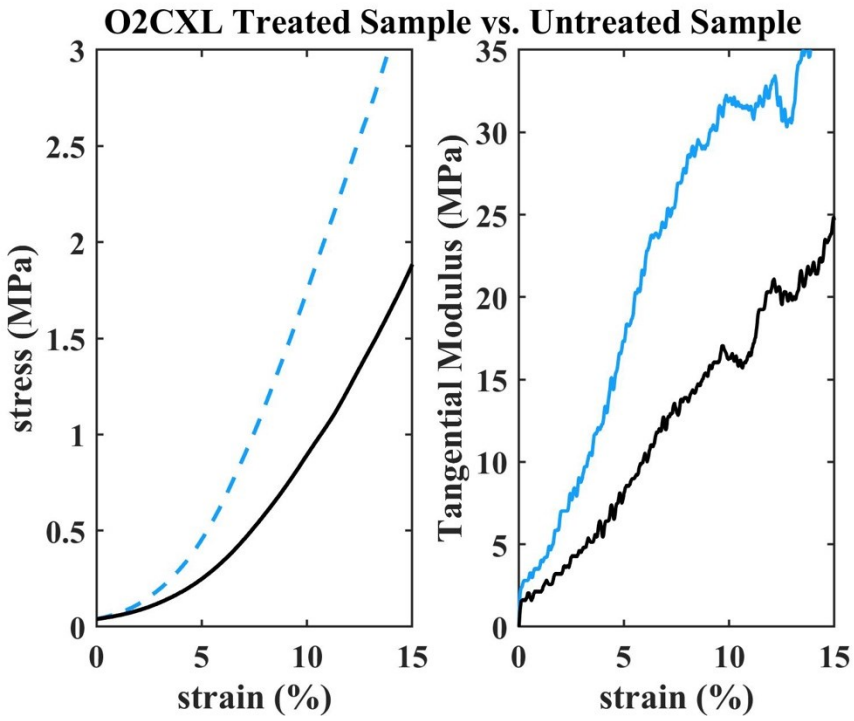

Figure 15. Raw data of O2CXL Treated Sample strip vs. Untreated Sample strip. Raw data of porcine eye #3 is shown above. There was an improvement of 95.84% when the O2CXL treated cornea strip was compared to the untreated sample (reference strip with no CXL treatment).

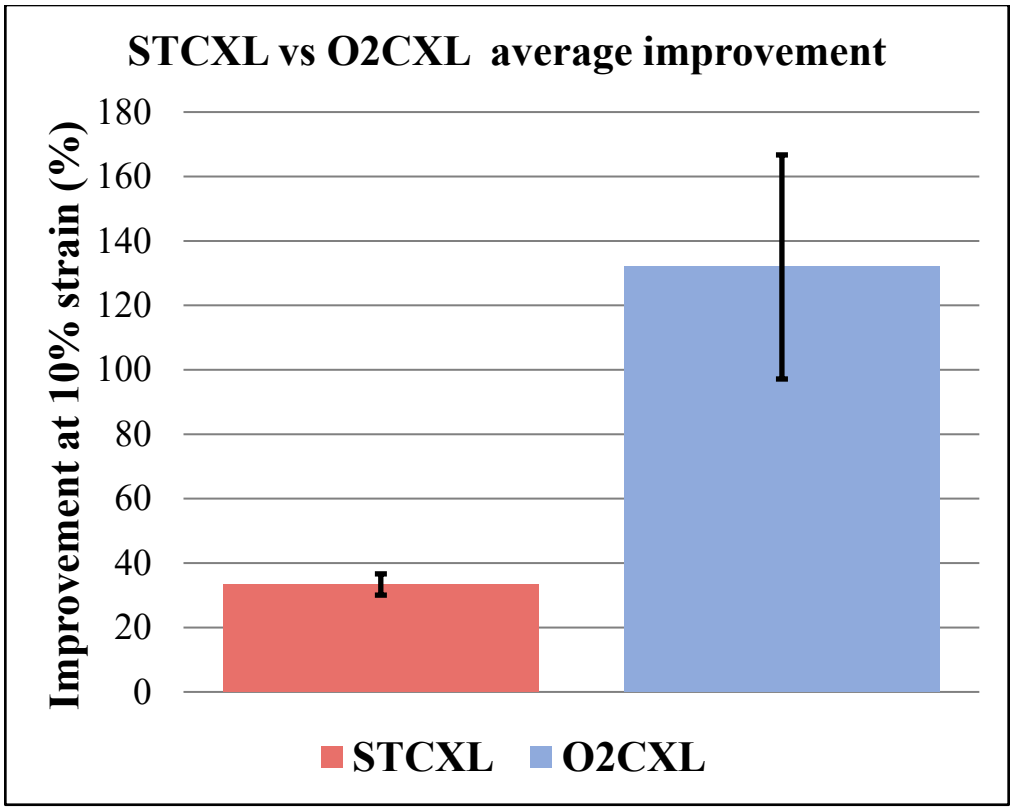

Figure 16. Average improvement of STCXL compared to O2CXL. O2CXL models are 295.5% more effective than the standard CXL.

CHAPTER 6. Discussion

In previous studies, keratoconic corneas displayed that the corneas are much weaker and less rigid when compared to normal corneas. CXL has proved to be a successful treatment alternative to manage and stop the progression of KCN. The Type-II pathway is ultimately limited in the CXL mechanism due to the depletion of oxygen during the procedure. Our study confirms oxygen's ability to successfully increase the porcine cornea's biomechanical stiffness, showing an impressive increase of nearly 300% in biomechanical rigidity—exceeding the expected 70% stiffness rate seen in previous research. This result could be attributed to the oxygen integrated into the procedure, further validating the earlier research study, claiming that oxygen is a fundamental qualification in CXL treatments. Interestingly, for the O2CXL cornea strips, four improvements were in the high 100-400% enhancement range. Even by removing all the outliers, such as the high enhancements seen in O2CXL, the O2CXL continued to show a significant difference in stiffness compared to all 12 STCXL eyes. Ultimately, the results indicate that oxygen-assisted CXL can be a useful method if incorporated into real-life practice. Finally, the results obtained from this research study could serve as valuable information and a guideline to further improve and optimize the current standard CXL treatment plans in the future.

Since it has been successfully confirmed that dissolved oxygen is necessary, further research can be conducted to improve the dissolved oxygen-assisted CXL methods. Ideally, treatment time needs to be reduced to prevent discomfort in real-life practice. Since incorporating dissolved oxygen in this study did not reduce the treatment time, a time-course study could be helpful to identify the prime CXL treatment time or the amount of time oxygen is introduced. Since dissolved oxygen exhibits a significant enhancement, treatment time could be reduced by increasing the UV-A exposure to see if additional oxygen can produce similar results

shown in this study. Potentially, with the help of more research, an accelerated version of CXL can be used with the incorporation of oxygen, such as 9 mW/cm^2 for 10 minutes or 18 mW/cm^2 for 5 minutes instead of 3 mW/cm^2 for 30 minutes. In the future, clinical studies should be further researched to find an ideal and safe method to incorporate oxygen into medical practice.

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