Corneal Cross-linking in the Management of Fungal Keratitis

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

2017

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BAC</td>
<td>Benzalkonium Chloride</td>
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<tr>
<td>CXL</td>
<td>Corneal Cross-linking</td>
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<tr>
<td>d</td>
<td>Day</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>h</td>
<td>Hour</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin Staining</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium Bicarbonate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>PACK-CXL</td>
<td>Photo-Activated Chromophore Corneal Cross-linking for Infectious Keratitis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Potato Dextrose Broth</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SAM</td>
<td>Scanning Acoustic Microscopy</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>TEX</td>
<td>Trans-epithelial Corneal Cross-linking</td>
</tr>
<tr>
<td>UV-A</td>
<td>Ultraviolet-A light</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
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<td>µl</td>
<td>Microlitre</td>
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Abstract

Fungal keratitis is a devastating corneal infection with a high level of ocular morbidity. Combined riboflavin/UV-A light-induced corneal cross-linking (PACK-CXL) has emerged as a potential primary or adjuvant therapy for treating corneal infections, in particular for the antibiotic resistant forms. The aims of this project were to develop a novel ex vivo human corneal *Fusarium* infection model, and to investigate the effect of PACK-CXL procedure as a primary or adjuvant with antifungal (natamycin) therapy in the management of different severities of *Fusarium* infection, following various regimes of the PACK-CXL procedure.

In this project, an ex vivo human corneal *Fusarium* infection model was successfully established using donor post-mortem human corneas and a recombinant strain of *Fusarium oxysporum* expressing green fluorescent protein (GFP). The results obtained from using the PACK-CXL procedure as a primary therapy in treating the *Fusarium* infections showed a beneficial antifungal effect against *Fusarium* by suppressing the growth of hyphae and sporulation, and reducing the depth of hyphal penetration into the corneal tissues at the central (UV irradiated) region of the treated corneas. However, PACK-CXL procedure alone had a limited ability in controlling the progression of the *Fusarium* infections, along with a time-dependent decrease in its efficiency in reducing the load of the infection. Nevertheless, the PACK-CXL procedure as an adjuvant to antifungal natamycin therapy greatly controlled progression of the infection.

The in vitro results suggest that the PACK-CXL procedure as a monotherapy is insufficient in controlling the *Fusarium* keratitis. The dual therapy of PACK-CXL and natamycin can be considered as a highly effective treatment in the management of *Fusarium* keratitis. However, PACK-CXL treatment could be used as an initial intervention in cases of infectious keratitis with unknown causative pathogen(s) to reduce pathogen load within the corneal tissues until the causative organism(s) is identified and appropriate antimicrobial therapy can be administered.
Declaration

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Thesis Format

This thesis includes eight chapters which are arranged in a journal format. The second chapter has been published, whereas the other chapters, except the eighth, are being prepared for submission to peer-reviewed journals.
Dedication

To those who have liberated me with love, and gave me wings so I can fly ...

To my father and mother ...

Today, I have looked back to the distant past. I quickly thought of the details of my lost years and found that there was no one who loved me unconditionally except the two of you. Each time I stumbled, I always found you beside me, holding my hands, helping me to stand up, and flicking the dust of this life away. Every time I lost the way or made the wrong choice despite your advice, you forgave my ignorance, patted my heart, healed my soul, and whispered to me that the most beautiful is still waiting for me. You were not only my parents, but you were my friends and my spiritual companions. You were my light and my guidance. Today, I owe every beautiful thing that has ever happened to me or ever will be to you.

I now lay in your hands the outcome of three years of yearns and homesickness to you which originally was the result of your love and patience. I know it is unfair to dedicate only three years to you while you have devoted your whole lives to see me as I am today, but all I can do is to dedicate my heart to you which was and always will be beating with your love.
Acknowledgments

All gratitude goes to Allah for all his infinite blessings. It is because of Allah and him only I was blessed with studying at the prestigious University of Manchester for my PhD degree.

I would like to express my deepest gratitude to my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby, and Dr Susan Shawcross for all their constant support, guidance, supervision, cooperation, and encouragement of my project. I have been extremely privileged to have three outstanding supervisors who cared so much about my research and who have been so prompt to answer all my queries.

I would like to thank my sponsor: The ministry of Health in Saudi Arabia for funding my PhD project.

I would like to thank my advisor: Dr Emma Gowen for her support.

I would like to thank my senior: Khaled Alzahrani for all his constant support and encouragement.

I would also like to sincerely thank the people who have played important roles that were turning points in my life. Lawyer Khalid Alsaadoun who stood by my side at a very hard time of my life. Dr Hasan Hamami who assisted me to get the scholarship from the ministry of health in Saudi Arabia. Prof. David Henson who gave me the opportunity at the beginning to study for my master degree at the University of Manchester.

Most of all, I would like to thank my family whom without I would not have become the person I am today. My sister Abeer for being the calming and reassuring voice in times of doubts and hesitation and encouraging me every step of the way. My sisters: Samira and Amani who overwhelmed me with endless love, stood by my side each and every time I needed them, and always tried to find time in their hectic lives to visit me. My brother Khalid and my brothers in law: Mohammed and Fahad for all their constant support. My nieces and nephews: Amjad, Mohammed and Alaa, and the small angels: Rodaina, Jassar and Baylasan for brightening my life.

I would like to thank my friends in Manchester: Boudor, Halah and Aiah, who helped me get through the days and night when I was homesick and made me laugh even when I did not want to.

Finally, I would like to thank my friends outside U.K.: Asia, Jonela, Dhanya, Jessie, Mona, Faten and Monirah who lives thousands of miles away from me but I can still feel their love and concern.
Chapter 1

Introduction and Literature Review

Submission for Publication

The literature review section of this chapter is prepared as a manuscript which will be submitted for publication.

1.1 Normal Human Cornea

The cornea is a highly specialized transparent tissue and frontal cover of the eye, which functions as a barrier to infection and provides most of the refractive power. The cornea is composed of five distinct layers (Figure 1-1), which are divided into two groups: epithelium, stroma, and endothelium are the cellular components; whereas the Bowman’s and Descemet’s membranes are the acellular interfaces (Daniels et al., 2010).

Figure 1-1: The structure of the human cornea.

Histological cross section of a human cornea showing the corneal structure of five layers: epithelium, Bowman’s layer, stroma, Descemet’s membrane, and endothelium. Adapted from: (Daniels et al., 2010).
The corneal epithelial layer is the primary protective barrier which blocks potential environmental organisms from entering the eye (Nishida et al., 2017). The corneal epithelium has 4 to 6 layers and a thickness between 53 µm to 58 µm, which is easily regenerated (Reinstein et al., 2008). The epithelial cells have a life span of between 7 and 10 days, which function through continuous involution, developmental cell death and desquamation (Hanna et al., 1961). The Bowman’s membrane is an acellular condensate of the anterior stroma. It is a 12 µm smooth layer which aids in the maintenance of the corneal shape. However, unlike the corneal epithelium it does not regenerate and scarring is possible following damage (Nishida et al., 2017). The corneal stroma comprises the majority of the cornea’s structure, and constitutes approximately 90% of the overall corneal thickness (Nishida et al., 2017). The stroma is composed of a complex of collagen fibres in a highly organized formation, unlike other collagenous frameworks (Boote et al., 2003). The collagen fibres are arranged in parallel bundles referred to as fibrils, and these fibrils are organised in parallel into lamellae (Maurice, 1957). The precise organization of stromal fibrils actively contribute to the mechanical stiffness and transparency of the cornea (Maurice, 1957, Boote et al., 2003). The corneal stroma contains keratocytes; the main type of cells in the stroma (Nishida et al., 2017), which play a role in synthesizing collagen molecules and creating extracellular matrix (ECM) and matrix metalloproteinases (MMPs) (DelMonte and Kim, 2011). The Descemet’s membrane is a basement membrane of substantial thickness of 5-10 µm, which is produced by the corneal endothelium (Pavelka et al., 2010). Corneal endothelium is a monolayer boundary situated between the anterior chamber and the corneal stroma, and covers the full posterior corneal layer. The corneal endothelium has a dual function where it allows water to pump from the
stroma into the aqueous humor and at the same time it allows for nutrients to diffuse from the aqueous humor into the corneal layers. This function plays a principal role in maintaining optical transparency, since the cornea is avascular, and controlling the corneal hydration (Waring et al., 1982).

The structure of the cornea undergoes several changes with age most important being an increase in the cross-links within the collagen fibrils with age (Elsheikh et al., 2007) and thinning of the Bowman’s membrane (Germundsson et al., 2013). The corneal structure and thickness also change with hydration for example with overnight closure of the eye or contact lens wear (Manchester Jr, 1970). However, there is a relation between corneal hydration and its transparency where the decreased transparency may occur due to a damage to the corneal epithelial or endothelial layers which results in epithelial and stromal oedema (Zucker, 1966).

The cornea is prone to be affected by external infectious organisms as it is constantly exposed to the environment. As a result, the cornea has developed various mechanisms of defence such as tear film and blinking system (Gilmore et al., 2010). The tear film has antimicrobial factors, such as lysozyme, lactoferrin and lipocalin, which reduce access of organisms to the corneal epithelial layer along with the blink shear forces (McDermott, 2013). For corneal infection to develop, it is imperative to initially overcome the corneal defence mechanisms (Gilmore et al., 2010). Likewise, the compromised integrity of the epithelial layer may initiate the onset of infection. This can be caused by ocular surface disease or trauma, which permits organisms to enter the tissue. Hence, a detrimental inflammatory response is felt, which damages the entire integrity of the eye, as well as its function (Mravičić et al., 2012).
1.2 Infectious Keratitis

Infectious keratitis is a sight threatening disease with the potential for significant and permanent visual impairment (Otri et al., 2013). It can be caused by a variety of microbes, such as bacteria, fungi, viruses and parasitic organisms. The clinical features of infectious keratitis vary depending on the underlying cause. Usually, it is characterized by the appearance of infiltrates, which may be localized or scattered among the corneal epithelium and stroma (Figures 1-2 and 1-3). Severe infectious keratitis cases are indicated by destructive ulceration of the corneal epithelium and stroma, which is likely to degrade vision significantly despite being treated (Thomas and Geraldine, 2007, Gilmore et al., 2010, Otri et al., 2013).

Figure 1-2: Human bacterial keratitis.

This figure shows a stromal infiltrate and hypopyon caused by bacterial pathogens. Source: (Shetty et al., 2014).
**Figure 1-3: Human fungal keratitis.**

This figure shows a corneal ulcer caused by *Fusarium sp.* Source: (Escarião et al., 2013).

Details of *Fusarium* organism are given in appendix A.

Clinical features and patient history are the guide for the initial diagnosis, which can be carried out using non-invasive examinations and techniques, including confocal microscopy and slit-lamp microscopy (Gilmore et al., 2010). Diagnosis of specific type of infectious keratitis can be challenging. Cases are frequently misdiagnosed and consequently treated incorrectly (Claerhout et al., 2004). A definitive identity of the infectious agent(s) is established by examining corneal scrapings (Gilmore et al., 2010). Diagnosis can be further hampered by the time taken to culture the infectious agent and antimicrobial sensitivity testing. Since infectious keratitis is a grave ocular disease, it needs rapid intervention and aggressive treatment. The delay in treatment may allow the infection to progress and potentially result in vision loss. The standard treatment options include antibacterial, antifungal and antiviral medications which can be applied topically or systemically according to the causative agent and seriousness of the infection (Thomas and Geraldine, 2007, Gilmore et al., 2010). On the other hand,
certain types of microorganisms have acquired resistance to antimicrobial agents, despite their general effectiveness (Betanzos-Cabrera et al., 2009). Hence, the inflammatory process can develop through corneal melting, and perforation of the cornea or ulceration, even after the required treatment is administered (Srinivasan et al., 1997, Chew et al., 2011). Nonetheless, corneal surgeries can be undertaken following failed medical treatments (Khodadoust and Quinter, 2003, Joshi et al., 2012). The surgical procedures depend specifically on the severity of the disease, and usually aim to remove the infectious elements, as well as necrotic tissue and other debris, which can prove detrimental to lesion healing. The surgeries do not necessarily permit total eradication of the infecting agent, thus failure of treatment can result through re-establishment of infection by residual micro-organisms (Tayapad et al., 2013). Consequently, current studies revolve around effective treatments, which actively manage and treat infectious keratitis, especially relating to potential treatment of resistant forms of corneal infections.

1.3 Corneal Cross-linking Procedure (CXL)

Corneal cross-linking (CXL) is an ocular surface technique which was initially introduced to treat corneal ectasias such as keratoconus (Wollensak et al., 2003a). This technique is based on utilising ultraviolet-A light (UV-A) and riboflavin (vitamin B2) as a photosensitizer. When riboflavin is activated by UV-A light, reactive oxygen species (ROS) are generated (Baier et al., 2006), and these photochemical reactions increase the covalent bonds (cross-links) through stromal collagen fibrils (Spoerl et al., 1998, McCall et al., 2010). These cross-links are formed between the collagen and the
proteoglycans of the extracellular matrix (ECM) (Zhang et al., 2011) and/or within the proteoglycan at the collagen fibril surface (Hayes et al., 2013). Hence, the fundamental biomechanical properties inside the cornea change, which results in a rise in corneal stiffness (Wollensak et al., 2003b, Kohlhaas et al., 2006, Wollensak, 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2013c, Beshtawi et al., 2014, Beshtawi et al., 2016). The standard corneal cross-linking protocol is commonly referred to as the ‘Dresden protocol’, and was originally demonstrated by Wollensak et al. (2003a). It is undertaken using 370 nm wavelength UV-A light with 0.1% riboflavin. During the standard CXL procedure, the corneal epithelium is removed in the central 7-9 mm diameter zone post-application of topical anesthesia. Following this, 0.1% riboflavin drops are instilled on the cornea for 30 minutes, at intervals of 2-5 minutes. Next, for half an hour there is application of UV-A irradiance at 3mW/cm², which administers a dose of 5.4 J/cm², alongside 0.1% riboflavin application at 5 minutes intervals. A bandage soft contact lens is then applied after antibiotic drops, until the process of re-epithelisation has taken place. The application of riboflavin solution creates a 95% rise in the UV-A absorption in the cornea, which aids the creation of new collagen fibril bonds (Wollensak, 2006), and functions as a filter in order to protect the underlying tissues from potential harmful effects by UV-A (Kymionis et al., 2009). The use of the CXL technique for treatment of corneal ectasia and advanced keratoconus is well-established (Raiskup-Wolf et al., 2008). Of late, the indications of CXL procedure have expanded to include infectious keratitis (Iseli et al., 2008, Makdoumi et al., 2010b, Müller et al., 2012, Panda et al., 2012, Said et al., 2014), although its efficacy for the management of corneal infections remains to be established. Recently, the terminology of “photo-activated chromophore corneal cross-linking (PACK-CXL)” has
been proposed to be utilised in case of treating the infectious keratitis with combined riboflavin/UV-A light instead of the CXL term (Said et al., 2014) in order to distinguish the particular use of the combined riboflavin/UV-A light for treatment of infectious keratitis from other indications.

1.4 Treating Infectious Keratitis with Corneal Cross-linking (PACK-CXL)

In recent years, the antimicrobial effect of combined riboflavin/UV-A light-induced corneal cross-linking in the management of corneal infections is of great interest in the scientific community. The previously established antimicrobial application of riboflavin and UV-A light in different scientific areas initiated the possibility of using the CXL procedure as a form of treatment for corneal infections. Tsugita et al. (1965) were the first researchers to propose that riboflavin together with applied UV-A light created an inactivation of RNA tobacco mosaic virus in tobacco plants. This method has been used since the 1960s to eliminate microorganisms in cases where asepsis is viewed as adequate; for instance for the water sterilization (Hallmich and Gehr, 2010) and sterilisation of blood products (Goodrich, 1999, Corbin, 2002, Goodrich, 2011). It is possible for riboflavin to become inserted in-between nucleic acid chains after passing through the lipid membrane of the cell(s). The photo-activation of riboflavin using UV-A light stimulates reactive oxygen species which leads to the damage of DNA of microorganism and generates lesions in the chromosomal strands by an oxidation process (Cadet, 1999, Kumar et al., 2004). In addition, it has been shown that the combined riboflavin/UV-A light is capable of stiffening the cornea, which may
simultaneously reduce pathogens’ capability to penetrate within the stromal tissue. This is likely to reduce the possibility of corneal melt, and thus, perforations, which may be initiated by collagen digesting enzymes released by pathogenic microorganisms. It has been stated that besides the direct elimination of pathogens, the decrease in the susceptibility of the stroma to proteolysis by pathogens is a specific part of the therapeutic effect of combined riboflavin/UV-A light (Spoerl et al., 2004). Likewise, the PACK-CXL procedure induces apoptosis in the cornea, and this can result in a reduction in the inflammatory response during an infection and arrest the melting processes (Wollensak et al., 2004, Wang, 2008).

Infectious keratitis is a clinically challenging condition to diagnose and manage and can rapidly lead to irreparable damage to the eye. The efficacy of corneal cross-linking in the management of infectious keratitis has been investigated, both in vitro and in vivo, as the PACK-CXL approach may offer the possibility of managing this devastating ocular challenge.

### 1.4.1 Laboratory Studies

The antibacterial effect of the combined riboflavin/UV-A light was reported in an earlier in vitro study by Goodrich (1999) where the combined riboflavin/UV-A light was used in a decontamination process for blood components and showed bacterial clearance. Martins et al. (2008) evaluated the antimicrobial properties of the combined riboflavin/UV-A light against two groups of common pathogenic bacteria and the yeast *Candida albicans*, in vitro. The first group included drug sensitive bacteria while the second one included drug-resistant bacteria and the yeast. The
findings showed a significant growth inhibition using treatment of combined riboflavin/UV-A light for both groups of pathogens. The findings obtained by Martins et al. (2008) demonstrated that with combined riboflavin/UV-A light, there was a localized response to the UV-A irradiation area with well-defined borders of bactericidal activity, which suggested that combined riboflavin/UV-A light could be effectively applied as a treatment option against corneal infections. Subsequently, Schrier et al. (2009) and Makdoumi et al. (2010a) assessed the antimicrobial effect of combined riboflavin/UV-A light against bacterial pathogens in vitro, and reported that the bacteria were eradicated effectively with combined riboflavin/UV-A light. Richoz et al. (2014) performed accelerated PACK-CXL protocols using 18 mW/cm² irradiance for 5 minutes or 36 mW/cm² irradiance for 2.5 minutes and showed that the accelerated PACK-CXL had a similar antibacterial effect observed using the standard PACK-CXL protocol. However, the impact of combined riboflavin/UV-A light against fungal pathogens was outlined by Sauer et al. (2010) in an in vitro study with and without prior therapy with amphotericin B. The antimicrobial features of the combined riboflavin/UV-A light were assessed on three kinds of fungi (Candida, Aspergillus and Fusarium) chosen from severe instances of fungal keratitis (details of Fusarium sp. are given in appendix A). Sauer et al. (2010) illustrated that the combined riboflavin/UV-A light alone has no effect against these test organisms, whereas there was a considerable inhibition of growth with the integrated PACK-CXL therapy and amphotericin B. The in vitro experiments carried out by Del Buey et al. (2012) and Kashiwabuchi et al. (2013) to evaluate the efficacy of PACK-CXL technique against Acanthamoeba species isolates and fungi, respectively, showed no beneficial effect of the combined riboflavin/UV-A light against these pathogens. Recently, Alshehri et al.
(2016) investigated the effect of the combined riboflavin/UV-A light by treating ex vivo human corneas with fungal infection, in particular Fusarium keratitis. In this study, the antifungal effect of this combination was demonstrated; it induced an efficient inhibition of Fusarium hyphal growth and stromal invasion. Similarly, an in vitro study showed a beneficial bactericidal effect of combined riboflavin/UV-A light against both antibiotic-resistant and non-resistant bacterial strains (Makdoumi and Bäckman, 2016).

1.4.2 Animal Studies

There are some published studies aimed at testing the impact of PACK-CXL technique on infectious keratitis in animal corneas. The influence of PACK-CXL procedure on fungal keratitis has been investigated by Galperin et al. (2012) using a rabbit model. In this study, 24 rabbits were divided into two groups where the first group was treated with PACK-CXL technique three days post-infection whereas the second group served as control and did not receive any treatment. It was found that the treated group demonstrated significantly lower infection progression compared with the un-treated group. Spiess et al. (2014) demonstrated the beneficial effect of PACK-CXL treatment in three dogs and three cats with resistant corneal melting and ulcers caused by unknown pathogens. After PACK-CXL treatment, it was noted that the corneal melting progression was blocked within one day to three weeks, and re-epithelisation was achieved within one to six weeks. These outcomes are consistent with the research study by Pot et al. (2014) which was carried out on 49 eyes of dogs and cats with melting keratitis. In this trial, 19 eyes were treated with the combined PACK-CXL procedure and antimicrobial treatment whereas the rest of eyes remained as control
(antimicrobial treatment only). After the treatment, it has been shown that corneal ulcers were shallower in the combined PACK-CXL and antimicrobial treated eyes. Even though the PACK-CXL treatment in this experiment failed to arrest the melting in five eyes that eventually required a further rescue procedure, Pot et al. concluded that PACK-CXL procedure had a beneficial effect in the management of melting keratitis. Additional study on the effect of PACK-CXL treatment on fungal, bacterial and ulcerative keratitis in 9 horses also showed a beneficial effect of PACK-CXL treatment (Hellander-Edman et al., 2013). In another animal study, Famose (2014) evaluated the influence of rapid PACK-CXL procedure in the management of melting keratitis in a dog model. Eight dogs were treated using 0.1% riboflavin that was administered for 30 minutes followed by UV-A irradiation (370 nm) at 30 mW/cm² which lasted for only three minutes. The findings showed improvement in the pain symptoms within three days, epithelial healing within two weeks, resolution of the infiltration within a week post-PACK-CXL treatment, as well as no report of recurrent corneal infection. Next, Famose (2015) performed accelerated PACK-CXL to treat melting keratitis in ten cats using 30 mW/cm² irradiance for 3 minutes, and confirmed that this approach is a valuable option for the treatment of melting keratitis. However, the impact of PACK-CXL treatment on Acanthamoeba keratitis was examined by Berra et al. (2013) on rabbit model. It was found that the infection stage and protozoal count were greater in the treated group compared to the control, and concluded that the PACK-CXL procedure was incapable of reducing the severity of Acanthamoeba keratitis. Similarly, another animal study conducted by Kashiwabuchi et al. (2011) on a hamster model failed to demonstrate a valuable impact of combined riboflavin/UV-A light in the management of Acanthamoeba keratitis.
1.4.3 Clinical Studies

There are a series of in vivo studies that have investigated or reported the therapeutic effect of corneal cross-linking on infectious keratitis caused by various organisms, including bacteria, fungi, acanthamoeba, or unknown causative pathogens. The first reported clinical study to assess the effect of combined riboflavin/UV-A light was conducted by Schnitzler et al. (2000) to control non-infectious corneal melting of various causes in four patients. Schnitzler et al. reported that the treatment helped to arrest the corneal melting in three patients, and delay the need for surgical procedures. The next clinical study to investigate the effect of PACK-CXL procedure and the first trial to treat corneal melting exclusively of infectious origin was conducted by Iseli et al. (2008) on five cases with bacterial or fungal keratitis, which were not-responsive to intensive medication. It had been reported that corneal melting was arrested in all cases, and that there was no need for any emergency corneal transplantation. Micelli et al. (2008) and Morén et al. (2010) presented case reports of bacterial keratitis and Acanthamoeba keratitis, respectively. The case of bacterial keratitis reported by Micelli et al. (2008) showed a remarkable improvement in the symptoms after only one day post-PACK-CXL treatment. One month post-PACK-CXL treatment, there was a complete resolution of the corneal oedema and healing of the corneal ulcer. Morén et al. (2010) described a case report of severe keratitis, presumed to be caused by Acanthamoeba with unproven pathogenesis. This case was not responsive to the aggressive medical treatments, so was treated with PACK-CXL technique. Following PACK-CXL treatment, a complete corneal re-epithelialisation
occurred within a month, complete wound healing after two months and noticeable visual acuity improvement, as well as rapid pain relief.

The antimicrobial effect of PACK-CXL was investigated by Hafez (2014) who presented a series of five eyes with fungal corneal ulcers which were resistant to the clinical medications therapy. These cases were treated by PACK-CXL procedure and showed a beneficial effect for PACK-CXL procedure in the management of resistant fungal infections by improving of infection signs and completion of ulcer healing, although vision improvement was not achieved. In a clinical study carried out by Said et al. (2014), 40 infectious melting keratitis cases caused by bacteria, fungi, Acanthamoeba or mixed microorganisms were included. These cases were divided into two groups where the first group involved 21 cases and treated with PACK-CXL procedure along with the clinical medications, whereas the second group involved 19 cases and were treated with clinical medications only. Even though PACK-CXL treatment did not reduce the corneal healing time nor improve the visual acuity, it offered valuable benefits with no recurrence of the infection or incidences of corneal perforation observed, compared with the other group who received antimicrobial therapy alone. These findings are consistent with the study by Makdoumi et al. (2010b) which revealed that PACK-CXL procedure is an influential treatment in overcoming the aggressive infectious keratitis caused by bacteria or unidentified microorganisms. Post-PACK-CXL treatment, the improvement in symptoms was shown in 6 out of 7 eyes within 1 day. Also, this treatment helped to arrest corneal melting and complete epithelisation was achieved. Likewise, the previous indications have been strongly supported by a study which demonstrated the efficacy of PACK-CXL application as an
alternative therapy on 16 cases of bacterial keratitis (Makdoumi et al., 2012). Interestingly, the antimicrobial medications had never been used prior to applying PACK-CXL procedure and, encouragingly, all cases showed improvement in the infection symptoms and healing of the corneal epithelium, apart from one case which required surgical intervention. Panda et al. (2012) demonstrated the efficacy of the PACK-CXL procedure by conducting a clinical trial on 7 eyes with infectious keratitis caused by acanthamoeba, fungi or mixed microorganisms, associated with corneal ulcers, melting and resistant to the medical treatments. After PACK-CXL treatment, it was reported that the ulcers were healed, the corneal melting progression arrested, and surgical interventions were avoided in all cases (Panda et al., 2012). With the same objective, two in vivo case reports were published in the literature, which describe the effect of combined riboflavin/UV-A light-induced corneal cross-linking in the management of infectious keratitis caused by acanthamoeba, after failure to respond to a broad-spectrum of medical treatments (Garduño-Vieyra et al., 2011, Arance-Gil et al., 2014). Both studies demonstrated that PACK-CXL application is an effective tool for treating the corneal infections. Garduño-Vieyra et al. (2011) implemented the PACK-CXL procedure for an Acanthamoeba keratitis case which was resistant to prolonged (8 weeks) treatment with antimicrobial agents. This case showed a positive response as early as 1 day post-PACK-CXL treatment. Three weeks post-PACK-CXL treatment, the cornea healed completely and vision improved remarkably well. Arance-Gil et al. (2014) applied the PACK-CXL procedure for an Acanthamoeba keratitis case after an extreme resistance to several medical treatments over a period of 12 months. It was observed that PACK-CXL treatment was more effective than intensive medication against the organisms; it offered a significant improvement for the corneal appearance
and resolution for corneal symptoms. Although PACK-CXL treatment did not offer complete ulcer healing in this case, it was effective in the elimination of the *Acanthamoeba* cysts. In a clinical study by Li et al. (2013), eight cases with refractory fungal keratitis were treated with PACK-CXL procedure. It was reported that following the PACK-CXL treatment resolution of infection was achieved, the epithelial layer healed, and emergency surgery was avoided in all cases. A clinical study was conducted by Bamdad et al. (2015) to evaluate the therapeutic effect of PACK-CXL on 32 cases of moderate bacterial keratitis. The cases were divided into 2 groups where one group was treated with antimicrobial therapy and PACK-CXL procedure, while the second group served as control and treated with antimicrobial therapy alone. The findings obtained in this study supported the beneficial antimicrobial effect of PACK-CXL; the treatment accelerated the epithelialization, shortened the healing duration, and removed the need for additional surgeries. In a further case report, accelerated PACK-CXL using 9 mW/cm² irradiance for 10 minutes showed promising observations as a first-line treatment in the management of early fungal keratitis (Tabibian et al., 2014). Similarly, there is a body of clinical evidence that supports the efficacy of PACK-CXL procedure in the management of infectious keratitis and shows that the PACK-CXL technique is a promising, valuable treatment option for corneal infections (Al-Sabai et al., 2010, Anwar et al., 2011, Müller et al., 2012, Demirci and Ozdamar, 2013, Rosetta et al., 2013, Skaat et al., 2013, Sorkhabi et al., 2013, Chan et al., 2014, Labiris et al., 2014, Zamani et al., 2015, Chan et al., 2017, Khalili et al., 2017).

It has been observed in few studies that when the PACK-CXL treatment did not offer the desirable outcomes in the management of the corneal infections, the procedure
was repeated (Khan et al., 2011, Saglik et al., 2013). A series of three cases of refractory *Acanthamoeba* keratitis were presented in a study by Khan et al. (2011) where they have been treated twice with the PACK-CXL procedure. After the first PACK-CXL treatment, there was an improvement in the symptoms and reduction in the ulcer size. This was followed by a decline in the improvement, and then the second PACK-CXL treatment was given. In this series, all cases showed resolution in the inflammation signs, healing of the epithelial defects and complete healing of the ulcer after the second PACK-CXL session. However, penetrating keratoplasty was needed in two cases due to the dense corneal scars that had been left. Saglik et al. (2013) reported a case of resistant infection and suggested fungal microorganisms as an underlying cause for this case, judging from the clinical features. After administering the intensive medications for ten days with no response, the first PACK-CXL treatment was administered. Post-initial PACK-CXL treatment, it was shown that stromal infiltrates were inactivated for a week but reactivated within the second week; therefore a second PACK-CXL session was administered. Post-second PACK-CXL treatment, active stromal infiltrates disappeared, epithelial defect was cured and corneal ulcer healed, although unfortunately with a scar. Despite the futility of first session of PACK-CXL treatment, it has been concluded that PACK-CXL technique can be considered in the management of resistant corneal infection and ulceration.

A previously published *in vivo* study indicated the limited efficacy of the combined riboflavin/UV-A light after treating 40 cases of infectious keratitis with PACK-CXL technique (Price et al., 2012). It has been reported that PACK-CXL procedure has an effect in the management of bacterial keratitis or in cases with limited infection depth,
while it showed an overall poor effect on the fungal keratitis (Price et al., 2012). This finding is consistent with a published clinical trial on 11 eyes with resistant bacterial or fungal keratitis. In this trial, the PACK-CXL treatment showed a good effect in treating of infectious keratitis cases caused by bacteria but not in the management of fungal keratitis as all cases with fungal ulcers needed surgical treatment (Escarião et al., 2013). Additionally, Shetty et al. (2014) evaluated the effect of PACK-CXL procedure on advanced non-resolving corneal infections and carried out the study on six patients with fungal keratitis and an additional nine with bacterial keratitis. It was shown that three fungal keratitis cases and six bacterial keratitis cases were managed successfully, and the pain symptoms being relieved in all cases. However, the well-managed cases were suffering from superficial stromal involvement only, whereas the cases where PACK-CXL treatment failed were suffering from deep forms of infection. In addition, confounding outcomes have been found in a clinical study (Vajpayee et al., 2015) which included 41 moderate-stage of fungal keratitis cases divided into control (treated with antifungal medications only) and cross-linked (treated with PACK-CXL procedure and antifungal medications) groups. Vajpayee et al. (2015) found that there is no difference between the two study groups in regards to the average of final corrected vision and recovery time, and concluded that the PACK-CXL treatment has no advantage over the antifungal agent alone. Recently, the efficacy of PACK-CXL treatment was evaluated in the management of refractory-deep fungal keratitis in a clinical study that included 13 cases divided into control and cross-linked groups which treated with antifungal therapy only and PACK-CXL along with antifungal medications, respectively (Uddaraju et al., 2015). It was shown that the cross-linked group experienced a higher perforation rate than the control group, and concluded that the
PACK-CXL treatment is not effective in the management of deep fungal keratitis (Uddaraju et al., 2015). Furthermore, cases of herpetic keratitis or a history of herpetic eye disease are contraindications for PACK-CXL treatment (Price et al., 2012, Ferrari et al., 2013), as the UV-A light could be a stimulus to reactivate the latent virus (Kymionis et al., 2007). Recently, it has been reported that PACK-CXL was effective in some cases of refractory herpes simplex virus keratitis (Khalili et al., 2017).

### 1.5 Rationale for Applying the PACK-CXL Procedure in the Management of Infectious Keratitis

A series of *in vitro* and *in vivo* experiments investigated the influence of combined riboflavin/UV-A light on various pathogens, and demonstrated the antimicrobial effect of this combination. It is worth noting that the observations obtained in the laboratory experiments have not necessarily been reflected *in vivo*, and that the cornea in the majority of animals is distinctly different from the humans (Zeng et al., 2001, Elsheikh et al., 2008, Hovakimyan et al., 2011). Hence, the results of *in vitro* and animal studies may not be good indicators of effects of PACK-CXL procedure in human corneas. Clinical studies published relating to the use of PACK-CXL treatment for corneal infections showed mixed findings, and the research observations seem to be equivocal. In a review article by Chan et al. (2015), it has been reported that the role of PACK-CXL in the management of infectious keratitis remained unclear, and that the PACK-CXL procedure should be only considered as an adjuvant therapy in severe, unresponsive infectious keratitis before undertaking emergency keratoplasty. An additional systematic review of 21 clinical studies on the effect of PACK-CXL treatment on
infectious keratitis by Abbouda et al. (2016) came to similar conclusions. Nevertheless, a body of the clinical studies support the view that the combined riboflavin/UV-A light-induced corneal cross-linking has a broad antimicrobial effect and seems to be effective in reducing the severity of corneal infections, and tends to be a beneficial adjuvant treatment in the management of infectious keratitis. In systematic review with meta-analysis works conducted by Alio et al. (2013) and Papaioannou et al. (2016) to evaluate the effectiveness of the PACK-CXL treatment in the management of infectious keratitis, the previously published data from 12 and 25 clinical studies, respectively, have been analysed. It has been reported that PACK-CXL procedure has a beneficial effect on the arrest of corneal melting, and confirmed that the available evidence supports the use of PACK-CXL in the treatment of infectious keratitis, excluding viral infections. Abbouda et al. (2016) reported that the PACK-CXL treatment appears to be more effective at blocking corneal melting in the bacterial keratitis, followed by Acanthamoeba keratitis, and then fungal keratitis, according to the previous published clinical studies. However, the evidence to support the level of effectiveness of PACK-CXL procedure in cases of infectious keratitis in clinical practice is limited, as it has also shown no beneficial effect of the PACK-CXL treatment over the antimicrobial agent alone, or no beneficial effect against pathogens such as fungi. The limited efficacy of the PACK-CXL technique has also been reported where its success rate was lower for the deep infections than superficial infectious keratitis and it was effective with corneal infections caused by bacteria while it was not as effective in cases caused by fungi. More recently, the previous published studies were analysed in a review article to evaluate the level of evidence for using PACK-CXL in the management of infectious keratitis (Garg et al., 2017). It has been reported that there
was a wide variation between the published studies making it difficult to determine the level of effectiveness of PACK-CXL procedure in treating infectious keratitis. Nonetheless, Garg et al. (2017) concluded that PACK-CXL procedure seems a promising treatment in the management of corneal infections associated with superficial stromal involvement, and those caused by bacteria, whereas it would be better to avoid the PACK-CXL treatment in cases of fungal or *Acanthamoeba* keratitis. In reality, fungal organisms are more invasive of corneal tissues where around 50% of fungal keratitis cases progress to deep fungal infection (Vemuganti et al., 2002), while it has been documented that the effect of CXL treatment is limited only to the first 200-300 µm from the corneal surface (Wollensak et al., 2004, Kohlhaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016). Thereby, the infectious agents localized beyond the corneal depth of 300 µm may be protected from the antimicrobial effect of the PACK-CXL treatment. This statement may explain the reduced beneficial effect of PACK-CXL therapy on the deep penetrating fungi in some studies. Zhang (2013) suggested that the PACK-CXL procedure should be combined with existing clinical therapies, and in particular in cases of fungal keratitis, as the riboflavin penetration can be blocked by the impervious fungal membranes. It has been proposed that the membrane sterols of the fungi will interact with the polyene antifungals, which results in creating trans-membrane channels (Baginski et al., 2006) that allow the riboflavin to enter the microorganism’s cell, and then its activity is destroyed. Another possibility for managing severe infectious keratitis cases and eradicating refractory pathogens is a repetition of the PACK-CXL treatment (Khan et al., 2011, Saglik et al., 2013). The insufficient management of an infection by the initial session of the PACK-CXL treatment and the need to a further session may be
attributed to the residual spores and cysts post-first PACK-CXL session which could re-establish the infection.

In most of the clinical studies, the PACK-CXL procedure was performed mainly on medical treatment-resistant forms of infectious keratitis cases. In these cases, the antimicrobial medications were usually administered for a prolonged period in an attempt to manage the infections. The PACK-CXL procedure was performed only once the infections failed to respond to the clinical medications, and possibly progressed to advanced stages. Hence, the delay in applying the PACK-CXL procedure could have affected its efficacy in the management of these corneal infections. Makdoumi et al. (2012) and Tabibian et al. (2014) investigated the combined riboflavin/UV-A light as an alternative therapy in the management of infectious keratitis. In both studies, promising results were achieved by applying the PACK-CXL treatment as the primary, early treatment of infectious keratitis cases where previous antimicrobial therapy had not been implemented. The results obtained seem to support the idea that early intervention with PACK-CXL procedure improves the outcome of infectious keratitis, and that the stage of infection should be considered as a critical factor for success of the PACK-CXL treatment.

The antimicrobial property of the action of the combined riboflavin/UV-A light makes it a potentially useful option for treatment of corneal infections. However, although combined riboflavin/UV-A light-induced antimicrobial action is broad spectrum, its efficacy may vary dependent upon the pathogen type. On the other hand, CXL protocol utilised for treating keratoconus has been directly extrapolated by majority of researchers, where the total UV-A irradiation dose is under the limit known to damage
the endothelium. However, some factors are still not clearly defined, including the most beneficial concentration of riboflavin and the optimum period of UV-A exposure required to kill the pathogens. Moreover, the antimicrobial efficacy of combined riboflavin/UV-A light on different types of pathogen and the effective depth of UV-A light in infectious cornea with different severities of ulcer also remained to be elucidated in future studies, as suggested by Jiang et al. (2016). Hence, developing a new protocol is required to target the infectious keratitis cases, and probably this protocol needs to vary based upon the severity of the individual case. Tabibian et al. (2016) reported that protocol modifications of PACK-CXL protocol are now needed to tailor the treatment to a specific clinical situation or certain types of pathogens. Alterations to the standard protocol have already been introduced by some researchers. For example, in cases of infectious keratitis wherein there was already a large epithelial defect, the epithelial layer was not removed (Anwar et al., 2011, Shetty et al., 2014). Another approach has also been introduced where the epithelial debridement was not performed, as it may cause permanent epithelial defects and increase the risk of delayed epithelial healing. Instead, the riboflavin penetration was through the pre-existing epithelial defects and the hypo-osmolar 0.1% riboflavin solution was used to induce corneal swelling so as to avoid the risk of treating thin corneas and damaging the underlying structures (Rosetta et al., 2013). In another study, a 0.127g of trans-epithelial riboflavin phosphate, which is equivalent to 0.1% of basic riboflavin with enhancers (sodium edetate and tromethamine), was used instead of debriding the epithelial layer (Hafez, 2014). Optimization of the protocol’s parameters by reducing the treatment duration and increasing the UV-A irradiance have also been introduced where the accelerated PACK-CXL procedure appeared to be
a promising alternative treatment in the management of fungal keratitis (Tabibian et al., 2014). Further development of the PACK-CXL treatment can be also achieved by introducing additional modifications in the treatment protocol, such as changing the type of chromophore. In an *in vitro* study, the effect of Rose Bengal as photosensitizing agents was compared with riboflavin on fungal isolates and showed a growth inhibition of the fungal isolates in the irradiated area (Arboleda et al., 2014). Previous published studies have confirmed the safety of CXL treatment in the management of keratoconus (Wollensak et al., 2003a, Spoerl et al., 2007, Goldich et al., 2012). However, the safety profile of the PACK-CXL treatment needs to be established. It has not yet been demonstrated whether corneal defects resulting from infectious keratitis cases allow the same depth of UV-A penetration, and consequently, whether the PACK-CXL treatment, using the standard protocol, is a safe procedure in the management of infectious keratitis. Accordingly, further precaution is advised when applying any modification in the PACK-CXL protocol as it could result in undesired consequences, such as endothelial damage.

### 1.6 Rationale and Aims

In light of the current evidence in the literature, combined riboflavin/UV-A light-induced corneal cross-linking holds promise and future potential as an alternative or adjuvant to standard antibiotic therapy against infectious keratitis. However, there is a gap in the literature regarding the impact of PACK-CXL treatment in controlling infectious keratitis in human due to a lack of clinical studies which have similar criteria, such as same causative pathogen, PACK-CXL treatment regimes, severity of infection,
investigation method, duration of administrating the medical therapy, the definition of infection resolution, and outcome assessments, and by which the results can be compared and interpreted. Hence, laboratory-based investigations on human corneal models would be highly beneficial in order to attain more reliable evidence about the effect of PACK-CXL procedure in the management of infectious keratitis and establish the use of PACK-CXL procedure as an effective therapy for treating corneal infections in patients. If the beneficial therapeutic effects of PACK-CXL are confirmed, the PACK-CXL procedures may offer the possibility of managing a universally threatening ocular challenge and potentially secure its place among the most versatile therapies in ophthalmology.

The main purpose of this project was to establish an ex vivo human corneal infection model and to evaluate the efficacy of PACK-CXL treatment in the management of human corneal infections. The aims of this project are detailed below:

- To develop and establish a novel ex vivo human corneal fungal infection model which mimic in vivo human fungal keratitis using donor post-mortem human corneas.
- To use the model established for evaluating the antimicrobial effect of the combined riboflavin/UV-A light-induced corneal cross-linking in the management of corneal infections caused by a human fungal pathogen Fusarium oxysporum: details of this organism are given in appendix A.
- To investigate the efficacy of the PACK-CXL procedure as a primary therapeutic technique for controlling the early stage Fusarium infection using the standard PACK-CXL protocol.
• To evaluate the efficacy of the standard PACK-CXL procedure as a primary treatment for the management of late stage *Fusarium* infection.

• To compare the effectiveness of standard PACK-CXL to a modified PACK-CXL protocol (trans-epithelial PACK-CXL), and assess the effect of epithelium-on (intact) and epithelium-off (debrided) variations of PACK-CXL procedure in the management of *Fusarium* infection.

• To investigate the efficacy of standard PACK-CXL treatment with early, late or repeated interventions, and evaluate the effect of timing and repeating of PACK-CXL procedure in controlling the progression of *Fusarium* infections.

• To explore the effect of stiffened corneal biomechanical properties produced by pre-cross-linking the *ex-vivo* human corneas in resisting the spread of *Fusarium* infection.

• To evaluate the efficacy of the standard PACK-CXL procedure as an adjuvant to antifungal therapy (natamycin) compared with PACK-CXL treatment as a monotherapy and to evaluate the effects of intervention timing in the management of *Fusarium* infection.
Chapter 2


Contributions

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross. Confocal imaging and processing the images using Imaris software were done by Dr David Caballero-Lima. Prof. Fiona Carley and Mr. Arun Brahma regularly treat patients with infectious keratitis in the Manchester Royal Eye Hospital. Prof. Carly and Mr. Brahma met with the research team on a regular basis to discuss ideas and progress of the research studies.
Publication


Conferences Presentations


Vivo Human Corneal Model’. The Manchester Optometry Meeting. The University of Manchester, Manchester, UK.

Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.
- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the Fusarium strain.
2.1 Abstract

Purpose: Some previous reports have established the use of photoactivated chromophore-induced corneal cross-linking (PACK-CXL) in treating fungal keratitis. The results of these case reports have often been conflicting. To systematically study the effect of PACK-CXL in the management of Fusarium keratitis, we have developed an ex vivo model of human corneal infection using eye-banked human corneas.

Method: Sixteen healthy ex vivo human corneas were divided into four study groups: 1) untreated control, 2) cross-linked, 3) infected with fungal spores, and 4) infected with fungal spores then cross-linked. All infected corneas were inoculated with Fusarium oxysporum spores. The PACK-CXL procedure was performed 24h post-inoculation for group 4. For PACK-CXL treatment, the corneas were debrided of epithelium then 0.1% (w/v) isotonic riboflavin was applied dropwise at 5-minute intervals for 30 minutes and during the course of UV-A cross-linking for another 30 minutes. The corneas were imaged using a confocal microscope at 48h post-inoculation, and the Fusarium hyphal volume and spore concentration were calculated.

Results: The infected then cross-linked group had a significantly lower volume of Fusarium hyphae, compared to the infected (P = 0.001) group. In the infected then cross-linked group there was significant inhibition of Fusarium sporulation compared with the infected (P = 0.007) group.

Conclusion: A model of human corneal infection was successfully developed for the investigation the effects of PACK-CXL on fungal keratitis. A treatment regime of
combined UV-A/riboflavin light-induced corneal cross-linking appears to be a valuable approach to inhibit the growth and sporulation of \textit{Fusarium} and supress the progression of fungal keratitis.
2.2 Introduction

Fungal keratitis is a destructive corneal infection with a high level of ocular morbidity (Srinivasan, 2004, Brasnu et al., 2007), which is particularly common within the developing world and subtropical areas, in patients with compromised corneal integrity. In the developing world, the annual number of recorded corneal ulcers is swiftly nearing between 1.5 and 2 million, and the actual figure is likely to be more. For most of these infections the final outcome is usually corneal opacity, or possibly even more devastating results such as corneal perforation, endophthalmitis or phtisis (Whitcher et al., 2001). Fungal keratitis is viewed as a key blinding eye disease, in particular in agriculture-based geographical areas that have hot, humid, subtropical and tropical climates. Fungi have been reported to cause 44% of all central corneal ulcers in South India, 36% in Bangladesh and 17% in Nepal, in addition to 35% in South Florida and 37.6% in Ghana (Liesegang and Forster, 1980, Upadhyay et al., 1991, Sharma et al., 1993, Dunlop et al., 1994, Hagan et al., 1995, Leck et al., 2002).

Fungal keratitis has been documented to be caused by over 70 species covering 40 fungal genera (Thomas, 1994). The key etiologic agents of fungal keratitis are the filamentous fungi with *Fusarium sp.* comprising the most frequently occurring corneal keratitis-associated filamentous fungal genus (Nelson et al., 1994, Dignani and Anaissie, 2004, Dóczi et al., 2004, Hua et al., 2010, Kredics et al., 2015). *Fusarium sp.* are the key pathogens in 37 to 62% of instances of fungal keratitis (Panda et al., 1997, Garg et al., 2000, Gopinathan et al., 2002, Bharathi et al., 2003).
Fungal keratitis appears as ulcerative lesions (Thomas, 2003a) and is usually managed using topical antifungal medications, occasionally integrated with sub-conjunctival injections, although therapeutic keratoplasty may be necessary for patients whose corneal infection persists (Dóczi et al., 2004). This type of corneal infection presents a problem for the ophthalmologist as its control is limited by the availability of effective antimycotics and the level to which they may infiltrate the corneal tissue. In addition, fungal infection is inclined to mimic other kinds of infectious keratitis (Thomas, 2003a) where it is often misdiagnosed or only diagnosed at a very late stage, following the failure of extensive treatments for viral or bacterial keratitis. Unfortunately, this delay in diagnosis and treatment can cause an irreparable loss of sight (Brasnu et al., 2007). Additionally, some microorganisms have displayed resistance to antimicrobial medications (Dóczi et al., 2004), and therefore, the infections could progress through corneal ulceration leading to corneal melting or perforation, even after provision of the necessary treatment. Consequently, current research is aimed at finding an innovative treatment, which actively controls and manages infectious keratitis, particularly in infections where the microorganism is difficult to identify or those caused by drug-resistant microorganisms.

Ultraviolet (UV) light has been identified as an efficient method for disinfection, particularly of water, and as an alternative to chemical treatment; this has resulted a large demand for UV treatment globally. Regardless of variations in modes of inactivation, disinfection by UV is effective against a range of pathogens encompassing bacteria, protozoa and viruses (Hijnen et al., 2006, Hallmich and Gehr, 2010). Riboflavin comprises a natural substance and is part of the vitamin B group, which is
readily available in various foods. Photoactivated riboflavin has been utilized to minimize the microbial burden of liquids over many years. A good example is within the sphere of transfusion medicine, where concentrates of platelets are regularly treated with the combined riboflavin/UV-A light (Goodrich, 1999). The mixture of riboflavin and UV-A light results in irreparable damage to the DNA and RNA of pathogenic organisms, preventing genome replication and thus preventing infection (Kumar et al., 2004).

UV-induced corneal cross-linking (CXL) is a non-invasive procedure established for the management of keratoconus and corneal ectasia (Wollensak et al., 2003a, Raiskup-Wolf et al., 2008). This technique relies upon using the photosensitizer riboflavin and UV-A to create photochemical cross-links within the anterior stromal collagen fibrils, and thus raise the biochemical and mechanical stiffness of the cornea (Spoerl et al., 1998, Wollensak and Iomdina, 2009b, Goldich et al., 2012), which in keratoconus halts the progression of the corneal thinning.

Photoactivated riboflavin and UV-A light have been trialled as potential adjuvant treatment for the management of corneal infections, particularly for the antibiotic resistant forms of infectious keratitis. It may also be beneficial where corneal melting occurs. Corneal stiffening resulting from this treatment could reduce melting and reduce or prevent corneal perforation (Iseli et al., 2008, Makdoumi et al., 2010b, Said et al., 2014). The term of “photoactivated chromophore (PACK-CXL)” was utilised instead of simply CXL in order to distinguish between using the CXL for treatment of infectious keratitis and for keratoconus (Said et al., 2014).
The aim of this study was to establish an \textit{ex vivo} human corneal infection model by means of utilising donor \textit{post-mortem} human corneas, infecting them with a fluorescently labelled \textit{Fusarium oxysporum} then investigating the effect of PACK-CXL treatment in the management of fungal keratitis.

2.3 Materials and Methods

2.3.1 \textit{Ex Vivo} Human Cornea

Sixteen healthy \textit{post-mortem} human corneal buttons from 9 male and 7 female donors were used in this study. The corneas provided by the Manchester Eye Bank (NHS Blood and Transplant, UK) were pre-consented for research if they were not suitable for transplantation. They were from donors in the age range 58 – 83 years old (mean ± SD, 67.5 ± 7.6 years). At the Manchester Eye Bank, the corneas, along with a 3 mm scleral rim, were extracted from eyes less than 24 hours after death and placed into Eagle's Minimal Essential Medium containing 2\% (v/v) foetal bovine serum (FBS), 100 units/ml Penicillin, 0.1mg/ml Streptomycin and 0.25 \mu g/ml Amphotericin B (All Sigma-Aldrich, UK) and maintained at 34°C. The corneas used in this research had been released for research because the endothelial cell count was below that required for transplantation or for other reasons which did not directly affect the eye, such as inadequate medical history. The project had full ethical approval from the Manchester University Research Ethics Committees (No. 040811) and followed the terms of the Declaration of Helsinki.

The corneas were randomly assigned to one of the four groups with four corneas in each group as detailed below:
• C: Control (not infected, not treated with CXL procedure), (1 M and 3 F, 59-83 years old, mean ± SD, 70.3 ± 10 years)

• X: Cross-linked (not infected, treated with CXL), (3 M and 1 F, 59-71 years old, mean ± SD, 63.8 ± 5.3 years)

• I: Infected (infected, not treated with PACK-CXL), (2 M and 2 F, 59-76 years old, mean ± SD, 70.8 ± 7.9 years)

• IX: Infected then cross-linked (infected then treated with PACK-CXL), (3 M and 1 F, 58-71 years old, mean ± SD, 65.3 ± 6.8 years).

2.3.2 Corneal Tissue Culture

The human corneal buttons were transferred into 6-well cell culture plates (Costar®; Corning Ltd, Ewloe, UK) with the outer surface of the cornea uppermost, and submerged in fresh culture medium (Dulbecco’s Modified Eagle’s Medium, DMEM, 1000mg Glucose/L; Sigma-Aldrich Ltd, Poole, UK), supplemented with 10% (v/v) foetal bovine serum (FBS; Labtech International Ltd, Uckfield, UK), 2mM Glutamine, 1% (v/v) Penicillin with Streptomycin (all GE Healthcare Life Sciences, Little Chalfont, UK), and 0.1% (v/v) Hydrocortisone (H4126, Sigma-Aldrich, UK). The corneas were incubated at 34°C in 5% CO₂ for a minimum of 72 hours prior to the infection and CXL processes (Figure 2-1). The manipulation of corneal tissue, subsequent infection and CXL treatment processes were performed in a recirculating Class II Microbiological Safety Cabinet (Labcaire SC-R; Labcaire Systems Ltd, Clevedon, North Somerset, UK).
2.3.3 Fungal Strains

The infecting fungus used was *Fusarium oxysporum* f. sp. *lycopersici* strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP). The strain was kindly provided by Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) (Di Pietro et al., 2001). The *F. oxysporum* GFP strain was cultured in Potato Dextrose Broth (PDB) (BD Difco, Oxford, UK) for five days at 28°C. On the day of corneal inoculation, the spores (microconidia) were harvested. The fungal spore suspension was filtered using a filter cloth (Miracloth; Millipore Limited, Watford, Hertfordshire, UK), and a spore pellet was collected by centrifugation at 3260 xg for five minutes followed by washing with sterile distilled water and further centrifugation. The spore pellet was then resuspended in 1 ml of sterile distilled water, diluted 1/1000 with sterile distilled water and the concentration of this spore suspension was determined using a Fuchs-Rosenthal haemocytometer (Scientific Laboratory Supplies, Nottingham, UK). The spore concentration was adjusted to 5x10^5 spores per ml using fresh culture medium and 10 µl of this suspension was used to inoculate each of the scratched corneas as detailed below.
Figure 2-1: The main steps of the methodology.

The flow chart shows the main procedures, Culturing, Scratching, Infection, Cross-linking and Imaging processes, which were carried out for the different experimental groups, along with the time scale ( Procedure, Time scale).
2.3.4 Corneal Infection Process

Seventy-two hours post-corneal culturing, two of the human corneas, the I and IX, were inoculated with the spores of *F. oxysporum* expressing cytosolic GFP: the GFP fluorescence facilitated the tracking of the fungal hyphal invasion through the corneal layers.

As an *ex vivo* model of corneal insult and fungal infection, corneal buttons were mounted upon artificial anterior chambers (Moria SA, 92160 Antony, France; SD Healthcare, Manchester, UK), and held in place by the retainer and locking ring. Then, the anterior surface was scratched in an asterisk shape using a 0.6X25 mm-gauge needle (BD Microlance 3; BD Biosciences, Oxford, UK). They were transferred into a fresh, dry 6-well tissue culture dish and two of the groups previously assigned for infection (I and IX) were inoculated with $5 \times 10^5$ *Fusarium oxysporum* spores in 10 μL of culture medium: the viability of the spores was checked by inoculating a further 10 μl of spore suspension into growth medium. Inoculated corneas were left for few minutes to aid spore attachment to the corneal tissue. Next, a small volume of culture medium was added around the periphery of each cornea to prevent washing off the fungal spores. The corneas were incubated for 24h at 34°C prior to the initial assessment using confocal laser scanning microscopy and the treatment with the CXL procedure.

2.3.5 CXL Procedure

The corneas in the X and IX groups were treated with the corneal cross-linking technique according to a standard CXL protocol (Wollensak et al., 2003a). The X and IX
corneas were subjected to the cross-linking procedure 24h post-inoculation with spores.

The corneal button was mounted on an artificial anterior chamber as described above. Phosphate buffered saline (PBS) was infused through a tube, valve and syringe to fill the interior of the cornea to mimic the in vivo intraocular pressure and maintain corneal stability and shape throughout the CXL procedure (Figure 2-2).

Debridement of the corneal epithelial layer was performed to allow adequate riboflavin penetration to the corneal stroma. The central 7-9 mm diameter area of the corneal epithelium was debrided with a single-use surgical blade (size 10) (Swann-Morton, Sheffield, England) then wiped using a cotton eye spear (bviMerocel; Beaver Visitec, International Ltd., Abingdon, Oxfordshire, UK). Subsequently, isotonic riboflavin eye drops (Riboflavin 5′-phosphate sodium salt hydrate, R7774, Sigma-Aldrich, UK), (0.1% (w/w) riboflavin, 20% (w/w) dextran T500) were dropped onto the anterior surface of the cornea at 5 minute intervals for 30 minutes. The prepared cornea was irradiated with ultraviolet-A light utilizing a medical electrical UV-A light emitter (370 nm, irradiance of 3mW/cm²) (VEGA, C.S.O. srl, Florence, Italy), which delivered a total UV-A irradiation dose of 5.4 J/cm² to the corneal surface over a period of 30 minutes, along with application of further isotonic riboflavin, again at five minute intervals. Following this procedure, the cornea was rinsed several times with sterile PBS, and placed in a fresh 6-well tissue culture plate along with fresh culture medium and incubated at 34°C in 5% CO₂.
Figure 2-2: A human corneal button fixed onto an artificial anterior chamber.

The figure shows a human corneal button fixed onto an artificial anterior chamber and filled with PBS via a tube, valve, and syringe (A), to mimic intraocular pressure, which maintains the shape and stiffness of the corneal surface (B).

2.3.6 Imaging

Twenty-four hours post-inoculation, the corneas were inverted and placed in a 2-well chamber slide (Thermo Fisher scientific, Rochester, NY, USA) and the anterior stromal surface to a depth of 100 µm was imaged using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems Ltd., Breckland, Linford Wood, Milton Keynes, UK) with a x20 objective. At 48h post-inoculation, the corneas were imaged using a Canon camera (Canon Powershot S5iS, Zoom lens x12, 8.0 megapixel; Canon Ltd., Reigate, Surrey, UK) and the confocal microscope. The confocal microscopy images were analysed with the Surpass module of the Imaris v8.0 software (Bitplane Scientific software module; Bitplane AG, Zurich, Switzerland), and fungal hyphal volume data was automatically calculated by the software and displayed in an excel sheet. The percentage of the total volume occupied by the fungal hyphae was calculated in five different z-stack images, each of 100 µm thickness, for each study group from each
experimental set: the hyphae were visualised from the GFP signal within corneal tissues. After imaging, the number of fungal spores per ml of corneal culture medium at 48h post-inoculation was counted for all infected corneas.

2.3.7 Statistical Analysis

The analysis of *Fusarium* hyphal volume and spores concentration for the inoculated groups was performed using a one-way analysis of variance (ANOVA) to assess the difference in means produced as a result of treatment.

2.4 Results

*Fusarium* keratitis-like infection developed successfully in all inoculated corneas. The effect of PACK-CXL treatment on *Fusarium* was determined by measuring fungal hyphal volume within the corneal tissues.

Twenty-four hours after infection, all inoculated corneas developed *Fusarium* infection, whereas the C and X corneas remained intact and uninfected. Forty eight hours after the initial inoculation, the fungal infection had progressed in I group, while IX cornea showed less severe infection. Figure 2-3 shows the reduced progress of *Fusarium* infection at 48h post-inoculation in IX group (B) compared to I (A) group. The *Fusarium* infection can be seen as branching, green fluorescent fungal hyphae.

The percentage of the total volume occupied by the fungal hyphae, and the quantity of fungal spores in the culture medium from each infected group are shown in Figures 2-4 and 2-5, respectively. There is variation in the volume occupied by *Fusarium* hyphae between the infected groups at 48h after inoculation. The I group showed greater
amounts of *Fusarium* hyphae and, consequently, more severe fungal infections compared to the IX group. Analysis of variance (ANOVA) of the *Fusarium* colony volume data showed a significant difference between I and IX groups ($P = 0.001$). These results show that the PACK-CXL treatment controlled the fungal infection in the IX group, where the growth of fungus was inhibited by the PACK-CXL procedure.

There was also variation in the quantity of *Fusarium* spores that were present in the culture medium at 48h post-inoculation between the infected groups. The IX group shows a considerable decrease in the number of *Fusarium* spores present compared to the I group. ANOVA of the *Fusarium* spore quantity data shows that there is a significant difference in fungal spore density between I and IX groups ($P = 0.007$). These observations show that the combined riboflavin/UV-A light-induced cross-linking treatment had an inhibitory effect on the *Fusarium* sporulation.

**Figure 2-3**: Confocal microscopy images of infected corneal buttons 48h post-inoculation following PACK-CXL treatment.

Each image is a projection of a z-stack of confocal images (x20 magnification) which is 100 µm thick of the anterior stroma. The images of the different infected corneas
show the extent of the branching hypha growth of the fungus, which is expressing GFP in its cytoplasm at 48h post-inoculation. Extensive fungal growth is visible in I (A) group, while there are fewer hyphae evidence in IX group (B).

![Graph showing percentage of total Fusarium volume within corneal tissue](image)

**Figure 2-4: The percentage of the total volume occupied by the fungal hyphae.**

The graph shows percentage of the total volume occupied by the fungal hyphae, as visualised by the GFP signal from the fungal hyphae within the anterior 100 µm of stromal tissues for the different infected groups at 48h post-inoculation. The I group exhibited greater *Fusarium* hyphal volume compared to that of the IX group.
Figure 2-5: The quantity of *Fusarium* spores in the infected groups.

The graph shows the number of *Fusarium* spores per ml of corneal culture medium at 48h post-inoculation for the infected groups. The quantity of *Fusarium* spores of IX group was significantly less than that of the I group (*P* = 0.007).

2.5 Discussion

This study aimed to establish a model of human corneal fungal infection using human donor corneas infected with *F. oxysporum* expressing cytosolic green fluorescent protein, which permits the tracking of hyphal growth within the corneal tissue. With the model established, the combined riboflavin/UV-A light-induced corneal cross-linking as a primary therapeutic technique for the management of fungal keratitis
could be evaluated. To our knowledge, this study is the first ex vivo attempt to model the treatment of human fungal keratitis using this PACK-CXL technique.

*Fusarium oxysporum* was selected for this study due to its aggressive destruction of the cornea, and its regularity and frequency of appearance as a clinical pathogen. Fungal keratitis caused by *Fusarium spp.* is recognized as one of the most sight-threatening corneal infections (Thomas, 1994, Eliopoulos et al., 2002, Gopinathan et al., 2002, Bharathi et al., 2003, Marangon et al., 2004, Kawakami et al., 2015). The control of fungal keratitis presents a challenging problem mostly as a result of belated diagnosis and limited options for therapy (Vajpayee et al., 2015). As current antifungal medications display little corneal infiltration and restricted effectiveness, the treatment of ocular diseases caused by fungi is presently unsatisfactory (Srinivasan, 2004, Galperin et al., 2012). In addition, most antimycotics utilized in the management of *Fusarium* keratitis require a long course of treatment and frequently fail to preserve vision (Alexandrakis et al., 1998).

Introduction of a therapy based upon the photosensitizer riboflavin and UV-A light was first described by Wollensak et al. for the induction of corneal cross-linking for corneal ectasia (Wollensak et al., 2003a, Wollensak et al., 2003c). Recently, indications for the use of the CXL procedure have expanded to include Fuch’s corneal dystrophy (Hafezi et al., 2010), pseudophakic bullous keratopathy (Ghanem et al., 2010) as well as infectious keratitis (Iseli et al., 2008, Makdoumi et al., 2010b, Anwar et al., 2011, Makdoumi et al., 2012, Price et al., 2012). The use of the CXL technique for treatment of corneal ectasia and advanced keratoconus is well-established, while the efficacy of
the PACK-CXL procedure in the management of infectious keratitis is still subject to appraisal.

In this study, a human *ex vivo* corneal *Fusarium* keratitis model was established. The results show that there was a significant difference in the volume of invading fungus within the infected corneal tissue between the groups, with the infected corneas treated with PACK-CXL showing a significant decrease in the fungal volume compared to the other experimental group. This is a good indication that the PACK-CXL procedure is effective in suppressing the progression of fungal infection. The favourable outcomes obtained in this study are contradicted by some published studies, which report the PACK-CXL procedure is not effective in managing fungal keratitis (Escarião et al., 2013, Uddaraju et al., 2015, Vajpayee et al., 2015). Nevertheless, these observations are supported by numerous *in vivo* studies that attempted to examine the influence of combined riboflavin/UV-A light in the management of infectious fungal keratitis. It is suggested that this combination has positive antimicrobial effects, which assist in inhibiting the growth of pathogens and managing the infection (Iseli et al., 2008, Panda et al., 2012, Li et al., 2013, Hafez, 2014). The beneficial antimicrobial effect of the PACK-CXL procedure in suppressing infection by *Fusarium* is likely to be attributable to several mechanisms. Inactivation of ribonucleic acids of organisms may occur by the combined riboflavin/UV-A light-induced cross-linking that may have a cytotoxic effect on the pathogens (Tsugita et al., 1965, Goodrich, 1999). In addition, it has been shown that the PACK-CXL technique brings about cross-linking in the corneal collagen fibrils thus increasing the stiffness and simultaneously reducing its penetrability by fungal hyphae. Furthermore, the cross-linked collagen is more
resistant to enzymatic digestion by microbial pathogens, which in turn reduces corneal melting (Wollensak et al., 2003b, Spoerl et al., 2004). In this study, the direct antifungal activity of combined riboflavin/UV-A light is perhaps the greater contributory factor. It is also possible that cross-linking might entrap the fungal hyphae within the collagen matrix thereby reducing its growth rate still further. The proposal that the direct antifungal effect of the combined riboflavin/UV-A light is the major component in reducing corneal infection is supported by the fact that the IX group showed a significant reduction in the number of Fusarium spores present after the PACK-CXL treatment compared with the I group. This is indicative of the combined riboflavin/UV-A light having an inhibitory effect on Fusarium sporulation. On the other hand, debriding the epithelial layer post-inoculation and prior to the PACK-CXL treatment may have played a role in reducing the infection by removing some of the Fusarium hyphae growing within the corneal epithelial tissue. Nevertheless, the reduction in spore numbers in the culture medium post-PACK-CXL treatment lends some support to the hypothesis that combined riboflavin/UV-A light has an antifungal action.

Iseli et al. (2008) set out the first published clinical trial to investigate the effect of PACK-CXL application on a series of five cases, with infectious melting keratitis caused by pathogens including fungi that showed no response to intensive antibiotic therapies. They found that following PACK-CXL, corneal melting was arrested in all cases, and that there was no need for emergency corneal transplantation as would normally have been the case. Another clinical trial presented results which support the efficacy of the PACK-CXL procedure (Panda et al., 2012). The study looked at seven eyes with infectious keratitis associated with corneal ulcers and melting, some of
which were caused by fungi. After PACK-CXL treatment, it was noted that the corneal infections had been well controlled and in all cases progression of the corneal melting stopped and ulcers healed with no severe consequences and no further surgery was necessary. In recent studies, the therapeutic efficacy of the PACK-CXL technique was assessed by Hafez (2014) on a series of five eyes with resistant fungal corneal ulcers. Even though notable vision improvement was not achieved post-PACK-CXL treatment, the findings showed that the PACK-CXL procedure can be effective in the management of resistant corneal ulcers as all cases showed improvement on infection signs and complete ulcer healing. In addition, Li et al. (2013) presented the clinical findings in eight patients with fungal keratitis who received PACK-CXL treatment. After the PACK-CXL procedure, resolution of infection and ulcer healing were achieved in all cases, and none required a further surgical intervention. Li et al. suggested that PACK-CXL procedure is a viable option for treating fungal keratitis. However, confounding outcomes have been found in a clinical trial conducted by Escarião et al. (2013) on 11 eyes with bacterial or fungal keratitis that were non-responsive to the medications administered for at least a period of one week. The bacterial keratitis cases showed relief in the symptoms post-PACK-CXL treatment whereas in the fungal keratitis cases no improvement was observed, which indicated that the effectiveness of PACK-CXL procedure is related to the causative agent. This finding is consistent with a study by Vajpayee et al. (2015) in which 41 cases of fungal keratitis were divided into two groups. The first group was treated with the PACK-CXL technique along with antifungal therapy, whilst the second group received antifungal medications only. It was observed that the infection resolution was achieved in 90% and 85.7% of cases, the recovery time rate was 31±27 and 31±20 days, and the best corrected vision rate was 1.13±0.55

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and 1.25±0.46 (logMAR) in the first and second group, respectively. Moreover, keratoplasty surgery was carried out in two cases from the cross-linked group and in three cases from the control group. Vajpayee et al. concluded that there was no beneficial effect of the combined antifungal/PACK-CXL treatment over the antifungal agent alone. Additionally, Uddaraju et al. (2015) evaluated the efficacy of PACK-CXL treatment in resistant deep stromal keratitis caused by fungi, where 13 cases were included, which showed no response to antifungal medications administered for two weeks. The cases were randomly divided into cross-linked and control groups, where the cross-linked group was treated with combined riboflavin/UV-A light along with the medications, while the control group received antifungal therapy only. Uddaraju et al. concluded that PACK-CXL procedure is not effective treatment in the management of advanced fungal keratitis as the cross-linked group experienced a higher perforation rate than the control group. Notably, the majority of the clinical studies were carried out on resistant forms of corneal infections where the antimicrobial medications had been administrated for various durations and mostly in cases where the infections had failed to resolve. In such cases, the PACK-CXL technique was implemented only after the infections had progressed to late stages and had caused further serious damage to the cornea. Thus, late intervention could have resulted in a great reduction in the effectiveness of PACK-CXL procedure, minimization of the healing rate or even a treatment failure.
2.6 Conclusion

The treatment of *Fusarium* keratitis with combined riboflavin/UV-A light-induced corneal cross-linking is effective in inhibiting *Fusarium* sporulation and hyphal growth, and thus, reduces the intensity of infection.
Chapter 3

Efficacy of Corneal Cross-linking in the Management of

*Fusarium* Keratitis: An Investigation Using a Histological Staining Technique

Contributions

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross. Prof. Fiona Carley and Mr. Arun Brahma regularly treat patients with infectious keratitis in the Manchester Royal Eye Hospital. Prof. Carly and Mr. Brahma met with the research team on a regular basis to discuss ideas and progress of the research studies.
Abstract Publication

Part of the data in this chapter was published in an abstract.


Submission for Publication

The chapter is prepared as a manuscript which will be submitted for publication.


Conference Presentation

Part of the data in this chapter was presented at an international conference.

Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.

- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the *Fusarium* strain.
Keywords: Ex vivo human cornea, Fusarium, photo-activated riboflavin-mediated corneal cross-linking (PACK-CXL), funga...
**Conclusion:** The riboflavin-activated PACK-CXL therapy seems to be a valuable approach in decreasing the depth of *Fusarium* hyphal penetration in the cornea and in turn in improving the resolution of fungal infection.
3.2 Introduction

Corneal damage resulting from fungal infection presents a very real threat to eyesight as well as being a significant clinical challenge. Particularly within the developing world, fungal infections account for a considerable proportion of blinding conditions (Srinivasan, 2004). For treatment of fungal keratitis, the main control comprises antifungal agents, including topical antifungal drops (Qiu et al., 2015). Nonetheless, for serious fungal infections antifungal agents are ineffective (Srinivasan, 2004) because of their inadequate intraocular infiltration, stromal permeability and unfavourable clinical results and are usually incapable of restoring or maintaining eyesight (Thomas, 1994, Srinivasan, 2004). To date no gold standard treatment for fungal keratitis has been identified. Even though novel antifungal agents have been developed, which provide additional options for treatment of fungal infections, keratitis resulting from filamentous fungi is still a challenge to manage (Thomas and Kaliamurthy, 2013). Moreover, an additional challenge is presented by the resistance of *Fusarium* species to the majority of presently accessible antifungal compounds (Thomas, 1994, Eliopoulos et al., 2002). Thus, there are still unfavourable outcomes for many patients (Kawakami et al., 2015). For cases of non-responsiveness to medical treatment or serious complications, surgery can be carried out (Thomas, 1994). It has been shown by some studies that 23-32% of fungal infections caused by *Fusarium* species result in keratoplasty (Rosa et al., 1994, Vemuganti et al., 2002).

The exposure to combined riboflavin/UV-A light has been employed in corneal cross-linking (CXL) as a therapy for keratoconus (Wollensak et al., 2003a). As a result of successful utilization of the combined riboflavin/UV-A light in inactivation
microorganisms within blood (Goodrich, 1999), various research groups have investigated the use of CXL for the treatment of infectious keratitis, and it has been re-named as photo-activated chromophore for infectious keratitis (PACK-CXL) (Said et al., 2014). In addition to inactivating the microorganisms it has also been shown that combined riboflavin/UV-A light-induced corneal cross-linking leads to an increase in the stiffness of the corneal stroma (Wollensak et al., 2003b, Kohlhaas et al., 2006, Wollensak, 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2013c, Beshtawi et al., 2014, Beshtawi et al., 2016), which stabilizes it and raises stromal resistance to degradation by secreted microbial enzymes (Spoerl et al., 2004). However, the use of PACK-CXL as an adjuvant treatment for fungal keratitis has produced inconsistent clinical results (Iseli et al., 2008, Anwar et al., 2011, Escarião et al., 2013, Li et al., 2013, Hafez, 2014).

This study aims to evaluate the efficacy of combined riboflavin/UV-A light-mediated corneal cross-linking as a primary therapy in the management of *Fusarium* keratitis using an *ex vivo* human corneal model to investigate the effect of PACK-CXL on fungal hyphal penetration within the corneal tissues using a histological staining technique.

### 3.3 Materials and Methods

#### 3.3.1 Ex Vivo Human Corneas

Twenty healthy *post-mortem* human corneal buttons from 12 male and 8 female donors in the age range 58 – 83 years old (mean ± SD, 69.2 ± 7.7 years) were used in this study. The corneas, provided by the Manchester Eye Bank (NHS Blood and Transplant, UK), were pre-consented for research in cases where they were not
suitable for transplantation. The project had full ethical approval from the Manchester University Research Ethics Committees (No. 040811), and followed the Tenets of the Declaration of Helsinki.

The corneas were randomly assigned to one of the five groups, with four corneas in each group as detailed below:

- **C - Control**: not inoculated with *Fusarium oxysporum* spores, not treated with CXL procedure (59-83 years old, mean ± SD, 70.3 ± 10 years).
- **X - Not inoculated, treated with CXL procedure**: (59-71 years old, mean ± SD, 63.8 ± 5.3 years).
- **I-24h - Inoculated with *Fusarium oxysporum* spores only, not treated with PACK-CXL, fixed at the time of commencing the cross-linking procedure on the IX-48h group at 24h post-inoculation (74-81 years old, mean ± SD, 75.8 ± 3.5 years).
- **I-48h - Inoculated with *Fusarium oxysporum* spores, not treated with PACK-CXL, fixed at 48h post-inoculation (59-76 years old, mean ± SD, 70.8 ± 7.9 years).
- **IX-48h - Inoculated, treated with PACK-CXL at 24h post-inoculation, fixed at 48h post-inoculation (58-71 years old, mean ± SD, 65.3 ± 6.8 years).

### 3.3.2 Corneal Tissue Culture

The human corneal buttons were submerged in fresh culture medium (Dulbecco’s Modified Eagle’s Medium, DMEM, 1000mg Glucose/L; Sigma-Aldrich Ltd, Poole, UK) supplemented with 10% (v/v) foetal bovine serum (FBS; Labtech International Ltd, Uckfield, UK), 2mM Glutamine, and 1% (v/v) Penicillin with Streptomycin (all Sigma-
Aldrich Ltd, Poole, UK), and incubated at 34°C for 72h prior to inoculation and the cross-linking processes. The manipulation of corneal tissue, subsequent infection and cross-linking treatment processes (Table 3-1) were performed in a recirculating Class II Microbiological Safety Cabinet (Labcaire SC-R; Labcaire Systems Ltd, Clevedon, North Somerset, UK).

### 3.3.3 Fungal Strain

The infecting fungus used was *Fusarium oxysporum f. sp. lycopersici* strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP). The strain was kindly provided by Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) (Di Pietro et al., 2001). The GFP fluorescence facilitated the tracking of the fungal hyphal invasion of the corneal layers. The *Fusarium* spore suspension was prepared and spore concentration adjusted to $5 \times 10^5$ spores per ml using fresh culture medium; 10 µl of this suspension was used to inoculate each of the scratched corneas as detailed below.
Table 3-1: Timeline of experimental procedures.

An outline of the main procedures performed on the different experimental groups of corneas: scratching, inoculation, cross-linking, staining and imaging. C: Control; X: Cross-linked only; I-24h: Inoculated only then fixed at 24h post-inoculation; I-48h: Inoculated then fixed at 48h post-inoculation; IX-48h: Inoculated, cross-linked at 24h post-inoculation, then fixed at 48h post-inoculation; PACK-CXL: Corneal cross-linking with isotonic riboflavin.

<table>
<thead>
<tr>
<th>Time scale</th>
<th>C group (n=4)</th>
<th>X group (n=4)</th>
<th>I-24h group (n=4)</th>
<th>I-48h group (n=4)</th>
<th>IX-48h group (n=4)</th>
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<tbody>
<tr>
<td>-</td>
<td>Scratched</td>
<td>Scratched</td>
<td>Scratch and inoculated</td>
<td>Scratch and inoculated</td>
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<tr>
<td>24 h</td>
<td>In culture</td>
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<tr>
<td>At 24h post-inoculation</td>
<td>-</td>
<td>PACK-CXL</td>
<td>Processing and PFA-fixation</td>
<td>-</td>
<td>PACK-CXL</td>
</tr>
<tr>
<td>24 h</td>
<td>In culture</td>
<td>In culture</td>
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<tr>
<td>At 48h post-inoculation</td>
<td>Processing and PFA-fixation</td>
<td>Processing and PFA-fixation</td>
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<td>Processing and PFA-fixation</td>
<td>Processing and PFA-fixation</td>
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<tr>
<td>-</td>
<td>PBS-Sucrose incubation, OCT-embedded, sectioned, H&amp;E-stained and imaged</td>
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3.3.4 *Ex Vivo* Model of Corneal Insult and Inoculation

**Process**

Seventy-two hours after corneal culturing, three of the corneal groups, I-24h, I-48h and IX-48h, were inoculated with the spores of *F. oxysporum* expressing cytosolic GFP. The corneal buttons of all groups were mounted on an artificial anterior chamber (Moria
SA, 92160 Antony, France; SD Healthcare, Manchester, UK), and held in place by the retainer and locking ring. The anterior surface was scratched in the central region of the cornea (approximately 7 mm scratch diameter) in an asterisk shape using a 0.6X25 mm-gauge needle (BD Microlance 3; BD Biosciences, Oxford, UK). The scratched corneas were transferred into a fresh, dry 6-well tissue culture dish and those previously assigned for infection (I-24h, I-48h and IX-48h) were inoculated with $5 \times 10^5$ Fusarium oxysporum spores in 10 μL of culture medium. The inoculated corneas (I-24h, I-48h and IX-48h) were left for few minutes to aid spore attachment to the corneal tissue. Next, a small volume of culture medium was added around the periphery of each cornea to prevent washing off the fungal spores, but maintain humidity and nutritional support. Then, the corneal buttons of all groups were incubated at 34°C.

### 3.3.5 Corneal Cross-linking Procedure

At 24h post-inoculation, the corneas in the X and IX-48h groups were treated with the corneal cross-linking technique according to a standard CXL protocol (Wollensak et al., 2003a). During the cross-linking procedure, each cornea was mounted on the artificial anterior chamber, the interior was filled with sterile Phosphate Buffered Saline (PBS) to mimic the in vivo intraocular pressure and maintain corneal stability during the cross-linking procedure, the central epithelial layer (7-9 mm diameter area) was removed and isotonic riboflavin eye drops (Riboflavin 5′-phosphate sodium salt hydrate, R7774, Sigma-Aldrich, UK), (0.1% (w/w) riboflavin, 20% (w/w) dextran T500) were dropped onto the corneal surface at 5 minute intervals for 30 minutes. Subsequently, corneas were irradiated with ultraviolet-A light for 30 minutes (370 nm, irradiance of 3mW/cm², irradiation dose of 5.4 J/cm²) along with riboflavin application.
at 5 minute intervals. Then, the corneas were rinsed with PBS and placed in a fresh culture medium and incubated at 34°C.

### 3.3.6 Corneal Tissue Processing

At 24h post-inoculation, corneal buttons in I-24h group were transferred into a fresh, dry tissue culture dish and cut into halves. One half from each corneal button was fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C overnight followed by 72h incubation in PBS-sucrose. Next, they were embedded in OCT (KP-CryoCompound, Klinipath BV, The Netherlands) over liquid nitrogen. Corneal buttons in the other groups, C, X, I-48h and IX-48h, were processed, fixed and OCT-embedded as detailed above at 24h post-cross-linking treatment, 48h post-inoculation. The OCT-embedded corneal tissue was sectioned at 30 µm thickness, using a Bright OTF cryostat (Bright Instrument Company, Huntingdon, UK), onto Superfrost® Plus glass slides (VWR, Leuven, Belgium), dried overnight at 37 °C and stored at -80 °C. The other half of each cornea was fresh-frozen in OCT and stored at -80 °C for future use.

### 3.3.7 Haematoxylin and Eosin (H&E) Staining

The fixed, pre-cut 30 µm sections were rehydrated in deionised water for 2 minutes, haematoxylin and eosin stained (Harris’ Haematoxylin and Eosin Y solutions, Sigma-Aldrich, UK), dehydrated and mounted using DPX mounting medium (Sigma-Aldrich, UK). Images of the H&E stained sections were captured at various magnifications using a Zeiss Axiostar Plus HBO 50 microscope (Zeiss UK, Cambridge, UK), Micropublisher 5.0 RTV camera and Q-CapturePro7™ Software (both Media Cybernetics UK, Buckinghamshire, UK).
3.3.8 Statistical Analysis of the Hyphal Penetration Depths

The effects of PACK-CXL treatment upon the resolution of *Fusarium* infection were determined by measuring the depth of hyphal penetration within the corneal tissues in I-24h group at 24h post-inoculation and compared with the depth of hyphal penetration at 48h-post-inoculation in un-treated I-48h group and treated IX-48h group. The corneal thickness and the depth of fungal penetration within the stromal tissue were semi-automatically measured (µm) for all of the inoculated corneas (I-24h, I-48h, and IX-48h) using ImageJ software (Schneider et al., 2012); and the percentage of the total corneal depth penetrated by the fungal hyphae was calculated for each inoculated cornea. The statistical analysis to assess the change in mean was performed using the one-way analysis of variance (ANOVA) and *post hoc* tests.

3.4 Results

Images of the H&E stained corneal sections which were obtained from the C group are shown in Figure 3-1A, where the healthy corneal structure of epithelium and stroma layers can be seen: the stromal tissues are stained pink, the stromal keratocytes are stained purple and the epithelium is dark purple as it consists of several layers of cells. At higher magnification (Figure 3-1B), the corneal epithelial and stromal histology is visible and appears intact and free from fungal invasion. Figure 3-1C shows an H&E stained corneal section from the X group. This figure shows the corneal stromal layers where there is a loss of keratocytes in the anterior stroma. The lack of an epithelial layer is due to its removal as a part of the CXL protocol (Figure 3-1D). Fungal infection developed in all of the inoculated corneas where the *Fusarium* spores successfully
attached to the corneas, germinated and hyphae penetrated the tissues (Figure 3-1E).

Figure 3-1F shows a magnified image (x40) of the epithelial and anterior stromal region of an inoculated cornea (I-48h) in which the *Fusarium oxysporum* hyphae have invaded the epithelial layer and penetrated into the anterior stroma 48h post-inoculation.

Figure 3-2 shows central (A, C and E) and peripheral (B, D and F) views of H&E stained sections of corneas from I-24h, I-48h and IX-48h groups, respectively, where penetrating fungal hyphae can be seen. The IX-48h cornea had the lowest hyphal penetration depth at the central region compared to the other corneas. In addition, the density of the fungal hyphae within the tissues is much lower in IX-48h cornea compared to that in I-24h and I-48h corneas.

The deepest *Fusarium* penetrations observed within the central and peripheral corneal stroma were measured in the inoculated groups (I-24h, I-48h and IX-48h), and expressed as percentage of the total corneal depth. ANOVA of the hyphal penetration depth showed that there were significant differences in the percentage of the corneal depth penetrated by the fungal hyphae in the central region ($P < 0.001$), and in the peripheral region ($P = 0.036$) between the inoculated groups. For the most deeply penetrating hyphae in the central cornea, those in the I-48h (inoculated; fixed at 48h post-inoculation) group penetrated significantly deeper than those in the I-24h (inoculated; fixed at 24h post-inoculation) group ($P = 0.001$), and deeper than those in the IX-48h (inoculated; PACK-CXL treated; fixed at 48h post-inoculation) group ($P < 0.001$) (Figure 3-3). In the periphery of the cornea the percentage corneal penetration depth in the I-48h group was significantly greater than in the I-24h group ($P = 0.03$) and greater than in the IX-48h group, but not significantly so. However, there is
considerable variation in hyphal penetration depth within the IX-48h group, which is not seen in the I-24h and I-48h groups. Comparing the central versus peripheral corneal penetration by the fungal hyphae within I-24h, I-48h and IX-48h groups (Figure 3-3), the difference in penetration depth within the IX-48h group was significant ($P = 0.015$) with the periphery more deeply invaded than the centre. Whereas, in the I-24h and I-48h groups there was no significant difference between the hyphal penetrations in the centre compared to the periphery.
Figure 3-1: H&E stained corneal sections of control, cross-linked and inoculated corneas.

H&E stained sections of a control cornea showing the intact corneal epithelial and stromal layers along with keratocytes (yellow arrows) at x10 magnification (A). The epithelial cells are visible at x40 magnification (B). H&E stained corneal sections of cross-linked (not inoculated) cornea (C and D) show the corneal stromal structure at magnification x10 and x40, respectively. The epithelium is absent as it is removed as part of the CXL process, and there are fewer keratocytes present compared to the untreated control cornea. H&E stained sections of an inoculated only cornea (E and F) at magnification x10 and x40 showing the invasion of *Fusarium* hyphae (blue arrows) over the epithelium and through the stroma at 48h post-inoculation. C: Control; X: Cross-linked only; I-48h: Inoculated only and fixed at 48h post-inoculation.
Figure 3-2: H&E stained sections from the different inoculated groups.

H&E stained sections at the central (A, C and E) and peripheral (B, D and F) regions (x10) of the I-24h (A and B), I-48h (C and D) and IX-48h (E and F) corneas showing the *Fusarium* hyphal invasion (blue arrows), which appears to be more severe at the periphery of the corneas than at the centre. The figure shows less *Fusarium* hypha in the central region of the IX-48h cornea (E; blue arrow) compared to that in the I-24h (A) and I-48h (C) corneas. Also shown is the deepest level of penetration (µm) at which fungal hyphae could be seen in this figure. I-24h: Inoculated only then fixed at 24h post-inoculation; I-48h: Inoculated then fixed at 48h post-inoculation; IX-48h: Inoculated, cross-linked then fixed at 48h post-inoculation.
The percentage of the *Fusarium* hyphal penetration depth within stromal tissues at the central and peripheral regions of the corneas

![Graph showing hyphal penetration depth](image)

*Figure 3-3: The percentage of the total stromal depth penetrated by the *Fusarium* hyphae at the central and peripheral regions for the inoculated groups.*

The graph shows that the depth of *Fusarium* hyphal penetration was greater in the I-48h group compared to the I-24h and IX-48h groups at both the centre and periphery of the corneas. There is a significant difference in depth of hyphal penetration at the centre of the cornea between I-48h and I-24h groups ($P = 0.001$), and between I-48h and IX-48h groups ($P < 0.001$). There is a significant difference in depth of hyphal penetration at the periphery of the cornea between I-48h and I-24h groups ($P = 0.03$). There is a significant difference in penetration of the hyphae between the central and peripheral regions within the IX-48h group ($P = 0.015$). I-24h: Inoculated only then fixed at 24h post-inoculation; I-48h: Inoculated then fixed at 48h post-inoculation; IX-48h: Inoculated, cross-linked then fixed at 48h post-inoculation.
3.5 Discussion

Treatment of infectious keratitis resulting from fungal infection is problematic and challenging for two main reasons – the wide range of infecting fungi and the limited range of available antimycotic drugs (Kredics et al., 2015). A highly effective universal antimycotic treatment for *Fusarium* keratitis is yet to be found (Ansari et al., 2013). It has been documented that 70% of superficial keratitis cases caused by *Fusarium* responds to current antifungal agents, but they are ineffective against deeper infections (Alexandrakis et al., 1998, Kawakami et al., 2015). However, novel antimycotics are being developed all the time and may provide effective treatments in the future; meanwhile, other potential treatments for penetrating fungal keratitis should be sought.

Previously, a novel *ex vivo* human corneal infection model was developed and established by means of utilising donor *post-mortem* human corneas and infecting them with a fluorescently labelled strain of *Fusarium oxysporum* (Alshehri et al., 2016). The previous study describes results obtained with confocal microscopy which show the growth of the *Fusarium* in three dimensions over time. However, confocal microscopy is restricted to imaging the *Fusarium* hyphal growth in the anterior 100 µm of the corneal depth. Therefore, having a cross section through the entire cornea to show the depth of hyphal penetration is a critical step to further investigate the beneficial effect of PACK-CXL in suppressing the growth of *Fusarium* hyphae. Figure 3-4 highlights the difference between the observations obtained using confocal microscopy (Figure 3-4A and B) and histological staining (Figure 3-4C and D) for two inoculated corneas. As the spores of *F. oxysporum* used for inoculation the corneas are
expressing GFP in its cytoplasm, the *Fusarium* infection can be seen as branching, green fluorescent fungal hyphae using confocal microscopy. The confocal images show severe infection in cornea 1 (Figure 3-4A) which is demonstrated by greater number and density of *Fusarium* hyphae compared to cornea 2 (Figure 3-4B). However, the H&E stained images show that cornea 2 (Figure 3-4D) was penetrated by *Fusarium* hyphae more deeply than cornea 1 (Figure 3-4C), which could pose a greater threat of invasion of the corneal endothelium.

The work outlined in this study was directed toward further assessment of the effectiveness of combined riboflavin/UV-A light-mediated corneal cross-linking as a therapy for the management of corneal infections caused by *Fusarium oxysporum*. An *ex vivo* model of corneal infection and H&E staining technique were used to capture the full thickness of the cornea in fixed sections from a single time point and identify *Fusarium* hyphae within the corneal tissues. This technique allowed measurement of the full depth of fungal hyphal penetration and evaluation of the impact of PACK-CXL treatment. Since there was natural variation in the thicknesses among the corneal buttons used in this study, depth of fungal hyphal penetration was expressed as a percentage of the individual corneal thicknesses.
Figure 3-4: Comparison of confocal microscopy images and H&E stained corneal sections of two inoculated corneal buttons.

The *Fusarium* infection can be seen in cornea 1 and 2 as branching, green fluorescent fungal hyphae using confocal microscopy (A and B), while the penetrating fungal hyphae in the H&E stained corneal sections for corneas can be seen in purple (C and D). The confocal images show extensive fungal growth in cornea 1 (A), while there are fewer hyphae in evidence in cornea 2 (B). Each confocal image is a projection of a z-stack of confocal images which is 100 µm thick. H&E stained corneal sections (C and D) showing the *Fusarium* hyphal invasion, which is deeper within cornea 2 (D) than in cornea 1 (C). The blue arrows show the deepest level of penetration at which fungal hyphae could be seen in these images.
The increased penetration of *Fusarium* hyphae in the I-48h (inoculated then fixed at 48h post-inoculation) group compared to the I-24h (inoculated then fixed at 24h post-inoculation) group at both the centre and periphery of the corneas was expected as the infection would normally develop with prolonged incubation time. The PACK-CXL treatment did not achieve a complete eradication of the fungus from the treated IX-48h corneas. Nevertheless, the results show that in the central (UV irradiated) region of the cornea there is a significant reduction in the depth of penetration by the *Fusarium* hyphae in the IX-48h group compared to the I-48h group. Whereas at the periphery of the corneas, comparison between the I-48h and IX-48h groups shows that there is no significant difference in depth of penetration of the fungal hyphae. The UV beam of the corneal UV-A light emitter (cross-linker) is about 9 mm wide. Hence, the reduction in depth of penetration in central cornea is a direct result of cross-linking while the peripheral cornea was not cross-linked.

There is a slight reduction in the depth of penetration by the *Fusarium* hyphae in the central region in the IX-48h group compared to the I-24h group. The I-24h group served as a guide to assess the corneal depth that fungal hyphae have penetrated and reached at the time the PACK-CXL procedure was carried out. Thus, the I-24h group verified that the penetration of *Fusarium* hyphae was restricted by the PACK-CXL treatment at the central (UV irradiated) region in the IX-48h group, while the *Fusarium* hyphae continued their penetration within the tissues during the second (24–48 h) incubation period in the I-48h group. In addition, the fungal infection and penetration of the central cornea was significantly less compared to that at the periphery within the IX-48h group. The selective reduction in fungal infection, invasion and penetration
at the centre of the IX-48h corneas is as expected, with much less effect at the periphery. This is because the peripheral region of the corneas in inoculated groups was partially submerged in the corneal culture medium where there are large numbers of *Fusarium* spores, which, in turn, could re-infect that region of the cornea. However, *in vivo* the action of tear production and blinking is likely to reduce the spore load on the corneal surface, in contrast to the static *ex vivo* model used in this study. In addition, and more importantly, only the central nine millimetres of the cornea are irradiated with the highly collimated beam of UV-A produced by the cross-linker. The clinical cross-linker was originally designed for use in the treatment of keratoconus by cross-linking stromal collagen fibrils, but without damaging the corneal limbus, which is the niche occupied by the corneal epithelial stem cells. These stem cells repopulate the debrided corneal epithelium thus their protection from UV-A irradiation is paramount (Thorsrud et al., 2012, Meek and Hayes, 2013). Thus, the findings of this study demonstrate that the PACK-CXL treatment has a beneficial effect in suppressing *Fusarium* infection and penetration in the central cornea, but is less effective for more peripheral infections if the traditional cross-linker and standard procedure are used. Peripheral infections can perhaps be tackled by centring the cross-linker on the ulcer and reducing the aperture of the cross-linker to cover the infected area while avoiding damage to the entire limbal region thus protecting some of the stem cells. However, it is noteworthy that in this study the hyphal penetration depth measurement in the peripheral cornea has not been taken perpendicularly to the corneal surface due to corneal curvature. As a result, the measured depth may not equal the actual depth of penetration.
The antimicrobial effects of the UV-A have likely contributed to the suppression of the *Fusarium* growth in the IX-48h group in this study, and consequently the progression of the fungal infection and invasion. However, some fungal hyphae appear to have come from *Fusarium* spores which were present within the corneal tissues and not from the original infection (Figure 3-5), which suggests that hyphal growth is inhibited by combined riboflavin/UV-A light whereas the spores survive the treatment and go on to germinate.

![Figure 3-5: H&E stained section of an inoculated, cross-linked, and fixed at 48h post-inoculation cornea (IX-48h).](image)

H&E stained section at the central region (x10) of the IX-48h cornea showing a single *Fusarium* colony (blue arrow), which appears to have come from *Fusarium* spores which were present within the corneal tissues and not from the original inoculation.
The findings obtained in this study are in agreement with results shown in other published studies, although the methodology of this present study did not include the use of antimycotic agents as reported by most of others (Anwar et al., 2011, Li et al., 2013). However, the results of this study are inconsistent with some studies of corneal cross-linking for treating fungal keratitis, in which the combined riboflavin/UV-A light seemed not to be effective in the management of moderate (Vajpayee et al., 2015), or deep fungal keratitis (Uddaraju et al., 2015). The specific reasons for the different outcomes of PACK-CXL treatment for fungal keratitis between the various studies, including this study, are unclear at present. Possibly, the timing of the procedure is a critical factor for success of the PACK-CXL treatment. In terms of in vivo studies, cases of fungal keratitis were usually treated by the PACK-CXL procedure after a prolonged course of intensive clinical medications, and in particular in cases where resolution of the infections with administrated antimicrobial medication had failed. In such cases, the spread of fungal hyphae as well as penetration into the deep stromal layers is easily enabled. Fungal spores may be also present at such an advanced stage of infection as these could survive the PACK-CXL treatment. In addition, an abrupt release of foreign fungal antigens will be induced by the PACK-CXL therapy, resulting in a brief, but obvious, host inflammatory response (Pearlman et al., 2013) which could possibly enhance corneal melting. Therefore, the delay preceding the PACK-CXL procedure could have resulted in reduction in the effectiveness of the PACK-CXL procedure. In addition, it has been stated that in cases of infectious keratitis caused by fungi, deep penetration develops in almost 50% of instances (Vemuganti et al., 2002), while the effect of cross-linking treatment is limited only to a depth of around the first 300 μm from the corneal surface (Wollensak et al., 2004, Kohlhaas et al., 2006, Beshtawi et al.,
which means that the PACK-CXL treatment might be not effective on deeply penetrating fungi. On the other hand, the PACK-CXL protocol used in this study, as well as in most of the literature, is based upon the standard protocol for treating the keratoconus (Wollensak et al., 2003a), in which the total UV-A irradiation dose is under the limit known to impair the corneal endothelium. Thus, as the employed CXL protocol was not designed to manage the infectious keratitis, investigation of the effects various periods of UV-A irradiation or UV dose fractionation and different concentrations of riboflavin may be necessary for the treatment of infectious keratitis; and also the combined riboflavin/UV-A treatment regimen used could be dependent upon the pathogenic species being treated. Every genus or species is likely to have a different response to the killing or arresting effects of the PACK-CXL procedure, with differing sensitivity amid the various infecting organisms related to the length of the cell cycle, cell wall structure, spore pigmentation and UV resistance (Escarião et al., 2013).

3.6 Conclusion

A combined riboflavin/UV-A light-induced corneal cross-linking protocol appears to be beneficial in reducing *Fusarium* load and depth of hyphal penetration into the corneal tissues, and as a consequence it suppresses the progression of fungal infection. However, this inhibitory effect is seen only in the corneal tissue directly below the UV-A treatment beam.
Contributions

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross.
Submission for Publication

The chapter is prepared as a manuscript which will be submitted for publication.


Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.
- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the *Fusarium* strain.
- Dr Peter March, Dr Steven Marsden and Mr Roger Meadows from the Bioimaging Facility for their help with the confocal microscopy and Imaris software.
### 4.1 Abstract

**Purpose:** The aim of this study was to investigate the impact of trans-epithelial corneal cross-linking compared to standard cross-linking (PACK-CXL), and evaluate the effect of epithelium-on and -off variations of PACK-CXL in the management of *Fusarium* keratitis using an *ex vivo* human corneal infection model which is designed to closely mimic human fungal keratitis.

**Method:** Twenty human corneas were divided into 5 groups: control (C), inoculated only with *Fusarium oxysporum* spores (I), inoculated then cross-linked following the standard CXL protocol using isotonic riboflavin/UV-A and epithelium-off approach (IX), inoculated then cross-linked using trans-epithelial riboflavin/UV-A and epithelium-on approach (ITEX), and inoculated then cross-linked using trans-epithelial riboflavin/UV-A and epithelium-off approach (ITEX-OF). The IX, ITEX and ITEX-OF groups were treated with PACK-CXL at 48h post-inoculation. Corneas were imaged at 72h post-inoculation, and *Fusarium* hyphal volume and spore concentration were calculated. Corneas were sectioned, haematoxylin and eosin stained, imaged, and the hyphal penetration depth within stromal tissue was measured.

**Results:** The I group had a significantly greater hyphal volume compared to ITEX-EON (*P* = 0.002) and ITEX-EOF (*P* = 0.045) groups. There was a significant inhibition of *Fusarium* sporulation in ITEX-OF compared to I groups (*P* = 0.024). There is a significant reduction in the hyphal penetration depth at central corneal region in ITEX compared to I (*P* = 0.001) and IX (*P* = 0.013) groups, and a significant reduction in ITEX-EOF compared to I (*P* = 0.002) and IX (*P* = 0.031) groups.
**Conclusion:** Trans-epithelial PACK-CXL appears to have an inhibitory effect on fungi which is significantly higher than the standard PACK-CXL procedure for treating fungal infections.
4.2 Introduction

Fungal keratitis is one of the most severe corneal infections, requiring immediate therapeutic intervention to avoid significant irreversible damage to vision. The management of infectious keratitis caused by fungi is a challenging issue mostly due to the delayed diagnosis and restricted options for treatment (Srinivasan, 2004, Kredics et al., 2015).

Combined riboflavin/UV-A light-induced corneal cross-linking (CXL) is a minimally invasive procedure, which was first introduced for the treatment of keratoconus (Wollensak et al., 2003a). In recent years, the use of this technique in the treatment of infectious keratitis has been explored, and is known as photo-activated chromophore for infectious keratitis-corneal cross-linking (PACK-CXL) (Said et al., 2014). It is suggested that the combined riboflavin/UV-A light-induced corneal cross-linking enhances the biomechanical stiffness of the cornea (Wollensak et al., 2003b, Kohlhaas et al., 2006, Wollensak, 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2013c, Beshtawi et al., 2014, Beshtawi et al., 2016). In cases of infectious keratitis this increases stromal resistance to enzymatic digestion by pathogens (Spoerl et al., 2004), and thus reduces the development and progression of the corneal melting (Iseli et al., 2008, Said et al., 2014). In addition, it has been proposed that the combined riboflavin/UV-A light has a beneficial antimicrobial effect, which assists in suppressing the growth of pathogens and restricting the development of the infection (Iseli et al., 2008, Panda et al., 2012, Hafez, 2014). Nevertheless, treating corneal infections, in particular fungal keratitis, with PACK-CXL procedure has resulted in
conflicting findings (Iseli et al., 2008, Escarião et al., 2013, Li et al., 2013, Hafez, 2014, Uddaraju et al., 2015, Vajpayee et al., 2015, Alshehri et al., 2016).

Riboflavin plays a crucial role in enabling the cross-linking process as the UV-A light is absorbed by the riboflavin, which produces reactive oxygen species (ROS) (Baier et al., 2006), which in turn increase the number of covalent bonds between the stromal collagen molecules (McCall et al., 2010). Therefore, inadequate stromal absorption of riboflavin could lead to failure of the cross-linking process (Wollensak and Iomdina, 2009a). The standard riboflavin solutions (containing 0.1% isotonic riboflavin in 20% dextran) show limited ability to penetrate the intact corneal epithelium (Hayes et al., 2008). Accordingly, the standard corneal cross-linking protocol includes removing the epithelial layer as an essential step prior to applying the standard isotonic riboflavin (Wollensak et al., 2003a) in order to deliver a sufficient riboflavin concentration within stromal tissues and allow stromal diffusion. The standard cross-linking protocol following the epithelium-off approach has some disadvantages, such as post-operative pain, potential visual loss, corneal scarring, corneal haze, long recovery time, and potential risks of further infections (Koller et al., 2009, Pollhammer and Cursiefen, 2009, Raiskup et al., 2009, Dhawan et al., 2011). A modification of the standard cross-linking protocol has been introduced in which the procedure is carried out without epithelial debridement (Kissner et al., 2010, Barbara et al., 2012, Stojanovic et al., 2012, Hayes et al., 2016) to minimise risk of complications associated with epithelium-off protocol. As the standard formulation of riboflavin exhibits limited ability to penetrate the intact epithelium (Hayes et al., 2008), the use of trans-epithelial riboflavin has been proposed to enhance epithelial penetration and allow stromal
diffusion (Kissner et al., 2010, Barbara et al., 2012). Trans-epithelial riboflavin can be formulated by adding chemical agents, such as Benzalkonium Chloride (BAC) (Pinelli et al., 2009), in order to loosen the tight junctions between the corneal epithelial cells, and thus, enable riboflavin penetration into the stroma. This in turn allows trans-epithelial cross-linking without the need to remove the epithelial layer. However, the epithelium-on protocol may lead to heterogeneous or insufficient trans-epithelial riboflavin diffusion into the stroma, which could result in a reduction in the efficacy of corneal cross-linking treatment (Wollensak and Iomdina, 2009a, Bottós et al., 2011, Aldahlawi et al., 2016).

To understand the efficacy of PACK-CXL with epithelium-on and -off protocols in the management of infectious keratitis it is important to investigate the effectiveness of PACK-CXL variations in treating corneal infections. These studies are usually done on animal corneas, which are different from their human counterparts. Previous investigations on animals showed that there are collagen fibre organisational differences between human and rabbit corneas (Müller et al., 2001), and a contrasting thickness and elasticity among human and porcine corneas (Zeng et al., 2001, Elsheikh et al., 2008). Also, there is the question of the ability of animal endothelial cell division, a property that their human equivalents do not possess (Hovakimyan et al., 2011). On the other hand, application of the cross-linking procedure in the clinical trials must be done with caution to protect the patients from any potential risks or undesirable side effects. Therefore, laboratory-based investigations on human corneal models would be highly beneficial in order to establish an effective protocol for PACK-CXL to treat fungal keratitis. An ex vivo human corneal model of treating *Fusarium* infection with
combined riboflavin/UV-A light-induced corneal cross-linking has been described previously (Alshehri et al., 2016) and offers valuable opportunities to further explore and investigate the therapeutic effect of PACK-CXL procedure in the management of fungal keratitis.

This study aims to use the ex vivo human corneal infection model, which mimics in vivo human fungal keratitis cases, to evaluate the effect of trans-epithelial PACK-CXL procedure compared to standard PACK-CXL, and to assess the impact of epithelium-on and -off variations of PACK-CXL in the management of Fusarium infections using confocal microscopy and histological staining.

4.3 Materials and Methods

4.3.1 Ex Vivo Human Corneas

Twenty healthy post-mortem human corneal buttons from 12 male and 8 female donors in the age range 55 – 94 years old (mean ± SD, 72.4 ± 11.3 years) were used in this study. The corneas were provided by the Manchester Eye Bank (NHS Blood and Transplant, UK) and pre-consented for research. The project had full ethical approval from the Manchester University Research Ethics Committees (No. 040811) and followed the terms of the Declaration of Helsinki.

The corneas were randomly assigned to one of the five groups with four corneas in each group as detailed below:

- C – Control: not inoculated with Fusarium oxysporum spores, not treated with PACK-CXL procedure (55-86 years old, mean ± SD, 69.5 ± 13.8 years).
• I - Inoculated with *Fusarium oxysporum* spores only, not treated with PACK-CXL (61-94 years old, mean ± SD, 76.5 ± 13.5 years).

• IX - Inoculated then treated with PACK-CXL at 48h post-inoculation following the standard protocol: using the conventional isotonic riboflavin and epithelium-off (debrided) approach (63-94 years old, mean ± SD, 77.5 ± 13 years).

• ITEX - Inoculated then treated with PACK-CXL at 48h post-inoculation using trans-epithelial riboflavin solution and epithelium-on (intact) approach (64-78 years old, mean ± SD, 71.8 ± 7.3 years).

• ITEX-OF - Inoculated then treated with PACK-CXL at 48h post-inoculation using trans-epithelial riboflavin solution and epithelium-off approach (55-79 years old, mean ± SD, 67 ± 12.3 years).

4.3.2 Corneal Tissue Culture

The corneas were transferred into 6-well cell culture plates (Costar®; Corning Ltd, Ewloe, UK), and submerged in fresh culture medium (Dulbecco’s Modified Eagle’s Medium, DMEM, 5000mg Glucose/L; Sigma-Aldrich Ltd, Poole, UK) supplemented with 2mM Glutamine, 10% (v/v) foetal bovine serum (FBS; Labtech International Ltd, Uckfield, UK), and 1% (v/v) Penicillin with Streptomycin (all Sigma-Aldrich Ltd, Poole, UK). The corneas were incubated at 34°C for a minimum of 72 hours before the inoculation process and PACK-CXL treatment (Figure 4-1).
4.3.3 Fungal Strain

*Fusarium oxysporum* f. sp. *lycopersici* strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP) was kindly provided by Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) (Di Pietro et al., 2001) and used in this study. The green fluorescent protein facilitated the tracking of the fungal hyphal invasion of the corneal layers. The *F. oxysporum* GFP strain was cultured, spore (microconidia) suspensions were prepared, and spore concentration was adjusted to $1 \times 10^5$ spores per ml using fresh culture medium, and 10 µl of this suspension was used to inoculate each cornea in the inoculated groups.

4.3.4 Corneal Infection Process

Seventy-two hours after corneal culturing, each corneal button was mounted on an artificial anterior chamber (Moria SA, 92160 Antony, France; SD Healthcare, Manchester, UK), and the anterior surface was scratched in the central region of the cornea (approximately 7 mm scratch diameter) to assist the *Fusarium* invasion using a 0.6X25 mm-gauge needle (BD Microlance 3; BD Biosciences, Oxford, UK). The corneas were transferred into a fresh 6-well cell culture plates with the posterior surface of the cornea uppermost. The posterior corneal cavity was filled with an agar-gelatine gel (2% (w/v) agar (Oxiod, Nottingham, UK) and 2% (w/v) gelatine (BD Difco, Oxford, UK), supplemented with 7.4% (w/v) Sodium Bicarbonate (NaHCO$_3$) (Sigma-Aldrich Ltd, Poole, UK) and fresh culture medium) pre-inoculation process, in an attempt to minimize the fungal invasion of the lower corneal layers via spores in the culture medium. The molten agar-gelatine gel was allowed to cool at room temperature for a few minutes, and then, the corneas were inverted with the anterior surface of the
cornea uppermost. Four of the study groups, I, IX, ITEX and ITEX-OF, were inoculated with the *Fusarium* spores suspension. Next, a small quantity of the fresh culture medium was applied at the corneal periphery, and the corneas were incubated at 34°C for 48h prior to the PACK-CXL procedure.
Figure 4-1: Main experimental procedures.

The flow chart shows the main procedures performed on the different corneal groups: scratching, inoculations, PACK-CXL treatment, imaging, sectioning and H&E staining. C: Control; I: Inoculated only; IX: Inoculated then cross-linked with isotonic riboflavin with epithelium-off; ITEX: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-on; ITEX-OF: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-off; PACK-CXL: Cross-linking with isotonic riboflavin; TE-PACK-CXL: Cross-linking with trans-epithelial riboflavin.
4.3.5 PACK-CXL Procedure

The corneas in the IX, ITEX and ITEX-OF groups were subjected to the PACK-CXL procedure 48h post-inoculation with *Fusarium* spores. The corneal buttons in the IX group were treated with the PACK-CXL technique according to the standard CXL protocol (Wollensak et al., 2003a). For this group, each cornea was mounted on the artificial anterior chamber, the interior was filled with sterile Phosphate Buffered Saline (PBS) to mimic the *in vivo* intraocular pressure and maintain corneal stability during the PACK-CXL procedure. The epithelium-off approach was performed by debriding the central 7-9 mm diameter area, isotonic riboflavin eye drops (Riboflavin 5′-phosphate sodium salt hydrate, R7774, Sigma-Aldrich, UK), (0.1% (w/w) riboflavin, 20% (w/w) dextran T500) were dropped onto the corneal surface for 30 minutes at 5 minute intervals, and then the cornea was irradiated with ultraviolet-A light using a UV-A light emitter (VEGA, C.S.O. srl, Florence, Italy) (370 nm, irradiance of 3mW/cm², UV-A irradiation dose of 5.4 J/cm²) for 30 minutes, along with application of isotonic riboflavin at 5 minute intervals.

The corneal buttons in the ITEX group were treated with the PACK-CXL technique as detailed above for IX group except that the epithelium-on approach was followed and the isotonic riboflavin solution was replaced with trans-epithelial riboflavin solution (0.1% riboflavin in 0.44% Sodium Chloride (NaCl) (Sigma-Aldrich Ltd, Poole, UK) with 0.01% Benzalkonium Chloride (Sigma-Aldrich Ltd, Poole, UK). Lastly, the corneas in the ITEX-OF group were treated with the PACK-CXL technique as described for the IX group with a difference that the trans-epithelial riboflavin was used instead of isotonic riboflavin solution.
Following the PACK-CXL procedure, the corneas were rinsed with PBS, placed in a new 6-well tissue culture plate, filled with fresh agar-gelatine gel, provided with fresh culture medium and incubated at 34°C for further 24h prior to imaging with confocal microscopy. Figure 4-1 summarises the protocols described above.

4.3.6 Imaging and Fusarium Spore Concentration

At 72h post-inoculation and 24h post-PACK-CXL, the central region of each cornea was imaged using a Leica TCS SP5 AOBS inverted confocal microscope (Leica Microsystems Ltd., Breckland, Linford Wood, Milton Keynes, UK) with a x20 objective. The confocal images were analysed with the surpass module of the Imaris v8.4 software (Bitplane Scientific software module; Bitplane AG, Zurich, Switzerland). For each cornea the volume occupied by the *Fusarium* hyphae within anterior stromal tissues was automatically quantified in a tiled z-stack of 100 µm thickness by the software and displayed in an excel sheet, and then, the percentage of the total *Fusarium* hyphal volume was calculated for each inoculated group. At 72h post-inoculation, 24h post-PACK-CXL treatment, the number of *Fusarium* spores per ml of corneal culture medium was counted for all inoculated corneas.

4.3.7 Corneal Tissue Processing and Haematoxylin and Eosin (H&E) Staining

After confocal imaging and spores counting, corneal buttons were cut into halves. One half of each cornea was fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C overnight followed by 72h incubation in PBS-sucrose, embedded in OCT (KP-CryoCompound, Klinipath BV, The Netherlands) over liquid nitrogen, and sectioned at
30 µm thickness using a Bright OTF cryostat (Bright Instrument Company, Huntingdon, UK). The other half of each cornea was fresh-frozen in OCT and stored at -80 °C for future use. The fixed corneal sections were haematoxylin and eosin (H&E) stained, and images of the central and peripheral regions of the H&E stained sections were captured at x10 magnification using a Zeiss Axioskop Plus HBO 50 microscope (Zeiss UK, Cambridge, UK) and Q-CapturePro7TM Software (Media Cybernetics UK, Buckinghamshire, UK). The total corneal thickness and the depth of *Fusarium* hyphal penetration within the stromal tissues at the central and peripheral regions were semi-automatically measured (µm) for all corneas using ImageJ software (Schneider et al., 2012). The percentage of the total corneal depth penetrated by the *Fusarium* hyphae was calculated for each inoculated cornea.

### 4.3.8 Statistical Analysis

The analysis of *Fusarium* hyphal volume, spores concentration and hyphal penetration depth within corneal tissues for all of the inoculated groups was performed using a one-way analysis of variance (ANOVA) and post hoc tests. These statistical tests allowed comparison between groups to assess changes as a result of treatment.

### 4.4 Results

Inoculation of the corneas with *Fusarium oxysporum* spores in the I, IX, ITEX and ITEX-OF groups resulted in development of *Fusarium* infection, while the un-inoculated corneas in the C group remained intact. At 72h after inoculation and 24h after PACK-CXL treatment, the *Fusarium* infection, which can be seen as branching, green fluorescent fungal hyphae in Figure 4-2, had the greatest progression in the I cornea.
(Figure 4-2A), followed by the IX cornea (Figure 4-2B), and then the ITEX-OF (Figure 4-2D); whereas the ITEX cornea had the lowest progression of the infection (Figure 4-2C).

The percentages of the total *Fusarium* hyphal volume within corneal tissues at 72h post-inoculation and 24h post-PACK-CXL treatment for I, IX, ITEX-OF and ITEX groups are shown in Figure 4-3A. It can be seen that there is a variation in the percentage of the total volume occupied by *Fusarium* colony between the inoculated groups. The inoculated, non-cross-linked I group showed the most severe *Fusarium* infection, which is demonstrated by greater volume of *Fusarium* hyphae compared to the inoculated, cross-linked groups; whereas the ITEX group showed less severe infection compared to the other groups. Analysis of variance (ANOVA) of the *Fusarium* hyphal volume data showed that there is an overall significant difference among the different inoculated groups ($P = 0.003$). *Post hoc* tests (Tukey) showed a significant difference between I and ITEX groups ($P = 0.002$), and a significant difference between I and ITEX-OF groups ($P = 0.045$).

The *Fusarium* spore concentrations present in the culture medium at 72h post-inoculation and 24h post-PACK-CXL treatment for I, IX, ITEX-OF and ITEX groups are shown in Figure 4-3B. There was considerable difference in the concentration of *Fusarium* spore between the inoculated groups. The inoculated, non-cross-linked I group showed the largest spore concentration compared to the inoculated, cross-linked groups while the ITEX-OF group showed the lowest concentration of spores in comparison to the other groups. Analysis of variance showed an overall significant difference in *Fusarium* spore concentration between the inoculated groups ($P = 0.032$).
Post hoc tests (Tukey) showed that there is a significant difference between I and ITEX-OF groups ($P = 0.024$).

Images of the H&E stained corneal sections for corneas from all experimental groups are shown in Figure 4-4. The corneal epithelial layers are stained dark purple, the stromal tissues are stained pink, and the keratocytes and *Fusarium* hyphae are stained purple, distinguishable by the difference in their shape. The structure of the central and peripheral regions for a cornea from the C group can be seen in Figures 4-4A and 4-4B, respectively, where the corneal tissue is free from fungal invasion and the epithelium is intact. Figures 4-4C and 4-4D show central and peripheral views of the H&E stained corneal sections from the I group in which large fungal colonies have accumulated over the corneal surface and *Fusarium* hyphae have successfully invaded the tissues and penetrated deep into the corneal stroma at 72h post-inoculation. Figures 4-4E and 4-4F show central and peripheral views of the H&E stained corneal sections from the IX group, where penetrating fungal hyphae can be seen. Although the *Fusarium* hyphae penetrated deeply through the central and peripheral corneal regions in the IX cornea, the density of the *Fusarium* colonies is lower compared to that in the I cornea. Figures (4-4G and 4-4H) and (4-4I and 4-4J) show the central and peripheral views of the H&E stained corneal sections from the ITEX and ITEX-OF groups, respectively. It can be seen that the densities of the *Fusarium* hyphae within the stromal tissues in the ITEX and ITEX-OF groups are much lower when compared to that in both the I and IX groups.

Figure 4-5 shows the percentage of the total corneal depth penetrated by the *Fusarium* hyphae at the central and peripheral regions for all inoculated groups. The I
group has the deepest penetration of hyphae in both central and peripheral regions; whereas the ITEX group had the least depth of hyphal penetration compared to the other groups. Analysis of variance showed that there are overall significant differences in the percentage of the corneal depth penetrated by the fungal hyphae at the central region ($P < 0.001$) and at the peripheral region ($P = 0.033$) between the different inoculated groups. *Post hoc* tests (Tukey) showed significant differences in the percentage of the penetration depth of fungal hyphae at the central corneal region between the I and ITEX groups ($P = 0.001$), between I and ITEX-OF groups ($P = 0.002$), between IX and ITEX groups ($P = 0.013$), and between IX and ITEX-OF groups ($P = 0.031$). At the peripheral corneal region, the difference is only marginally significant between I and ITEX group ($P = 0.054$).
Figure 4-2: Confocal microscopy images of inoculated corneas at 72h post-inoculation and 24h following various PACK-CXL treatments.

Confocal images of different inoculated corneas show the extent of branching green fluorescent fungal hyphal growth at 72h post-inoculation and 24h post-PACK-CXL. Each image is a projection of a tiled z-stack (2x2 stacks, each stack is 775x775x100 µm) of confocal images (x20 magnification) which were captured from the anterior central corneal region. The most extensive fungal growth is visible in I group (A), *Fusarium* hyphae in ITEX group (C) are relatively fewer than in the other inoculated groups. I: Inoculated only cornea; IX: Inoculated then cross-linked with isotonic riboflavin with epithelium-off; ITEX: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-on; ITEX-OF: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-off.
Figure 4-3: The percentage of the *Fusarium* hyphal volume and the concentration of the *Fusarium* spores for the different inoculated groups.

The graphs show the percentage of the total volume occupied by the fungal hyphae within the anterior 100 µm of stromal tissues (A) and the number of *Fusarium* spores...
per ml of corneal culture medium (B) for the inoculated groups at 72h after inoculation process and 24h after different PACK-CXL approaches. The I group demonstrated greater *Fusarium* hyphal volume and *Fusarium* spore concentration compared to the treated groups, whereas the ITEX and ITEX-OF groups show considerable decrease in the *Fusarium* hyphal volume and the concentration of *Fusarium* spore, respectively, compared to the other groups. I: Inoculated only group; IX: Inoculated then cross-linked with isotonic riboflavin with epithelium-off; ITEX: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-on; ITEX-OF: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-off.
Figure 4-4: H&E stained corneal sections from the different study groups.

Central and peripheral corneal regions for the H&E stained corneal sections at x10 magnification from different study groups at 72h post-inoculation (24h post-PACK-CXL...
H&E stained sections for cornea from C group show the intact corneal epithelial and stromal layers along with keratocytes (A and B). H&E stained sections of cornea from I group show heavy infection, enormous fungal colonies and deep penetration of *Fusarium* hyphae within the stroma (C and D). H&E stained sections of cornea from IX group show the *Fusarium* hyphal penetration, which appears to be severe at both the centre and periphery of the cornea (E and F). H&E stained sections of corneas from ITEX (G and H) and ITEX-OF (I and J) groups show the reduced progress of *Fusarium* infection, where the depth of hyphal penetrations appears to be less when compared to the I and IX groups. Blue arrows indicate to the deepest level of penetration at which *Fusarium* hyphae could be seen in the figure. C: Control cornea; I: Inoculated only; IX: Inoculated then cross-linked with isotonic riboflavin with epithelium-off; ITEX: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-on; ITEX-OF: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-off.
Figure 4-5: The percentage of the total corneal depth penetrated by the *Fusarium* hyphae for the inoculated groups.

The graph shows the *Fusarium* hyphal penetration depth at the central and peripheral corneal regions of the inoculated groups at 72h post-inoculation and 24h post-PACK-CXL treatment. The I group had the greatest penetration depth by *Fusarium* hyphae at both central and peripheral corneal regions, followed by IX group, while the ITEX group had the lowest *Fusarium* hyphal penetration depth compared to the other groups. I: Inoculated only group; IX: Inoculated then cross-linked with isotonic riboflavin with epithelium-off; ITEX: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-on; ITEX-OF: Inoculated then cross-linked with trans-epithelial riboflavin with epithelium-off.
4.5 Discussion

Corneal cross-linking procedure has been recommended as a treatment for the management of infectious keratitis (Iseli et al., 2008, Panda et al., 2012, Hafez, 2014, Said et al., 2014). Standard PACK-CXL procedure relies on using the combined isotonic riboflavin and UV-A light, and has a beneficial antimicrobial effect which assists in controlling corneal infections. Alteration to the standard protocol of PACK-CXL, such as using a trans-epithelial riboflavin which removes the need to debride the epithelium pre-PACK-CXL, have been introduced where it showed a beneficial therapeutic effect of PACK-CXL in the management of fungal keratitis (Hafez, 2014).

The present study shows that PACK-CXL treatment using trans-epithelial riboflavin has a significantly higher impact on controlling Fusarium infection when compared to the standard PACK-CXL using isotonic riboflavin. The standard PACK-CXL procedure used for treatment of IX group in this study resulted in a considerable decrease, but not significant, in the volume of fungal hyphae and the number of spores present in the culture medium compared to the un-treated group (I). The groups treated with trans-epithelial PACK-CXL procedure (ITEX and ITEX-OF) showed a significant reduction in the volume of fungal hyphae and the number of spores when compared to un-treated group (I), and a considerable decrease in the number of spores when compared to the group treated with standard PACK-CXL (IX).

The use of trans-epithelial riboflavin would negate the need to debride the corneal epithelium prior to PACK-CXL procedure which reduces the side effects caused by removing the epithelium. Trans-epithelial riboflavin enhances epithelial penetration
and allows stromal diffusion (Kissner et al., 2010, Barbara et al., 2012, Hayes et al., 2016). The trans-epithelial riboflavin formulation used in this study contains 0.01% of Benzalkonium Chloride (BAC) as recommended by Raiskup et al. (2012). BAC is a chemical substance and one of the most widely used preservative agents in medicine. It has been reported that BAC has a toxic effect on the corneal epithelium (Debbasch et al., 2000, Cha et al., 2004). It is possible that it could enhance the antimicrobial effect of PACK-CXL in the treatment of corneal infections.

Debriding the epithelial layer prior to performing the PACK-CXL procedure would assist in removing a large quantity of the infectious agents. However, the surviving fungal spores can germinate and new hyphae can then re-invade and grow further within the exposed stromal tissues after removing the epithelium. Hence, removing the need to debride the corneal epithelium in the trans-epithelial PACK-CXL procedure would help to impede the progression of the infection as the intact epithelium may work as a protective barrier to invasion of the deep corneal structures. In this study it was found that removing the epithelium before the PACK-CXL treatment in the ITEX-OF group reduced the spore count within the growth medium; conversely, by leaving the epithelium intact before treatment the volume of fungal hyphae within the stromal tissues in the ITEX group was reduced.

At 72h post-inoculation, an average of 99% of the corneal thickness was penetrated by Fusarium hyphae in the un-treated group (I). However, in the treated groups with trans-epithelial PACK-CXL (ITEX and ITEX-OF) the Fusarium hyphal penetration depth within central corneal tissues (exposed to UV-A light) was reduced to average of 22% and 30%, respectively; whereas it was reduced to an average of 77% in the group
treated with standard PACK-CXL procedure. This effect shows a significant advantage of the trans-epithelial PACK-CXL over the standard PACK-CXL procedure in suppressing the progression of fungal infection into the corneal tissue.

In addition, in the two groups treated with trans-epithelial PACK-CXL the hyphal penetration depth at the corneal periphery was considerably less compared to that seen after standard PACK-CXL treatment. The diameter of the central corneal region which was irradiated by UV-A light was approximately 9 mm, which means that the *Fusarium* agents at the peripheral cornea would not be affected by the UV-A light. Therefore, in this case the inhibition of hyphal growth at the periphery of the cornea could be attributed to the inhibitory effect of the trans-epithelial riboflavin on the fungal agents rather than to the combined riboflavin/UV-A light-induced corneal cross-linking. Removing the need to debride the corneal epithelium in the trans-epithelial PACK-CXL treatment would further limit the spread of corneal infections due to the intact epithelium protecting the stroma from new spores’ invasion and hyphal penetration. It is noteworthy that the hyphal penetration depth measurement in the peripheral cornea has not been taken perpendicularly to the corneal surface due to corneal curvature. As a result, the measured depth may not equal the actual depth of penetration.

Despite the overall reduction in hyphal penetration depth, both at the centre and periphery of the cornea in the treated groups there are large variations in the percentages of the corneal thickness penetrated by *Fusarium* hyphae. In addition to the variability in hyphal penetration depth in all three treated groups there was significant penetration within corneal tissue. Hence, PACK-CXL treatment alone, with
either of the two riboflavin formulations, cannot be considered as an effect therapy in controlling the *Fusarium* infections. In the literature, there is variation in the outcomes obtained by the clinical studies on the effect of PACK-CXL treatment in the management of corneal infections caused by fungi. While some clinical studies demonstrated a beneficial effect for PACK-CXL in controlling the fungal keratitis (Iseli et al., 2008, Li et al., 2013, Hafez, 2014), others were consistent with the present study and stated the limited efficacy of PACK-CXL procedure in the management of fungal infections (Escarião et al., 2013, Uddaraju et al., 2015, Vajpayee et al., 2015). This variability in the effect of PACK-CXL treatment in the management of fungal keratitis may due to stage of fungal infection when the PACK-CXL was performed in the different studies, as once the fungal hyphae have reached the periphery of the cornea they will not be supressed by the PACK-CXL treatment.

The present study utilised a human corneal infection model, which mimics *in vivo* human fungal keratitis cases more closely than in previous work (Alshehri et al., 2016). This was achieved by introducing some modifications to the previous *ex vivo* human *Fusarium* infection model, such as using a lower concentration of *Fusarium* spores for inoculating the corneas (1x10⁵ spores/ml), along with longer incubation periods (48 hours). The previous *Fusarium* keratitis model was initially designed using an inoculum of 5,000 *Fusarium* spores per inoculated cornea. This number of spores is far greater than would be needed to initiate a clinical infection. Therefore, the fungal infection that occurred 24h post-inoculation for that model was often far greater than would be observed 24h post-infection in patients. On the other hand, despite the greater fungal load in the previous model compared to *in vivo* cases, the 24 hours infection time was
quite brief before the PACK-CXL treatment; whereas in patients it would take longer to seek and receive treatment. Thus, the spread of infection would be incomparable as the *Fusarium* infection observed in the previous model was comprised by many small *Fusarium* hyphae rather than the few mature ones that would be observed in clinics. The results obtained in this study by applying the standard PACK-CXL in the IX group to control the *Fusarium* infection are not in line with those obtained by Alshehri et al. (2016). Failure of the standard PACK-CXL procedure to suppress the *Fusarium* infection in this study may due to several factors: the longer infection time in this study which may allow the *Fusarium* hyphae to grow and mature prior to the PACK-CXL treatment. The mature slower growing hyphae may be harder to eradicate, and they are thought to have a larger surface area over which to secrete digestive enzymes, and likely produce more proteases. This would change the collagen structures, and thus, change the effectiveness of the PACK-CXL treatment as a disorganised matrix may reduce or block UV-A penetration. Another factor to consider is the treated corneas were continually prone to attacks by the fungal spores existing in the corneal culture medium, which were able to cause new fungal infections. In addition, although the *Fusarium* spore concentration has been reduced in this study to $1 \times 10^5$ spores/ml, and thus, the number of spores used for inoculating each cornea was around 1,000 spores, this number of spores is still far greater than those in the *in vivo* cases: infection in patients can result from only a single spore. Consequently, the *Fusarium* infection developed at 48h post-inoculation and pre-PACK-CXL in this study was also larger than would be observed at 48h post-infection in patients.
4.6 Conclusion

The standard PACK-CXL treatment suppresses the growth of *Fusarium* hyphae and sporulation, but is not as effective as trans-epithelial PACK-CXL treatment. However, neither standard nor trans-epithelial PACK-CXL treatments alone are effective in fully controlling the *Fusarium* infection.
Chapter 5

Does Increasing the Corneal Biomechanical Stiffness Through Cross-linking Influence the Severity of Subsequent Corneal Fungal Infection?

Contributions

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross.
Submission for Publication

The chapter is prepared as a manuscript which will be submitted for publication.


Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.
- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the Fusarium strain.
- Dr Peter March, Dr Steven Marsden and Mr Roger Meadows from the Bioimaging Facility for their help with the confocal microscopy and Imaris software.
5.1 Abstract

**Aims:** To evaluate the effect of stiffened corneal biomechanical properties produced by pre-treating *ex vivo* human corneas with corneal cross-linking (CXL) procedure, with both an intact epithelium or with the epithelium removed, in resisting the spread of *Fusarium* infection.

**Methods:** Twelve *ex vivo* human corneas were divided into three groups: inoculated with *Fusarium oxysporum* (I) only, cross-linked following the standard protocol using isotonic riboflavin/UV-A and epithelium-off (debrided) protocol, then inoculated (XI), cross-linked using trans-epithelial riboflavin/UV-A and epithelium-on (intact) protocol, then inoculated (TEXI). The XI and TEXI groups were cross-linked seven days pre-inoculation, and then the corneas were inoculated with *Fusarium* spores. Corneas were imaged by confocal microscopy at 3 days post-inoculation, and *Fusarium* hyphal volume and spore concentration were calculated. Corneas were fixed, sectioned, haematoxylin and eosin stained, imaged, and the hyphal penetration depth within stromal tissue measured.

**Results:** There was no significant difference in the hyphal volume among the different inoculated groups (*P* = 0.371). There was a significant inhibition of *Fusarium* sporulation in XI group (*P* = 0.047) compared to I group. There is a significant reduction in the hyphal penetration depth in TEXI at central corneal region (*P* = 0.022) and at peripheral corneal region (*P* = 0.039) compared to I. There is a significant reduction in the hyphal penetration depth at the periphery compared to the centre within XI group (*P* = 0.034).
**Conclusion:** Pre-treatment with epithelium-on CXL seems to be effective in reducing the *Fusarium* hyphal penetration depth within corneal tissue. However, both epithelium-on and -off CXL alone are not adequate to prevent the development of the *Fusarium* infection.
5.2 Introduction

Infectious keratitis caused by fungi has potentially disastrous visual consequences (Srinivasan, 2004). Combined riboflavin and ultraviolet A-light-induced corneal cross-linking (CXL) was initially introduced to treat corneal ectasia (Wollensak et al., 2003a). Corneal cross-linking procedure increases the cross-links between the stromal collagen fibrils and, consequently, leads to around 300 % rise in the biomechanical rigidity of the human cornea (Wollensak et al., 2003b). Recently, it has been suggested that corneal cross-linking could be a valuable approach for treating fungal keratitis (Li et al., 2013, Alshehri et al., 2016). The beneficial impact of the cross-linking procedure in the management of infectious keratitis may be due to increasing the stiffness of bonds within the stromal collagen fibrils (Wollensak et al., 2003b, Kohlhaas et al., 2006, Wollensak, 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016) which, in turn, increases the resistance to enzymatic digestion (Spoerl et al., 2004). In addition, it has been established that the combined riboflavin/UV-A light has an antimicrobial effect which helps in suppressing the growth of pathogen thus controlling infection progression (Iseli et al., 2008, Li et al., 2013, Alshehri et al., 2016).

During the CXL process the role played by riboflavin is a crucial one as it involves the absorption of the UV-A resulting in the production of reactive oxygen species (ROS) (Baier et al., 2006). On the other hand, riboflavin also plays the role of a filter offering protection to the underlying tissue from the possible undesirable effects of UV-A light (Kymionis et al., 2009). The failure of the corneal cross-linking process could arise from
insufficient stromal absorption and penetration of the riboflavin (Wollensak and Iomdina, 2009a). Corneal epithelium is the primary barrier to the environment which plays a fundamental role in protecting the corneas from the invading pathogens (DelMonte and Kim, 2011). The standard CXL protocol involves removing the epithelium prior to application of the standard isotonic riboflavin solution (Wollensak et al., 2003a) in order to facilitate its penetration into the stroma (Hayes et al., 2008). The epithelial debridement step in this protocol may result in complications including corneal infections post-CXL treatment (Pollhammer and Cursiefen, 2009). In order to have the benefits of CXL treatment and overcome the associated complications of epithelial removal, some modifications to the standard CXL protocol have been made to allow a trans-epithelial cross-linking procedure (Kissner et al., 2010, Barbara et al., 2012, Hayes et al., 2016). Trans-epithelial CXL is based on enhancing riboflavin penetration and allowing stromal diffusion without a need to remove the epithelial layer. One of the proposed methods to facilitate the riboflavin penetration through the epithelium is using a trans-epithelial riboflavin solution instead of the conventional isotonic solution. It has been demonstrated that epithelium-on CXL using trans-epithelial riboflavin has similar effectiveness in stiffening the cornea to epithelium-off CXL approach (Filippello et al., 2012, Magli et al., 2013). However, it has been reported that trans-epithelial cross-linking reduced the biochemical stiffening effect to around 20% of that found using the standard cross-linking protocol (Wollensak and Iomdina, 2009a). The reason behind the reduced effect of trans-epithelial CXL could possibly be linked to the heterogeneous and/or inadequate diffusion of the trans-epithelial riboflavin into the stroma (Aldahlawi et al., 2016).
The aim of this study was to evaluate the effectiveness of CXL procedure in reducing the spread and severity of corneal fungal infections that develop subsequently and to assess if the increased biomechanical stiffness of the cornea has a protective effect against the development of subsequent fungal infections.

5.3 Materials and Methods

5.3.1 Ex Vivo Human Corneas

Twelve healthy post-mortem human corneal buttons from 7 male and 5 female donors in the age range 35 – 94 years old (mean ± SD, 72.4 ± 15.5 years) were used in this study. The corneas were provided by the Manchester Eye Bank (NHS Blood and Transplant, UK), and pre-consented for research. The project had full ethical approval from the Manchester University Research Ethics Committees (No. 040811) and followed the terms of the Declaration of Helsinki.

The corneas were randomly assigned to one of the three groups with four corneas per group as detailed below:

- I: Inoculated with *Fusarium oxysporum* spores, not treated with CXL procedure (61-94 years old, mean ± SD, 76.5 ± 13.5 years).

- XI: Treated first with CXL procedure, following the standard protocol: using the conventional isotonic riboflavin and epithelium-off (debrided) approach, and then inoculated by *Fusarium oxysporum* spores with no further treatment for any potential *Fusarium* infection that may develop (61-85 years old, mean ± SD, 71 ± 10.9 years).
• TEXI: Treated with CXL procedure, using trans-epithelial riboflavin solution and epithelium-on (intact) approach, and then inoculated by *Fusarium oxysporum* spores with no further treatment (35-85 years old, mean ± SD, 69.8 ± 23.4 years).

### 5.3.2 Corneal Tissue Culture

The corneas were transferred into 6-well cell culture plates (Costar®; Corning Ltd, Ewloe, UK), and submerged in fresh culture medium (Dulbecco’s Modified Eagle’s Medium, DMEM, 5000mg Glucose/L; Sigma-Aldrich Ltd, Poole, UK) supplemented with 2mM Glutamine, 10% (v/v) foetal bovine serum (FBS; Labtech International Ltd, Uckfield, UK), and 1% (v/v) Penicillin with Streptomycin (all Sigma-Aldrich Ltd, Poole, UK). The corneas were incubated at 34°C for a minimum of 3 days prior to the CXL treatment.

### 5.3.3 CXL Procedure

The corneal buttons in the XI and TEXI groups were subjected to the CXL procedure at 7 days pre-inoculation with *Fusarium* spores (Figure 5-1). The corneas in the XI group were treated with the CXL technique according to the standard CXL protocol (Wollensak et al., 2003a). For this group, each cornea was mounted on the artificial anterior chamber (Moria SA, 92160 Antony, France; SD Healthcare, Manchester, UK), the interior was filled with sterile Phosphate Buffered Saline (PBS) to mimic the *in vivo* intraocular pressure and maintain corneal stability during the CXL procedure, the epithelium-off approach was performed by debriding the central 7-9 mm diameter area, isotonic riboflavin eye drops (Riboflavin 5’-phosphate sodium salt hydrate,
R7774, Sigma-Aldrich, UK), (0.1% (w/w) riboflavin, 20% (w/w) dextran T500) were dropped onto the corneal surface at 5 minute intervals for a total of 30 minutes, and then, cornea was irradiated with ultraviolet-A light using a UV-A light emitter (VEGA, C.S.O. srl, Florence, Italy) (370 nm, irradiance of 3mW/cm², UV-A irradiation dose of 5.4 J/cm²) for 30 minutes, along with application of isotonic riboflavin at 5 minute intervals. The corneas in the TEXI group were treated with the CXL technique as detailed above for XI group except that an epithelium-on approach was followed with the isotonic riboflavin solution replaced by trans-epithelial riboflavin solution (0.1% riboflavin in 0.44% Sodium Chloride (NaCl) (Sigma-Aldrich Ltd, Poole, UK) with 0.01% Benzalkonium Chloride (BAC) (Sigma-Aldrich Ltd, Poole, UK).

Following the CXL procedure, the corneas were rinsed with PBS, placed in a new 6-well tissue culture plate, provided with fresh culture medium daily and incubated at 34°C for 7 days prior to the inoculation process.

5.3.4 Fungal Strain

The fungus used for infection was *Fusarium oxysporum* f. sp. *lycopersici* strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP). The strain was kindly provided by Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) (Di Pietro et al., 2001). The *F. oxysporum* GFP strain was cultured, spore (microconidia) suspensions were prepared and spore concentration adjusted to 1x10⁵ spores per ml using fresh culture medium; 10 µl of this suspension was used to inoculate each cornea in the I, XI and TEXI groups.
Figure 5-1: Main experimental procedures along with timeline.

The flow chart shows the main procedures performed on the different corneal groups: CXL treatment, scratching, inoculations, imaging and H&E staining. I: Inoculated only; XI: Cross-linked with isotonic riboflavin and epithelium-off then inoculated; TEXI: Cross-linked with trans-epithelial riboflavin and epithelium-on then inoculated; TE-CXL: Trans-epithelial cross-linking; d: Day.
5.3.5 Corneal Infection Process

Seven days post-CXL procedure, each corneal button from the three study groups, I, XI and TEXI, was mounted on an artificial anterior chamber, and the anterior surface was scratched in the central region of the cornea (approximately 7 mm scratch diameter) to assist the *Fusarium* invasion using a 0.6X25 mm-gauge needle (BD Microlance 3; BD Biosciences, Oxford, UK). The corneas were transferred into a fresh 6-well cell culture plates with the posterior surface of the cornea uppermost. The posterior corneal cavity was filled with an agar-gelatine gel (2% (w/v) agar (Oxoid, Nottingham, UK) and 2% (w/v) gelatine (BD Difco, Oxford, UK), supplemented with 7.4% (w/v) Sodium Bicarbonate (NaHCO₃) (Sigma-Aldrich Ltd, Poole, UK) and fresh culture medium) pre-inoculation process, as an attempt to minimize the fungal invasion of the lower corneal layers via spores in the culture medium. The molten agar-gelatine gel was allowed to cool at room temperature for few minutes, and then the corneas were inverted with the anterior surface of the cornea uppermost. Finally, the corneas were inoculated with the *Fusarium* spore suspension. A small volume of the fresh culture medium was applied at the corneal periphery, and the corneas were incubated at 34°C for 3 days prior to the imaging by confocal microscopy.

5.3.6 Imaging and *Fusarium* Spore Concentration

At 10 days post-CXL (3 days post-inoculation), the central region of each cornea was imaged using a Leica TCS SP5 AOBS inverted confocal microscope (Leica Microsystems Ltd., Breckland, Linford Wood, Milton Keynes, UK) with a x20 objective. The confocal images were analysed with the Surpass module of the Imaris v8.4 software (Bitplane Scientific software module; Bitplane AG, Zurich, Switzerland). For each cornea the
volume occupied by the *Fusarium* hyphae within anterior stromal tissues was automatically quantified in a tiled z-stack of 100 µm thickness by the software and displayed in an excel sheet, and then, the percentage of the total *Fusarium* hyphal volume was calculated for each inoculated group. At 3 days post-inoculation, the number of *Fusarium* spores per ml of corneal culture medium was counted for all inoculated corneas.

### 5.3.7 Corneal Tissue Processing and Haematoxylin and Eosin (H&E) Staining

After confocal imaging and spores counting, the corneal buttons were dissected diametrically into halves. One half of each cornea was fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C overnight followed by 72h incubation in PBS-sucrose, embedded in OCT (KP-CryoCompound, Klinipath BV, The Netherlands) over liquid nitrogen, and sectioned at 30 µm thickness using a Bright OTF cryostat (Bright Instrument Company, Huntingdon, UK). The other half of each cornea was fresh-frozen in OCT and stored at -80 °C for future use. The fixed corneal sections were haematoxylin and eosin (H&E) stained (Harris’ Haematoxylin and Eosin Y solutions, Sigma-Aldrich, UK), and images of the central and peripheral views of the H&E stained sections captured at x10 magnification using a Zeiss Axiostar Plus HBO 50 microscope (Zeiss UK, Cambridge, UK), Micropublisher 5.0 RTV camera and Q-CapturePro7TM Software (both Media Cybernetics UK, Buckinghamshire, UK). The total corneal thickness and the depth of *Fusarium* hyphal penetration within the stromal tissues at the central and peripheral regions were semi-automatically measured (µm) for all corneas using ImageJ software (Schneider et al., 2012). The percentage of the total
corneal depth penetrated by the *Fusarium* hyphae was calculated for each inoculated cornea.

### 5.3.8 Statistical Analysis

The analysis of *Fusarium* hyphal volume, spores concentration and hyphal penetration depth within corneal tissues for I, XI and TEXI groups was performed using a one-way analysis of variance (ANOVA) and *post hoc* tests.

### 5.4 Results

Inoculation of the corneas with *Fusarium oxysporum* spores in the I, XI and TEXI groups resulted in development of *Fusarium* infection. At 10 days post-CXL (3 days post-inoculation), the *Fusarium* infection, which can be seen as branching, green fluorescent fungal hyphae in Figure 5-2, had the greatest progression in the XI cornea (Figure 5-2B), followed by the I cornea (Figure 5-2A), whereas the TEXI cornea had the least progression of the infection (Figure 5-2C).

The percentages of the total *Fusarium* hyphal volume within corneal tissues at 10 days post-CXL (3 days post-inoculation) for the three groups are shown in Figure 5-3A. The XI group showed the most severe *Fusarium* infections, which is demonstrated by greater volumes of *Fusarium* hyphae compared to the other groups; whereas the TEXI group showed less severe infection compared to the other groups. ANOVA of the *Fusarium* hyphal volume data showed that there is no significant difference among the groups (*P* = 0.371).
The *Fusarium* spore concentrations present in the culture medium at 3 days post-inoculation for the three groups are shown in Figure 5-3B. It can be seen that there is a variation in the quantity of *Fusarium* spores between the un-treated I group and treated XI and TEXI groups. The I group showed the greatest spore concentrations in the growth media compared to the two treated groups. ANOVA of the fungal spore concentration data showed an overall significant difference in *Fusarium* spore concentration among the different inoculated groups ($P = 0.032$). Post hoc tests (Tukeys) showed that there is a significant difference between I and XI groups ($P = 0.047$), but the difference between I and TEXI groups did not reach significance ($P = 0.06$).

Images of the H&E stained corneal sections for corneas from inoculated groups are shown in Figure 5-4. The stromal tissue is stained pink, and the corneal epithelial layers, keratocytes and *Fusarium* hyphae are stained dark purple. Figures (5-4A and 5-4B) and (5-4C and 5-4D) show the central and peripheral views of the H&E stained corneal sections from the I and XI groups, respectively. At 3 days post-inoculation dense *Fusarium* colonies have accumulated over the corneal surface and fungal hyphae have invaded the tissues and penetrated deep into the corneal stroma. Figures 5-4E and 5-4F show central and peripheral views of the H&E stained corneal sections from the TEXI group. It can be seen that the density of the *Fusarium* hyphae on the corneal surface and within the stromal tissues in the TEXI group is lower compared to that in the I and XI groups.

Figure 5-5 shows the percentage of the total corneal depth penetrated by the *Fusarium* hyphae at the central and peripheral regions for all three groups. It can be
seen that the I group had the deepest penetration of hyphae in both the central and peripheral regions; whereas the TEXI group had the lowest hyphal penetration depth compared to the other groups. There is also a variation between the treated XI and TEXI groups in terms of the central infection versus the peripheral infection. ANOVA of the hyphal penetration depth showed that there were overall significant differences in the percentage of the corneal depth penetrated by the fungal hyphae in the central region ($P = 0.024$), and in the peripheral region ($P = 0.033$) between the different inoculated groups. Post hoc test (Tukeys) showed significant differences in the percentage of the penetration depth of fungal hyphae between the I and TEXI groups at the central corneal region ($P = 0.022$), and at the peripheral corneal region ($P = 0.039$). In terms of the central infection versus peripheral infection, there is a significant difference between centre and periphery of the XI group ($P = 0.034$).
Confocal microscopy images of inoculated corneas at 10 days following various preventative CXL treatments (3 days post-inoculation).

Confocal images of I, XI and TEXI corneas showing the extent of the branching green fluorescent fungal hyphal growth at 10 days post-CXL (3 days post-inoculation). Each 2D image is a projection of a tiled z-stack (2x2 stacks, each stack is 775x775x100 µm) of confocal images (x20 magnification), which were captured from the anterior central corneal region. The most extensive *Fusarium* growth is visible in the XI group (B), while the *Fusarium* growth in the TEXI group (C) is less severe compared to the other inoculated groups. I: Inoculated only; XI: Cross-linked with isotonic riboflavin and epithelium debrided then inoculated; TEXI: Cross-linked with trans-epithelial riboflavin and epithelium intact then inoculated.
Figure 5-3: The percentage of the *Fusarium* hyphal volume and the concentration of the *Fusarium* spore for the different groups.

The graphs show the percentage of the total volume occupied by the fungal hyphae within the anterior 100 µm of stromal tissues (A) and the number of *Fusarium* spores per ml of corneal culture medium (B) for the I, XI and TExI groups at 10 days after
different preventative CXL treatments (3 days after inoculation). The TEXI group had
the least *Fusarium* hyphal volume compared to the I and XI groups; whereas the XI
group had the greatest *Fusarium* hyphal volume compared to the other groups. The
treated XI show significant decrease in the number of *Fusarium* spores compared to
the un-treated I group, whereas the difference between TEXI and I groups did not
reach significance ($P = 0.06$). I: Inoculated only; XI: Cross-linked with isotonic riboflavin
and epithelium debrided then inoculated; TEXI: Cross-linked with trans-epithelial
riboflavin and epithelium intact then inoculated.
Figure 5-4: H&E stained corneal sections from inoculated groups.

The figure shows the central and peripheral corneal regions of the H&E stained sections at x10 magnification from different study groups at 10 days post-CXL treatment (3 days post-inoculation). The H&E stained sections of corneas from the I (A and B) and XI (C and D) groups show heavy infection, large fungal colonies and deep penetration of Fusarium hyphae within the stroma. The H&E stained sections of cornea from TEXI (E and F) group show the Fusarium hyphal penetration, which appears to be deep at both the centre and periphery of the cornea, but the density of the Fusarium colonies seems lower compared to those in the I and XI groups. The blue arrows
indicate the deepest level of penetration at which *Fusarium* hyphae could be seen in the images. I: Inoculated only; XI: Cross-linked with isotonic riboflavin and epithelium debrided then inoculated; TEXI: Cross-linked with trans-epithelial riboflavin and epithelium intact then inoculated.

**Figure 5-5:** The percentage of the total corneal depth penetrated by the *Fusarium* hyphae for the inoculated groups.

The graph shows the *Fusarium* hyphal penetration depth at the central and peripheral corneal regions of the I, XI and TEXI groups at 10 days post-CXL treatment (3 days post-inoculation). The I group had the greatest depth of penetration by *Fusarium* hyphae in both central and peripheral corneal regions, followed by XI group, while the TEXI group had the lowest *Fusarium* hyphal penetration depth compared to the other groups. I: Inoculated only; XI: Cross-linked with isotonic riboflavin and epithelium debrided then inoculated; TEXI: Cross-linked with trans-epithelial riboflavin and epithelium intact then inoculated.
5.5 Discussion

This study aimed to assess the beneficial impact of epithelium-on (intact) and epithelium-off (debrided) CXL in terms of protecting the cornea and preventing, and/or reducing, the severity of subsequent infections caused by *Fusarium oxysporum*. The antifungal contribution of the combined riboflavin/UV-A light in suppressing the *Fusarium* keratitis was not assessed in this study, as the CXL procedure was carried out before inoculation of the corneas with fungal spores. Consequently, the role of the CXL technique in terms of increasing the stromal resistance against fungal invasion is the focus of this study.

It has been demonstrated in previous published studies that epithelium-on CXL using trans-epithelial riboflavin has similar effectiveness in stiffening the cornea to epithelium-off CXL approach (Filippello et al., 2012, Magli et al., 2013). Nonetheless, some studies reported less apparent effects for epithelium-on CXL (Wollensak and Iomdina, 2009a, Bottós et al., 2011, Kocak et al., 2014, Aldahlawi et al., 2016). In this study, the XI group was treated following the standard corneal cross-linking and epithelium-off protocol. As the epithelial layer is the most important protective barrier against the pathogens, the corneas were placed in corneal culture medium post-CXL procedure for one week as an attempt to allow re-epithelialisation prior to the inoculation process. The TExI group was treated with trans-epithelial cross-linking following epithelium-on protocol and using trans-epithelial riboflavin. The trans-epithelial riboflavin formulation used in this study contains 0.01% of Benzalkonium Chloride as is recommended by Raiskup et al. (2012). The results obtained from confocal imaging show that there were no significant differences in the *Fusarium*
hyphal volume among the different inoculated groups. This indicates that pre-treating the un-inoculated corneas with the CXL procedure prior to the infection is ineffective in terms of protecting the corneas and preventing subsequent infections caused by fungus. Moreover, the XI group had a greater volume of *Fusarium* hyphae than I and TEXI groups. This is probably due to the fact that the epithelial layer was not regenerated successfully during the one week incubation period post-CXL and pre-inoculation. This meant that the stroma was exposed to invasion by the fungus, and consequently, enhanced the growth of *Fusarium* and increased the intensity of the infection. However, the confocal images are inadequate to assess the depth of *Fusarium* hyphal penetration within stromal tissue. Therefore, the H&E staining technique was used for further investigation and to establish if the *Fusarium* hyphal growth is extensive only on the corneal surface. The H&E staining results show a reduction in the depth of penetration by the *Fusarium* hyphae in the central and peripheral regions of the XI and TEXI groups in comparison to the I group. The reduction in the depth of hyphal penetration was significant in the TEXI group compared to the I group. This indicates a potentially beneficial effect of the CXL procedure for corneal stability in suppressing the spread of *Fusarium* infection accompanied by the substantial role that a healthy epithelial layer plays in protecting the corneas from the invading fungus. Nevertheless, the present results show that the treated XI and TEXI groups had greater penetration depth by the *Fusarium* hyphae in the central region, which was exposed to UV-A light, compared to the un-irradiated peripheral region, and the difference in the fungal penetration depth within XI group was significant. These observations were unexpected, and could possibly be owing to the corneal defects produced pre-inoculation by scratching to assist the fungal
invasion of the corneas at the central region which probably increased the capability of fungal invasion and hyphal penetration through the stromal tissues at the scratched central regions. In addition, epithelial layer debridement in the XI is possibly the reason for making the difference significant within this group. However, it is noteworthy that the hyphal penetration depth measurement in the peripheral cornea has not been taken perpendicularly to the corneal surface due to corneal curvature. As a result, the measured depth may not equal the actual depth of penetration. Nonetheless, changed collagen structure following the CXL procedure might reduce the Fusarium hyphal movement across the cornea allowing the spread of infection downward under the inoculation site instead of laterally, and thus, reduced the stromal penetrability by fungal hyphae at the periphery. For fungi to move, they secrete proteases which infiltrate between the collagens and break them down, and thus, allow the fungal hyphae to move further and penetrate within corneal tissues. After the CXL treatment, the cross-links pull the collagens close together which would block fungal enzymes infiltration between collagens which would disrupt hyphal movement. However, it was shown in a previous study that the changes in the corneal structure post-cross-linking are due to changes in the hydration of corneal tissues rather than being an outcome of the combined riboflavin/UV-A light-induced corneal cross-linking per se (Hayes et al., 2011).

The results showed that the XI and TEXI groups had a significant decrease in the number of Fusarium spores present in the culture medium at 10 days post-CXL treatment (3 days post-inoculation) compared with the I group. As the direct antifungal activity of combined riboflavin/UV-A light was not applicable in this study,
inhibition of *Fusarium* sporulation perhaps resulted from enhancing resistance of the corneal stroma to digestion by enzymes following CXL treatment, and entrapment of the fungal agents by the cross-linked stromal collagen, which resulted in restricting the rate of spore release.

Corneal cross-linking following the epithelium-on protocol showed a more beneficial effect over the epithelium-off protocol in term of reducing the depth of *Fusarium* penetration within corneal tissues. The depth of *Fusarium* penetration observed within stromal tissues of treated corneas in both groups, XI and TEXI, is relatively deep. It is likely that the *Fusarium* hyphae would continue to penetrate more deeply within corneal tissues over time, due to the limited effects of CXL treatment in the anterior region of the cornea, and then, invade the deep corneal structure. Consequently, the findings in this study indicate that the increase of stiffness of the stromal collagen alone with combined riboflavin/UV-A light does not lead to control the *Fusarium* infection, regardless of which of the two CXL protocols are used.

5.6 Conclusion

Increasing the stromal tissue stiffness by pre-treating the cornea with epithelium-off or epithelium-on UV-A cross-linking seems insufficient for controlling subsequent development of *Fusarium* infection; although the epithelium-on CXL is effective in decreasing the fungal hyphal penetration depth within corneal tissue.
Chapter 6

Corneal Cross-linking in the Management of *Fusarium* Keratitis:

Effect of Timing and Repeated Treatment

Contributions

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross. Prof. Fiona Carley regularly treats patients with infectious keratitis in the Manchester Royal Eye Hospital. Prof. Carly met with the research team on a regular basis to discuss ideas and progress of the research studies.
Submission for Publication

The chapter is prepared as a manuscript which will be submitted for publication.


Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.
- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the Fusarium strain.
- Dr Peter March, Dr Steven Marsden and Mr Roger Meadows from the Bioimaging Facility for their help with the confocal microscopy and Imaris software.
6.1 Abstract

**Purpose:** Corneal cross-linking (PACK-CXL) procedure has been used for the management of un-responsive corneal infections with some success. This study aims to investigate the impact of PACK-CXL treatment in the management of different severity of *Fusarium* infections by evaluating the effect of timing of intervention and repeat treatment.

**Method:** Thirty-two *ex vivo* human corneas were divided into 8 groups: C: Control; X: Cross-linked; I: Inoculated with *Fusarium oxysporum* spores; I-EOF: Inoculated, epithelium debrided; I-24h-X: Inoculated, cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated, cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated, cross-linked at 48h post-inoculation; I-72h-X: Inoculated, cross-linked at 72h post-inoculation. Corneas were imaged daily with confocal microscopy post-inoculation over a 7-day experiment time, and *Fusarium* hyphal volume and spore concentration were calculated. Corneas were sectioned, haematoxylin and eosin stained, imaged, and the hyphal penetration depth within stromal tissue measured.

**Results:** Timing and repeating of the PACK-CXL procedure had a significant effect on the fungal hyphal volume and *Fusarium* sporulation over the duration of the experiment ($P < 0.001$), and by the end of the experiment on day 7 ($P < 0.001$). The early/single intervention with PACK-CXL treatment resulted in a considerable reduction in the load of fungal infection in the I-24h-X group, while the early/twice intervention with PACK-CXL resulted in a significant reduction in the load of infection in the I-24h-36h-X group compared to the late/single-treatment I-48h-X and I-72h-X groups. The
percentage of total hyphal penetration depth within corneal tissues on day 7 was 100% in all treated groups.

**Conclusion:** Early timing and repeating of the PACK-CXL procedure has a significant effect in suppressing the growth of fungal hyphae and *Fusarium* sporulation, and thus, reducing the load of the infection. However, using the PACK-CXL procedure alone appears insufficient in reducing the depth of hyphal penetration into the corneal tissues and controlling the progression of the infection. Therefore, the PACK-CXL procedure would work better in a dual therapy rather than as a monotherapy.
6.2 Introduction

Infectious keratitis is an ophthalmic emergency with a heightened risk of vision loss (Otri et al., 2013). Broad-spectrum topical antimicrobials are often used to manage majority of infectious keratitis cases (Thomas and Geraldine, 2007, Gilmore et al., 2010). However, the appearance and proliferation of organisms that have the ability to resist antimicrobial agents presents a clinical and public health challenge (Neu, 1992, DeMuri and Hostetter, 1995). Fungal keratitis is one of the most severe corneal infections, which should be treated immediately in order to reduce the risk of permanent vision loss (Srinivasan, 2004). Antifungal agents such as topical antifungal drops are the primary means used in the management of fungal keratitis (Qiu et al., 2015). Corneal infection usually facilitates the inflammatory state, which is often responsible for the melting and degradation of the cornea (Gilger et al., 2007). Corneal melting is a serious condition which usually happens in advanced corneal infection as a result of the upregulation of certain proteinases (Said et al., 2014). It is induced by the release of collagenolytic matrix metalloproteinase (MMP), and an imbalance between these proteolytic enzymes and the proteinase inhibitors, which are found in the cornea and tear film (Ollivier et al., 2007). Advanced cases of fungal keratitis, including corneal melting, tend to be extremely challenging to manage and can lead to a total loss of vision (Srinivasan, 2004, Kredics et al., 2015). As a result, there is a need for an effective antifungal therapy that could control the progression of fungal infections, and prevent associated corneal melting.

Corneal cross-linking technique utilising combined riboflavin/UV-A light (CXL) introduces additional cross-links in the corneal stroma and increases the corneal
stiffness (Wollensak et al., 2003b, Kohlhaas et al., 2006, Wollensak, 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2013c, Beshtawi et al., 2014, Beshtawi et al., 2016). These cross-links are formed between the collagen and the proteoglycans of the extracellular matrix (ECM) (Zhang et al., 2011) and/or within the proteoglycan at the collagen fibril surface (Hayes et al., 2013). The corneal cross-linking technique is re-termed photoactivated chromophore corneal cross-linking (PACK-CXL) (Said et al., 2014) and used in controlling the resistant forms of infectious keratitis (Iseli et al., 2008, Hafez, 2014). The use of combined riboflavin/UV-A light as a therapy for corneal infections stems from its established antimicrobial activity (Goodrich, 1999, Corbin, 2002, Goodrich, 2011), along with enhancing stromal resistance against microbial enzymes (Spoerl et al., 2004), which in turn decreases the progression of the corneal degradation and melting (Iseli et al., 2008, Said et al., 2014).

PACK-CXL procedure has been shown to be successful in the management of fungal keratitis associated with corneal melting (Iseli et al., 2008, Panda et al., 2012); however, the results have been mixed and some clinical studies failed to demonstrate a valuable impact of the PACK-CXL treatment in the management of fungal keratitis (Price et al., 2012, Escarião et al., 2013, Uddaraju et al., 2015, Vajpayee et al., 2015). It is likely that the fungal keratitis with deep corneal infiltrations might not be effectively managed by the PACK-CXL procedure (Shetty et al., 2014, Uddaraju et al., 2015) due to the limited penetration of UV-A light through the anterior 300 µm of the corneal stroma (Wollensak et al., 2004, Kohlhaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016). Thus, the infectious agents
localized beyond the corneal depth of 300 µm may be protected from the antimicrobial effect of the PACK-CXL treatment.

In a clinical setting, it is not feasible to study the effect of PACK-CXL procedure in managing fungal infection at different severity levels under controlled conditions. Hence, the present study aims to investigate the impact of the PACK-CXL procedure in the management of superficial and deep forms of fungal infections in-vitro using a previous established ex vivo human corneal model of Fusarium infection (Alshehri et al., 2016). The study will evaluate the effect of timing of treatment and the role of repeating the PACK-CXL procedure to control the progression of the infection, using confocal microscopy and histological staining. As the infection progresses, and the efficacy of PACK-CXL treatment is likely reduce with increasing the severity of the infection, it is hypothesised that the treatment will fail and become ineffective in treating the fungal infection when the depth of the infection exceeds the depth of UV-A penetration.

6.3 Materials and Methods

6.3.1 Ex Vivo Human Corneas

Thirty-two healthy post-mortem human corneal buttons from 20 male and 12 female donors in the age range 48 – 92 years old (mean ± SD, 73.2 ± 9.7 years) were used in this study. The corneas were provided by the Manchester Eye Bank (NHS Blood and Transplant, UK) and pre-consented for research. The project had full ethical approval from the Manchester University Research Ethics Committees (No. 040811) and followed the terms of the Declaration of Helsinki.
The corneas were randomly assigned to one of the eight groups with four corneas in each group as detailed below:

- **C**: Control - not inoculated with *Fusarium oxysporum* spores, not treated with CXL procedure (60-92 years old, mean ± SD, 73.3 ± 13.7 years).
- **X**: Not inoculated, treated with CXL procedure (60-81 years old, mean ± SD, 69.3 ± 10 years).
- **I**: Inoculated with *Fusarium oxysporum* spores, not treated with PACK-CXL (74-89 years old, mean ± SD, 80.5 ± 6.4 years).
- **I-EOF**: Inoculated, then epithelial layer debrided, not treated with PACK-CXL (68-81 years old, mean ± SD, 73.5 ± 6.1 years).
- **I-24h-X**: Inoculated, treated with PACK-CXL at 24h post-inoculation (62-69 years old, mean ± SD, 65.3 ± 3.3 years).
- **I-24h-36h-X**: Inoculated, treated with PACK-CXL at 24h post-inoculation, then the treatment was repeated at 36h-post-inoculation (at 12h post-first-PACK-CXL treatment) (68-78 years old, mean ± SD, 72 ± 4.5 years).
- **I-48h-X**: Inoculated, treated with PACK-CXL at 48h post-inoculation (74-81 years old, mean ± SD, 78 ± 3.6 years).
- **I-72h-X**: Inoculated, treated with PACK-CXL at 72h post-inoculation (48-92 years old, mean ± SD, 74.3 ± 18.8 years).

### 6.3.2 Corneal Tissue Culture

The corneas were placed in 6-well cell culture plates (Costar®; Corning Ltd, Ewloe, UK), and submerged in fresh culture medium (Dulbecco’s Modified Eagle’s Medium, DMEM, 5000mg Glucose/L; Sigma-Aldrich Ltd, Poole, UK) supplemented with 2mM Glutamine,
10% (v/v) foetal bovine serum (FBS; Labtech International Ltd, Uckfield, UK), and 1% (v/v) Penicillin with Streptomycin (all Sigma-Aldrich Ltd, Poole, UK). The corneas were incubated at 34°C for three days prior to the inoculation process and cross-linking treatment.

6.3.3 Fungal Strain

*Fusarium oxysporum* f. sp. *lycopersici* strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP) was kindly provided by Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) (Di Pietro et al., 2001) and used in this study. The green fluorescent protein facilitated the tracking of the fungal hyphal invasion of the corneal layers. The *F. oxysporum* GFP strain was cultured in Potato Dextrose Broth (PDB) (BD Difco, Oxford, UK), spores suspensions was prepared, and spore concentration was adjusted to $1 \times 10^5$ spores per ml using fresh culture medium, and 10 µl of this suspension was used to inoculate each cornea from the different inoculated groups.

6.3.4 Corneal Scratch and Inoculation Process

After culturing the corneas, the experiment continued for 7 days (Table 6-1). The corneal scratch and inoculation process was performed on day 1 post-corneal tissue culture. Each corneal button was mounted on an artificial anterior chamber (Moria SA, 92160 Antony, France; SD Healthcare, Manchester, UK), and the anterior surface was scratched in the central region of the cornea (approximately 7 mm scratch diameter) to assist the *Fusarium* invasion using a 0.6X25 mm-gauge needle (BD Microlance 3; BD Biosciences, Oxford, UK). The corneas were transferred into a fresh 6-well cell culture
plates with the posterior surface of the cornea uppermost. The posterior corneal cavity was filled with agar-gelatine gel (2% (w/v) agar (Oxide, Nottingham, UK) and 2% (w/v) gelatine (BD Difco, Oxford, UK), supplemented with 7.4% (w/v) Sodium Bicarbonate (NaHCO₃) (Sigma-Aldrich Ltd, Poole, UK) and fresh culture medium) pre-inoculation process, as an attempt to minimize the fungal invasion of the lower corneal layers via spores in the culture medium. The molten agar-gelatine gel was allowed to cool at room temperature for a few minutes, and then, the corneas were inverted with the anterior surface uppermost. Six of the corneal study groups, I, I-EOF, I-24h-X, I-24h-36h-X, I-48h-X and I-72h-X, were inoculated with *Fusarium* spore suspension. Next, a small quantity of the fresh culture medium was applied at the corneal periphery, and the corneas were incubated at 34°C for 24h prior to undertaking the first session of cross-linking.

### 6.3.5 Corneal Cross-linking Procedure

Five of the corneal study groups, X, I-24h-X, I-24h-36h-X, I-48h-X and I-72h-X, were treated with the combined isotonic riboflavin/UV-A light according to the standard CXL protocol (Wollensak et al., 2003a). The corneas in the X and I-24h-X groups were subjected to the cross-linking treatment on day 2 (at 24h post-inoculation). The corneas in the I-24h-36h-X group were treated twice with the PACK-CXL procedure; these corneas were subjected to the first PACK-CXL session on day 2 (at 24h post-inoculation), and then, subjected to the second PACK-CXL session on the same day at 12h after the first PACK-CXL session (at 36h post-inoculation). The corneas in the I-48h-X and I-72h-X groups were subjected to the PACK-CXL procedure on day 3 (at 48h post-inoculation) and day 4 (at 72h post-inoculation), respectively. During the cross-linking
procedure, each cornea was mounted on the artificial anterior chamber, the interior was filled with sterile Phosphate Buffered Saline (PBS) to mimic the intraocular pressure and maintain corneal stability during the procedure, the central 7-9 mm diameter area of epithelial layer was removed, isotonic riboflavin solution (Riboflavin 5'-phosphate sodium salt hydrate, R7774, Sigma-Aldrich, UK), (0.1% (w/w) riboflavin, 20% (w/w) dextran T500) was dripped on the corneal surface every 5 minutes for half an hour, the corneal surface was exposed to the ultraviolet-A irradiation (370 nm, irradiance of 3mW/cm², UV-A irradiation dose of 5.4 J/cm²) for 30 minutes, using a UV-A light emitter (VEGA, C.S.O. srl, Florence, Italy), in combination with continued riboflavin solution dripping at 5 minute intervals. The corneas in the I-EOF group were not treated with the combined riboflavin/UV-A light, but only the epithelial layer was debrided on day 2.

6.3.6 Imaging and Fusarium Spore Concentration

Imaging of the corneas and calculating spore concentration per ml of corneal culture medium was performed every 24h, from the day 2 through to day 7 of the experiment. The central region of each cornea was imaged using a Leica TCS SP5 AOB5 inverted confocal microscope (Leica Microsystems Ltd., Breckland, Linfood Wood, Milton Keynes, UK) with a x20 objective. The imaging was always centered in the central corneal zone directly beneath the scratch. The confocal images were analysed with the Surpass module of the Imaris v8.4 software (Bitplane Scientific software module; Bitplane AG, Zurich, Switzerland). The volume occupied by the Fusarium hyphae within anterior stromal tissues for each inoculated cornea was automatically quantified daily post-inoculation over the experimental period in a tiled z-stack (4x4 z-stacks, each z-
stack is 775x775x100 µm) by the software and displayed in an excel sheet. The percentage of the total Fusarium hyphal volume was calculated at each imaging time-point for the inoculated corneas. On day 7, the gross morphology of corneal buttons was imaged. Following the imaging and/or cross-linking procedure on each day of the experiment, all corneas were rinsed with PBS, placed in a new 6-well tissue culture plate, filled with fresh agar-gelatine gel, provided with fresh culture medium and incubated at 34°C.

6.3.7 Corneal Tissue Processing and Haematoxylin and Eosin (H&E) Staining

On day 7, the corneal buttons were cut into halves. One half of each cornea was fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C overnight followed by 72h incubation in PBS-sucrose, embedded in OCT (KP-CryoCompound, Klinipath BV, The Netherlands) over liquid nitrogen, and sectioned at 30 µm thickness using a Bright OTF cryostat (Bright Instrument Company, Huntingdon, UK). The other half of each cornea was fresh-frozen in OCT and stored at -80 °C for future use. The fixed corneal sections were haematoxylin and eosin (H&E) stained, and the images of the central views of the H&E stained sections were captured at x10 magnification using a Zeiss Axiostar Plus HBO 50 microscope (Zeiss UK, Cambridge, UK), Micropublisher 5.0 RTV camera and Q-CapturePro7TM Software (both Media Cybernetics UK, Buckinghamshire, UK). The total depth of Fusarium hyphal penetration within the stromal tissues at the central regions was semi-automatically measured (µm) for all corneas using ImageJ software (Schneider et al., 2012). The percentage of the total corneal depth penetrated by the Fusarium hyphae was calculated for each inoculated cornea.
Table 6-1: Timeline of experimental procedures.

The main experimental procedures performed on the different groups of corneas. C: Control; X: Cross-linked; I: Inoculated only; I-EOF: Inoculated, epithelium removed; I-24h-X: Inoculated, cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated, cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated, cross-linked at 48h post-inoculation; I-72h-X: Inoculated, cross-linked at 72h post-inoculation; Epi-Off: Epithelium debrided; PACK-CXL: Cross-linking with isotonic riboflavin and UV-A light.

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PBS-Sucrose incubation, OCT-embedded, sectioned, H&E-stained and imaged
6.3.8 Statistical Analysis

The analysis of *Fusarium* hyphal volume and spores concentration for all of the inoculated groups was performed using a repeated analysis of variance (ANOVA) and post hoc tests to assess changes over time. The analysis of hyphal penetration depth within corneal tissues for all groups was performed using a one-way analysis of variance and post hoc tests to assess mean difference.

6.4 Results

The percentages of the total *Fusarium* hyphal volume calculated from the confocal images within the anterior 100 µm of the corneal tissue on each day post-inoculation are shown in Figure 6-1. The percentage of the hyphal volume over the duration of the experiment reflects the progression of the infection over time. All inoculated corneas developed *Fusarium* infection, while the un-inoculated corneas remained intact and uninfected on day 2. In the inoculated/un-treated groups, there is a steady increase in *Fusarium* hyphal volume from day 2 to day 7 showing that the severity of infection increases with duration after inoculation. In each of the four inoculated and cross-linked groups, the *Fusarium* hyphal volume decreased considerably 24h after PACK-CXL treatment. However, the *Fusarium* hyphal volume in these treated groups continued to increase on subsequent days at a similar rate to that seen in un-treated inoculated groups. There is a large variation in *Fusarium* hyphal volume between the groups on day 7. The inoculated group treated with repeated PACK CXL on day 2 at 24h and 36h post inoculation showed the lowest hyphal volume, followed by the groups receiving a single treatment of PACK CXL on days 2, 3 and 4 post-inoculation (at 24h, 48h and 72h
respectively). Conversely, the un-treated/epithelium-intact I and un-treated/epithelium-debrided I-EOF groups showed the greatest *Fusarium* hyphal volume. Repeated analysis of variance of the hyphal volume data over the duration of the experiment showed a significant difference between the inoculated groups ($P < 0.001$). *Post hoc* tests (Tukey) showed significant differences between the early/twice-treatment I-24h-36h-X group and un-treated I and I-EOF ($P \leq 0.001$), late/single-treatment I-48h-X ($P = 0.028$) and I-72h-X ($P = 0.008$) groups. There is also a significant difference between the early/single-treatment I-24h-X and un-treated I ($P = 0.002$) groups. Analysis of variance showed a significant difference on day 7 between the inoculated groups ($P < 0.001$). *Post hoc* tests (Tukey) showed that the early/twice-treatment I-24h-36h-X group had a significantly less hyphal volume on day 7 compared to un-treated I and I-EOF ($P \leq 0.001$), late/single-treatment I-48h-X ($P = 0.011$) and I-72h-X ($P = 0.040$) groups. The early/single-treatment I-24h-X group had a significantly less hyphal volume compared to un-treated I ($P = 0.012$) and I-EOF ($P = 0.05$) groups.

The concentration of *Fusarium* spores present in the culture medium on each day post-inoculation for inoculated groups are shown in Figure 6-2. The change in *Fusarium* spore concentration in the culture medium over the duration of the experiment reflects the progression of the infection and maturation of the *Fusarium* to sporulation over time. The spore concentration in the culture medium increased with time in all inoculated groups. Although spore concentration decreased 24h after the PACK-CXL treatment in the inoculated and treated groups, it resumed increasing at a similar or slightly lower rate to un-treated groups on subsequent days. Repeated analysis of variance of the *Fusarium* spore concentration data over the days of the experiment
showed a significant difference between the inoculated groups \((P < 0.001)\). Post hoc tests (Tukey) showed significant differences between the early/twice-treatment I-24h-36h-X group and un-treated I and I-EOF \((P < 0.001)\), late/single-treatment I-48h-X \((P = 0.022)\) and I-72h-X \((P = 0.005)\) groups. There are also significant differences between the early/single-treatment I-24h-X and un-treated I and I-EOF \((P < 0.001)\) groups, between the late/single-treatment I-48h-X and un-treated I \((P = 0.003)\) and I-EOF \((P = 0.044)\) groups, and between the late/single-treatment I-72h-X and un-treated I \((P = 0.016)\) groups. Analysis of variance showed a significant difference in *Fusarium* spore concentration on day 7 between the inoculated groups \((P < 0.001)\). Post hoc tests (Tukey) showed a significant inhibition of *Fusarium* sporulation on day 7 in the early/twice-treatment I-24h-36h-X compared to un-treated I \((P < 0.001)\), un-treated I-EOF \((P < 0.001)\), late/single-treatment I-48h-X \((P = 0.035)\) and late/single-treatment I-72h-X \((P = 0.004)\) groups. There is a significant inhibition of sporulation in the early/single-treatment I-24h-X compared to un-treated I \((P < 0.001)\), un-treated I-EOF \((P < 0.001)\), late/single-treatment I-72h-X \((P = 0.037)\) groups. There is a significant inhibition of sporulation in the late/single-treatment I-48h-X compared to un-treated I \((P = 0.035)\) and I-EOF \((P = 0.02)\) groups.

The *Fusarium* infection can be seen as branching, green fluorescent fungal hyphae using fluorescence confocal microscopy (Figure 6-3). The greatest infection progression can be seen as a high density of *Fusarium* hyphae within the 16 z-stack of confocal images in the un-treated I and I-EOF corneas on day 7 (Figure 6-3A and C). Focusing on one z-stack of confocal images (Figure 6-3B and D), details of *Fusarium* hyphae can be seen more clearly combined with a large quantity of spores in the I and I-EOF corneas.
The density of hyphae is reduced in the late/single-treatment I-48h-X and I-72h-X corneas (Figure 6-3I and K); while the early/single-treatment I-24h-X and early/twice-treatment I-24h-36h-X corneas had the lowest progression of the infection on day 7 (Figure 6-3E and G).

The anterior surface images of corneal buttons on day 7 are shown in Figure 6-4. The fungal infection can be observed with the naked eye in the inoculated corneas where *Fusarium* colonies were observed as a creamy colouration over the corneal surface. The early/twice-treatment I-24h-36h-X cornea (Figure 6-4F) showed less severe infection compared to the other inoculated corneas. In the early/single-treatment I-24h-X cornea (Figure 6-4E) and late/single-treatment I-48h-X and I-72h-X corneas (Figure 6-4G and H), the fungal infection had spread over the corneal surface, with more localized infection observed in the central cornea where the corneal scratches were made and the epithelium was debrided. However, the fungal infection had greatly progressed leading to severe infection in the un-treated I and I-EOF corneas (Figure 6-4C and D, respectively), where the *Fusarium* colonised the majority of the corneal surface.

Images of the H&E stained corneal sections for corneas from all groups are shown in Figure 6-5. The corneal epithelial layers are stained dark purple, the stromal tissues are stained pink, and the keratocytes and *Fusarium* hyphae are stained purple, distinguishable by the difference in their shape. The structure of the central region for corneas from the C and X groups can be seen in Figures 6-5 (A and B), where the corneal tissue is free from fungal invasion with intact stromal layers populated with keratocytes. Figures 6-5 (C and D) show central view of the H&E stained corneal
sections from the un-treated I and I-EOF corneas in which large fungal colonies have accumulated over the corneal surface and a high density of *Fusarium* hyphae have penetrated deep within the corneal stroma by day 7. Figures 6-5 (E-H) show central view of the H&E stained corneal sections from the early/single-treatment I-24h-X, early/twice-treatment I-24h-36h-X, late/single-treatment I-48h-X and I-72h-X groups, respectively, where several fungal hyphae can be seen. However, the density of the *Fusarium* hyphae within the stromal tissues in the early/twice-treatment I-24h-36h-X group (Figure 6-5F) is lower compared to that in the other groups. Regardless of this, the percentage of the total corneal depth penetrated by the *Fusarium* hyphae by day 7 was 100% for all inoculated corneas: the hyphae had penetrated the full thickness of the corneas and reached the endothelial layer in all inoculated corneas.
Figure 6-1: The percentage of the *Fusarium* hyphal volume for the study groups.

The graph shows the mean percentage of the total volume occupied by the fungal hyphae within the anterior 100 µm of corneal tissues for the corneal groups on each day post-inoculation. The error bars represent ±1 standard error. *P* values are derived from post hoc tests (Tukey) for ANOVA for day 7 data. C: Control; X: Cross-linked only; I: Inoculated only; I-EOF: Inoculated then epithelium removed; I-24h-X: Inoculated then cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated then cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated then cross-linked at 48h post-inoculation; I-72h-X: Inoculated then cross-linked at 72h post-inoculation.
Figure 6-2: The concentration of the *Fusarium* spores for the inoculated groups.

The graphs show the mean number of *Fusarium* spores per ml of corneal culture medium for the inoculated groups on each day post-inoculation. The error bars represent ±1 standard error. *P* values are derived from *post hoc* tests (Tukey) for ANOVA for day 7 data. I: Inoculated only; I-EOF: Inoculated then epithelium removed; I-24h-X: Inoculated then cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated then cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated then cross-linked at 48h post-inoculation; I-72h-X: Inoculated then cross-linked at 72h post-inoculation.
Figure 6-3: Confocal microscopy images of inoculated corneas on day 7 of the experiment.

Confocal images of inoculated corneas show the extent of the branching, green fluorescent fungal hyphal growth within the anterior central corneal region on day 7. Each image on the left (A, C, E, G, I and K) is a projection of a tiled z-stack of confocal images (x20 magnification). The projection of one z-stack of each tiled z-stack image (indicated by a yellow square) is shown on the right (B, D, F, H, J and L). The most extensive fungal growth is visible in the un-treated I (A) and I-EOF (C) groups, while Fusarium hyphae in the early/single-treatment I-24h-X (E) and early/twice-treatment I-24h-36h-X (G) groups are relatively fewer than in the other late/single-treatment I-48h-X and I-72h-X groups (I and K). I: Inoculated only; I-EOF: Inoculated then epithelium removed; I-24h-X: Inoculated, cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated, cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated, cross-linked at 48h post-inoculation; I-72h-X: Inoculated, cross-linked at 72h post-inoculation.
Images of corneal buttons on day 7 show that the C and X corneas (A and B) were uninfected and intact. The *Fusarium* infection developed and progressed in all inoculated corneas (C, D, E, F, G and H). Large *Fusarium* colonies are seen in un-treated I and I-EOF corneas (C and D). Infection was less severe in the early/twice-treatment I-24h-36h-X cornea (F) compared to the early/single-treatment I-24h-X (E) and late/single-treatment I-48h-X (G) and I-72h-X (H) corneas. C: Control; X: Cross-linked only; I: Inoculated only; I-EOF: Inoculated then epithelium removed; I-24h-X: Inoculated then cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated then cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated then cross-linked at 48h post-inoculation; I-72h-X: Inoculated then cross-linked at 72h post-inoculation.
Figure 6-5: H&E stained corneal sections from the different study groups.

The figure shows the central corneal region for the H&E stained corneal sections at x10 magnification from different groups on day 7. H&E stained sections for corneas from C
and X groups show the intact stromal layers along with keratocytes (A and B). H&E stained sections of corneas from un-treated I and I-EOF groups show heavy infection, enormous *Fusarium* colonies above the corneal surface and within the stroma (C and D). H&E stained sections of cornea from early/single-treatment I-24h-X and early/twice-treatment I-24h-36h-X groups (E and F) show the *Fusarium* infection, which appears to be severe but with reduced load of *Fusarium* compared to the late/single-treatment I-48h-X and I-72h-X groups (G and H). C: Control; X: Cross-linked only; I: Inoculated only; I-EOF: Inoculated then epithelium removed; I-24h-X: Inoculated then cross-linked at 24h post-inoculation; I-24h-36h-X: Inoculated then cross-linked twice at 24h and 36h post-inoculation; I-48h-X: Inoculated then cross-linked at 48h post-inoculation; I-72h-X: Inoculated then cross-linked at 72h post-inoculation.

### 6.5 Discussion

The present study shows that there is a time-dependent decrease in the efficiency of PACK-CXL procedure in reducing the volume of fungal hyphae and number of spores. Early intervention with PACK-CXL treatment has a significantly greater impact on suppression of the fungal infection caused by *Fusarium* when compared to relatively late PACK-CXL treatment. The repetition of PACK-CXL treatment has a further significant effect in the management of fungal hyphal invasion and suppression of *Fusarium* sporulation. This is probably due to suppression of the growth of the residual *Fusarium* hyphae which survived the first session of PACK-CXL treatment, similarly as reported by Saglik et al. (2013), along with further suppression of the *Fusarium* sporulation, therefore decreasing the number of spores in the culture medium which could re-invade the corneal tissues. Groups treated late with PACK-CXL at 48 or 72h post-inoculation had a considerable, but not significant, decrease in the volume of
fungal hyphae compared to the un-treated groups and again this highlights the benefit of prompt intervention with PACK-CXL in the management of fungal infection. Delay in PACK-CXL treatment can result in a great decline in the treatment efficacy or even lead to treatment failure. In a previous clinical study, the effect of PACK-CXL as an early/alternative therapy in the management of infectious keratitis suggests that early intervention with PACK-CXL treatment improves the outcome of infectious keratitis (Makdoumi et al., 2012). Repeating the corneal cross-linking procedure has also been conducted by some studies where repeated PACK-CXL showed a beneficial effect in the management of resistant corneal infection (Khan et al., 2011, Saglik et al., 2013). Some of the treatment effect with PACK-CXL could be due to the removal of a proportion of fungal hyphae and spores with epithelial debridement instead of PACK-CXL procedure itself as is evident from the inoculated group which had epithelium debrided post-inoculation but no PACK-CXL treatment.

The beneficial effect of PACK-CXL treatment in reducing the growth of fungal hyphae and Fusarium spores in the treated groups was apparent 24h post-treatment but disappeared over time and had no control on progression of the fungal infection. This is possibly because that the effect of PACK-CXL treatment was due to the direct elimination of pathogen by combined riboflavin/UV-A light (Tsugita et al., 1965, Goodrich, 1999, Corbin, 2002, Goodrich, 2011) rather than enhancement of the biomechanical rigidity of the cornea and decreasing the efficiency of enzymatic degradation of the collagen (Wollensak et al., 2003b, Spoerl et al., 2004). In the literature, it has been shown that PACK-CXL treatment reduces the capability of pathogens to penetrate within the stromal tissue, and therefore, assists in arresting
the corneal melting caused in infectious keratitis (Iseli et al., 2008, Makdoumi et al., 2010b, Müller et al., 2012, Panda et al., 2012, Said et al., 2014). However, PACK-CXL treatment in the present study was not effective enough to reduce the depth of hyphal penetration into the corneal tissues and rescue the endothelium as the percentage of the total corneal depth penetrated by the *Fusarium* hyphae by day 7 was 100% for all treated groups. This could have been due to the severe infection being induced in the model from day 1 due to a large scratch (approximately 7 mm diameter) and a relatively large number of *Fusarium* spores being used. However, presentation of *Fusarium* keratitis in clinical cases often tends to be at a relatively advanced stage (Vemuganti et al., 2002, Shetty et al., 2014, Uddaraju et al., 2015).

The results obtained in the present study are consistent with some clinical studies which failed to demonstrate a valuable impact of the PACK-CXL treatment in the management of fungal keratitis (Price et al., 2012, Escarião et al., 2013, Shetty et al., 2014, Uddaraju et al., 2015, Vajpayee et al., 2015). The failure of PACK-CXL treatment in controlling the *Fusarium* infection in this study is probably due to the following factors. Firstly, the short-coming of *ex vivo* human corneal model of *Fusarium* infection in that it lacks a tear production and drainage system to mimic the *in vivo* human lacrimal system. This resulted in accumulation of the *Fusarium* spores in the corneal culture medium where corneas were incubated overnight. A tear drainage system would be a fundamental factor in washing the corneal surface and removing the fungal agents, which in turn, assists in reducing the potential of re-invasion of the corneal tissue and results in new post-PACK-CXL infection by surviving spores that are present in the corneal culture medium. Secondly, although debriding the epithelium in the
treated corneas as a part of PACK-CXL may assist in removing some of the *Fusarium* agents, incubation of the treated corneas for prolonged times after epithelium removal along with the high spore count in the medium make the exposed stromal tissues continually prone for further attacks by germinating *Fusarium* spores and hyphae. The increased severity of the infection could lead to reduction in the efficacy of PACK-CXL treatment, or make it ineffective in treating the fungal infection due to the depth of the infection exceeding the depth of UV-A penetration. It has been shown that the effect of corneal cross-linking treatment is limited to the anterior 300 μm of the corneal stroma (Wollensak et al., 2004, Kohlhaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016), whereas the fungal agents deep within the corneal stroma lie beyond this limit and are unlikely to be affected by the PACK-CXL treatment. Lastly, the high density at which the *Fusarium* hyphae grew and penetrated within tissue, particularly in groups treated late, could act as a block for riboflavin diffusion throughout the entire corneal stroma or be a barrier for UV-A absorption by the tissues; both effects would reduce the efficacy of PACK-CXL treatment. However, for the moment these ideas are speculative and there may be other factors affecting the antifungal efficacy that have not yet been identified.

According to the results obtained from this study, the PACK-CXL procedure cannot be considered as an effective monotherapy in the management of *Fusarium* infection. However, PACK-CXL treatment would be useful in a dual therapy or in cases of infectious keratitis with unknown underlying cause as an initial intervention until the
causative microorganism has been identified and appropriate antimicrobial therapy administered.

### 6.6 Conclusion

Early intervention with PACK-CXL treatment has a significant effect in reducing the load of the *Fusarium* hyphae and spores. Repetition of the PACK-CXL treatment has a further significant effect in suppressing the growth of fungal hyphae and *Fusarium* sporulation. However, the PACK-CXL treatment alone appears ineffective in controlling the progression of the infection as the percentage of hyphal penetration depth within corneal tissues by day 7 was 100% in treated groups. Hence, it would be better to use the PACK-CXL procedure in a dual therapy rather than as a monotherapy.
Chapter 7

Efficacy of the Dual Therapy of Corneal Cross-linking and Natamycin in the Management of *Fusarium* Keratitis

Contributions

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross. Prof. Fiona Carley and Mr. Arun Brahma regularly treat patients with infectious keratitis in the Manchester Royal Eye Hospital. Prof. Carly and Mr. Brahma met with the research team on a regular basis to discuss ideas and progress of the research studies.
Submission for Presentation

The abstract is submitted for presentation in an international conference.


Submission for Publication

The chapter is prepared as a manuscript which will be submitted for publication.


Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.
- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the *Fusarium* strain.
- Bodour Rajab (PhD student, Division of Cardiovascular Sciences, Faculty of Biology, Medicine and Health, University of Manchester) for assisting with administration of natamycin over the period of the experiment.
• Dr Peter March, Dr Steven Marsden and Mr Roger Meadows from the Bioimaging Facility for their help with the confocal microscopy and Imaris software.
7.1 Abstract

**Purpose**: to investigate the efficacy of dual therapy of corneal cross-linking (PACK-CXL) and natamycin in the management of *Fusarium* infections and to evaluate the effect of intervention timing with PACK-CXL or natamycin in the combined treatment vs. monotherapy with natamycin.

**Method**: Twenty-four *ex vivo* human corneas were divided into 6 groups: C: Control; I: Inoculated with *Fusarium oxysporum* spores; IN: Inoculated, treated with natamycin at 24h post-inoculation; IXN: Inoculated, treated with combined PACK-CXL and natamycin at 24h post-inoculation; IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, then treated with natamycin at 48h post-inoculation; IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation, treated with PACK-CXL at 72h post-inoculation. The corneas were imaged daily by confocal microscopy and *Fusarium* hyphal volume was calculated for the duration of the experiment (7 days) following inoculation. Corneas were sectioned, haematoxylin and eosin stained, imaged, and the hyphal penetration depth within the stromal tissue was measured.

**Results**: All treated groups (IN, IXN, IX-48h-N and IN-72h-X) had a significant reduction in both *Fusarium* hyphal volume and hyphal penetration depth on day 7 compared to the un-treated group (I) (*P* < 0.001). The PACK-CXL/natamycin treatment had a higher impact in controlling the progression of fungal infection and completely eradicating of the *Fusarium* agents in the IXN group (which had early intervention at 24h post-inoculation with both natamycin and PACK-CXL) and IN-72h-X group (which had early intervention of natamycin at 24h but late PACK-CXL treatment at 72h), when
compared to either the treatment in the IX-48-N group (which had early intervention of PACK-CXL at 24h but late natamycin at 48h) or IN group (which had natamycin only from 24h post-inoculation). However, the differences between the treated groups were not statistically significant.

**Conclusion:** The dual therapy of PACK-CXL with natamycin is more effective in the management of corneal infection caused by *Fusarium* than treatment with natamycin alone. Delaying the natamycin treatment reduces dual therapy efficacy more than delaying the PACK-CXL treatment.
7.2 Introduction

Fungal keratitis is one of the most challenging corneal infections to diagnose and treat (Srinivasan, 2004, Sara et al., 2016). *Fusarium* spp., which is a genus of filamentous fungi, is one of the leading causes of fungal infections (Srinivasan et al., 1997, Dóczi et al., 2004, Hua et al., 2010, Kredics et al., 2015). Compared to other conical infections, fungal keratitis is more difficult to manage owing to the limited range and prohibitive costs of antifungal agents, thus a gold standard for the management of fungal infections is yet to be identified (Thomas, 2003b, Srinivasan, 2004, FlorCruz and Peczon, 2008, FlorCruz et al., 2012). The main antifungal agents used include amphotericin B, natamycin and voriconazole. A good prognosis and management of fungal keratitis depends upon the early diagnosis and intervention in a timely manner with appropriate antifungal therapy (Xie et al., 2008). However, most of the currently available medications appear to have limited efficacy in treating fungal infections and reducing vision loss, which in turn makes therapeutic penetrating keratoplasty a necessity for a significant number of patients (Srinivasan, 2004, Qiu et al., 2015).

The clinical effectiveness of any antifungal agent deployed in the treatment of fungal infections relies upon the level of concentration attained within the corneal tissue, its spectrum of activity, and how safe it is to use (Manzouri et al., 2001, Thomas, 2003b).

In the past, amphotericin B was the preferred treatment method for management of fungal keratitis due to its ability to meet the first two requirements (O'Brien, 1999). The high toxicity of amphotericin B led to the drug being replaced by natamycin as the preferred antifungal agent for treatment of filamentous fungal infections (O'Brien, 1999, Thomas, 2003a, Thomas, 2003b). Natamycin is now considered to be the most
effective agent for treating infections caused by *Fusarium* (Thomas, 2003b, Qiu et al., 2015); however, it is less effective in the management of infections caused by yeast (Sara et al., 2016).

Natamycin is available as 5% suspension for topical administration. It functions by binding to the ergosterol within the fungal cell wall and inhibiting transport across the plasma membrane (Medoff and Kobayashi, 1980, Te Welscher et al., 2008, Qiu et al., 2015). When the corneal epithelial layer is intact, natamycin penetrates poorly due its size (molecular mass 665.75 Da), which makes diffusion difficult (Manzouri et al., 2001, Thomas, 2003b). Therefore the corneal epithelium has to be debrided before natamycin therapy (Sharma et al., 2013). The poor penetration of topical natamycin has resulted in it being deployed as a monotherapy in superficial fungal infections (Srinivasan, 2004), and in combination with oral azole antifungals as a dual therapy in the treatment of deep fungal infections (Rosa et al., 1994). A further complicating factor is that natamycin is expensive and not easy to access in the third world where fungal keratitis is most prevalent (Srinivasan, 2004). An alternative antifungal, voriconazole, can be administered in a number of ways, and better penetrates the corneal epithelial layer (O'day et al., 1986, Hariprasad et al., 2008) with broader antifungal coverage against both filamentous fungi and yeast. Use of voriconazole as a monotherapy in cases of filamentous fungal infections is not recommended, however, as it has been linked to heightened rates of corneal perforation and therapeutic penetrating keratoplasty (Prajna et al., 2013, FlorCruz and Evans, 2015). In the absence of a gold standard therapy for fungal keratitis, dual treatment has been recommended to reduce the risk of antimicrobial resistance (Tuli, 2011). One of the most used
combinations in the treatment of fungal infections has been a combined topical natamycin and systemic voriconazole (Iselin et al., 2017).

Studies conducted to compare the effectiveness of natamycin and other antifungal agents in the treatment of fungal keratitis did not produce consistent results (Srinivasan, 2004, Kalavathy et al., 2005). FlorCruz et al. (2012) conducted a systematic review in which they concluded that there is no single drug, or drug combination, that is more effective than any other in the management of fungal keratitis. Conversely, in a subsequent review the same authors suggest that in the treatment of fungal ulcers, natamycin is the most effective agent (FlorCruz and Evans, 2015). Another systematic review also reported that natamycin is preferable in the management of fungal keratitis, particularly during the early stages of corneal infection caused by Fusarium (Qiu et al., 2015).

As a result of the multidrug-resistant corneal infections associated with failed medical management, the combined riboflavin/UV-A light-induced corneal cross-linking (PACK-CXL) has emerged as an adjuvant treatment for infectious keratitis (Said et al., 2014). The exposure to combined riboflavin/UV-A light suppresses the growth of pathogens and restricts the infection development on the one hand (Iseli et al., 2008, Makdoumi et al., 2010b, Said et al., 2014, Alshehri et al., 2016), and enhances stromal resistance against microbial enzymes on the other (Spoerl et al., 2004). The current literature supports a promising role of PACK-CXL in the management of infectious keratitis. The results in cases of fungal keratitis, however, are variable (Iseli et al., 2008, Price et al., 2012, Hafez, 2014, Shetty et al., 2014).
The aims of this study were to investigate the efficacy of a dual antifungal therapy comprising PACK-CXL and natamycin in the treatment of *Fusarium* infection, compared with natamycin treatment alone, and to evaluate the effect of intervention timing with PACK-CXL or natamycin in controlling the progression of fungal infection.

### 7.3 Materials and Methods

#### 7.3.1 Ex Vivo Human Corneas

Twenty-four healthy *post-mortem* human corneal buttons from 11 male and 13 female donors in the age range 55 – 92 years old (mean ± SD, 74.3 ± 9.8 years) were used in this study. The corneas were provided by the Manchester Eye Bank (NHS Blood and Transplant, UK) and pre-consented for research. The project had full ethical approval from the Manchester University Research Ethics Committees (No. 040811) and followed the terms of the Declaration of Helsinki.

The corneas were randomly assigned to one of the six groups with four corneas per group as detailed below:

- **C**: Control - not inoculated with *Fusarium oxysporum* spores, not cross-linked, and not treated with natamycin (60-92 years old, mean ± SD, 73.3 ± 13.7 years).
- **I**: Inoculated with *Fusarium oxysporum* spores, not cross-linked, and not treated with natamycin (74-89 years old, mean ± SD, 80.5 ± 6.4 years).
- **IN**: Inoculated, treated with natamycin only at 24h post-inoculation and over the course of the experiment (55-81 years old, mean ± SD, 71.8 ± 11.5 years).
• IXN: Inoculated, treated with combined PACK-CXL and natamycin at 24h post-inoculation, treatment with natamycin continued over the course of the experiment (71-82 years old, mean ± SD, 76 ± 5.4 years).

• IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, and then treated with natamycin at 48h post-inoculation, treatment with natamycin continued over the course of the experiment (66-82 years old, mean ± SD, 74.5 ± 7.3 years).

• IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation and over the course of the experiment, treated with PACK-CXL at 72h post-inoculation (55-89 years old, mean ± SD, 70 ± 14.3 years).

7.3.2 Corneal Tissue Culture

The corneas were placed in 6-well cell culture plates (Costar®; Corning Ltd, Ewloe, UK), and submerged in fresh culture medium (Dulbecco’s Modified Eagle’s Medium, DMEM, 5000mg Glucose/L; Sigma-Aldrich Ltd, Poole, UK) supplemented with 2mM Glutamine, 10% (v/v) foetal bovine serum (FBS; Labtech International Ltd, Uckfield, UK), and 1% (v/v) Penicillin with Streptomycin (all Sigma-Aldrich Ltd, Poole, UK). The corneas were incubated at 34°C for three days prior to the inoculation process.

7.3.3 Fungal Strain

Fusarium oxysporum f. sp. lycopersici strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP) was kindly provided by Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) (Di Pietro et al., 2001) and used in this study. The green fluorescent protein facilitated the tracking of the fungal hyphal
invasion of the corneal layers. The *F. oxysporum* GFP strain was cultured in Potato Dextrose Broth (PDB) (BD Difco, Oxford, UK), spore suspensions were prepared, and spore concentration adjusted to $1 \times 10^5$ spores per ml using fresh culture medium, and 10 µl of this suspension was used to inoculate each cornea in the different inoculated groups.

### 7.3.4 Corneal Scratch and Inoculation Process

After culturing the corneas, the experiment continued for 7 days (see table 7-1). The corneal scratch and inoculation process was performed on day 1 post-corneal tissue culture.

Each corneal button was mounted on an artificial anterior chamber (Moria SA, 92160 Antony, France; SD Healthcare, Manchester, UK), and the anterior surface was scratched in the central region of the cornea (approximately 7 mm scratch diameter) to assist the *Fusarium* invasion using a 0.6X25 mm-gauge needle (BD Microlance 3; BD Biosciences, Oxford, UK). The corneas were transferred into a fresh 6-well cell culture plates with the posterior surface of the cornea uppermost. The posterior corneal cavity was filled with agar-gelatine gel (2% (w/v) agar (Oxoid, Nottingham, UK) and 2% (w/v) gelatine (BD Difco, Oxford, UK), supplemented with 7.4% (w/v) Sodium Bicarbonate ($\text{NaHCO}_3$) (Sigma-Aldrich Ltd, Poole, UK) and fresh culture medium) pre-inoculation process, as an attempt to minimize the fungal invasion of the lower corneal layers via spores in the culture medium. The molten agar-gelatine gel was allowed to cool at room temperature for few minutes, and then the corneas were inverted with the anterior surface uppermost. Five of the corneal groups, I, IN, IXN, IX-48h-N and IN-72h-X, were inoculated with the *Fusarium* spore suspension. A small volume of the fresh
culture medium was applied at the corneal periphery, and the corneas were incubated at 34°C for 24h prior to administrating the natamycin and undertaking the first session of cross-linking.

### 7.3.5 Corneal Cross-linking Procedure

During the PACK-CXL procedure, each cornea was mounted on the artificial anterior chamber, the interior was filled with sterile Phosphate Buffered Saline (PBS) to mimic intraocular pressure and maintain corneal stability during the procedure, the central 7-9 mm diameter area of the epithelial layer was removed and isotonic riboflavin solution (Riboflavin 5′-phosphate sodium salt hydrate, R7774, Sigma-Aldrich, UK), (0.1% (w/w) riboflavin, 20% (w/w) dextran T500) was dripped on the corneal surface every 5 minutes for half an hour. The corneal surface was exposed to the ultraviolet-A irradiation (370 nm, irradiance of 3mW/cm², UV-A irradiation dose of 5.4 J/cm²) for 30 minutes, using a UV-A light emitter (VEGA, C.S.O. srl, Florence, Italy), in combination with continued riboflavin solution dripping at 5 minute intervals.

Table 7-1 shows the timeline of the experimental process including PACK-CXL procedure. Three of the inoculated groups, IXN, IX-48h-N and IN-72h-X, were treated with the combined isotonic riboflavin/UV-A light according to the standard CXL protocol (Wollensak et al., 2003a), on various days post-inoculation process. The corneas in the IXN and IX-48h-N groups were subjected to the PACK-CXL procedure on day 2. The corneas in the IN-72h-X group were subjected to the PACK-CXL procedure on day 4.
7.3.6 Antifungal Therapy: Natamycin Administration

Four of the inoculated groups, IN, IXN, IX-48h-N and IN-72h-X, were treated with topical natamycin (Natamycin ophthalmic suspension 5%, Alcon, Texas, USA). The corneas in the IN, IXN and IN-72h-X groups were subjected to the treatment with natamycin on day 2 (Table 7-1). The corneas in the IX-48h-N group were subjected to the treatment with natamycin on day 3. During the course of antifungal therapy, natamycin suspension was dripped on the corneal surface every 2 hours. This was followed by dripping PBS on the corneal surface at 10 minutes post-natamycin administration; as an attempt to mimic the role of the ocular tears, minimise accumulation of natamycin suspension over the corneal surface, and then, avoid the over-effect of the antifungal agent. Administration of natamycin continued until day 7 in all groups subjected for antifungal therapy.
Table 7-1: Timeline of experimental procedures.

An outline of the main experimental procedures performed on the different groups of corneas. C: Control; I: Inoculated only; IN: Inoculated, treated with natamycin only at 24h post-inoculation; IXN: Inoculated, treated with PACK-CXL and natamycin at 24h post-inoculation; IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, treated with natamycin at 48h post-inoculation; IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation, treated with PACK-CXL at 72h post-inoculation; PACK-CXL: Corneal cross-linking with isotonic riboflavin and UV-A light.

<table>
<thead>
<tr>
<th>Experimental time scale</th>
<th>Donor Corneas (n=24)</th>
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<tr>
<td></td>
<td>C Group (n=4)</td>
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<td>Day 1</td>
<td>Scratched</td>
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<td></td>
<td>In culture</td>
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<td>Day 2</td>
<td>at 24h post-inoculation</td>
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<td>In culture</td>
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<td>Day 3</td>
<td>at 48h post-inoculation</td>
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<td>In culture</td>
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<td>Day 4</td>
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<td></td>
<td>In culture</td>
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<td>Day 5</td>
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<td>In culture</td>
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<td>Day 6</td>
<td>at 120h post-inoculation</td>
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<td>In culture</td>
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<td>Day 7</td>
<td>at 144h post-inoculation</td>
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<td>Processing and PFA-fixation</td>
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<td></td>
<td>PBS-Sucrose incubation, OCT-embedded, sectioned, H&amp;E-stained and imaged</td>
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7.3.7 Imaging

The central region of each cornea was imaged using a Leica TCS SP5 AOBS inverted confocal microscope (Leica Microsystems Ltd., Breckland, Linford Wood, Milton Keynes, UK) with a x20 objective. Imaging of the corneas was performed daily on day 2 through to day 7 of the experiment. The confocal images were analysed with the Surpass module of the Imaris v8.4 software (Bitplane Scientific software module; Bitplane AG, Zurich, Switzerland). The volume occupied by the *Fusarium* hyphae within anterior stromal tissues for each inoculated cornea was automatically quantified daily post-inoculation over the experimental period in a tiled z-stack (4x4 z-stacks, each z-stack is 775x775x100 µm) by the software and displayed in an excel sheet. The percentage of the total *Fusarium* hyphal volume was calculated at each imaging time-point for the inoculated corneas. Following the imaging and/or cross-linking procedure on each day of the experiment, all corneas were rinsed with PBS, placed in a new 6-well tissue culture plate, re-filled with fresh agar-gelatine gel, provided with fresh culture medium and incubated at 34°C.

7.3.8 Corneal Tissue Processing and Haematoxylin and Eosin (H&E) Staining

On day 7, corneal buttons were cut into halves. One half of each cornea was fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C overnight followed by 72h incubation in PBS-sucrose, embedded in OCT (KP-CryoCompound, Klinipath BV, The Netherlands) over liquid nitrogen, and sectioned at 30 µm thickness using a Bright OTF cryostat (Bright Instrument Company, Huntingdon, UK). The other half of each cornea was fresh-frozen in OCT and stored at -80 °C for future use. The fixed corneal sections were
haematoxylin and eosin (H&E) stained, and images of the central and peripheral views of the H&E stained sections were captured at x10 magnification using a Zeiss Axiostar Plus HBO 50 microscope (Zeiss UK, Cambridge, UK), Micropublisher 5.0 RTV camera and Q-CapturePro7TM Software (both Media Cybernetics UK, Buckinghamshire, UK). The total depth of *Fusarium* hyphal penetration within the stromal tissues at the central and peripheral regions was semi-automatically measured (µm) for all inoculated corneas using ImageJ software (Schneider et al., 2012). The percentage of the total corneal depth penetrated by the *Fusarium* hyphae was calculated for each inoculated cornea.

### 7.3.9 Statistical Analysis

The analysis of *Fusarium* hyphal volume for all of the inoculated groups was performed using a repeated analysis of variance (ANOVA) and post hoc tests. The analysis of hyphal penetration depth within corneal tissues for all groups was performed using a one-way analysis of variance and post hoc tests.

### 7.4 Results

The percentages of the total *Fusarium* hyphal volume within corneal tissues on each day post-inoculation for the all groups are shown in Figure 7-1. All inoculated corneas in the I, IN, IXN, IX-48h-N and IN-72h-X groups developed *Fusarium* infection by day 2, while the un-inoculated corneas in the C group remained intact and uninfected (Figure 7-1A). The percentage of the hyphal volume over the duration of the experiment reflects the progression of the infection over time with high hyphal volumes being linked to more severe infection. The infection progressed over the duration of the
experiment in the un-treated (I) group and showed severe *Fusarium* infection on day 7; whereas the progression of the infection was significantly supressed in all treated groups. There is a significant difference in the percentage of the total volume occupied by *Fusarium* colonies between the un-treated (I) and treated (IN, IXN, IX-48h-N and IN-72h-X) groups on day 7 (Figure 7-1A). There are also differences in the *Fusarium* hyphal volume, though not large, between treated groups following various regimens of PACK-CXL/natamycin treatment (Figure 7-1B). The treated IXN and IN-72h-X groups showed the lowest density of hyphae on day 7 compared to the other treated IN and IX-48h-N groups. Repeated measures analysis of variance of the hyphal volume data over the duration of the experiment showed a significant difference between the inoculated groups (*P* < 0.001). *Post hoc* tests (Tukey) showed a significant increase in the *Fusarium* hyphal volume in the un-treated group (I) compared to each of the other treated groups (*P* < 0.001). Analysis of variance of the hyphal volume data on day 7 showed a significant difference between the inoculated groups (*P* < 0.001). *Post hoc* tests (Tukey) showed a significant increase in the *Fusarium* hyphal volume on day 7 in the un-treated group (I) compared to each of the other treated groups (*P* < 0.001).

Figure 7-2 shows fluorescence confocal microscopy images where the *Fusarium* infection can be seen as branching, green fluorescent fungal hyphae. The greatest infection progression can be seen as a high density of *Fusarium* hyphae within the 16 z-stack of confocal images in the un-treated I cornea on day 7 (Figure 7-2A). The density of hyphae is significantly reduced in the treated IN and IX-48h-N corneas (Figure 7-2C and G). The treated IXN and IN-72h-X corneas appeared to be free of any *Fusarium* hyphae on day 7 (Figure 7-2E and I). Focusing on one z-stack of confocal images, details
of *Fusarium* hyphae can be seen more clearly in the I, IN and IX-48h-N corneas (Figure 7-2B, D and H, respectively), whereas no fungal hyphae can be seen on day 7 in the IXN and IN-72h-X corneas (Figure 7-2F and J).

Images of the H&E stained corneal sections from all study groups are shown in Figure 7-3. The central and peripheral regions for a cornea from the C group can be seen in Figures 7-3A and B, where the corneal tissue is free from fungal invasion with intact stromal layers populated with keratocytes. Figures 7-3C and D show central and peripheral views of the H&E stained corneal sections from the un-treated I group in which large fungal colonies have accumulated over the corneal surface and a high density of *Fusarium* hyphae have penetrated deep within the corneal stroma by day 7. Figures (7-3E and F) and (7-3I and J) show central and peripheral views of the H&E stained corneal sections from the treated IN and IX-48h-N groups, respectively. In these two treated corneas, only few fungal hyphae can be seen where they have barely penetrated the epithelial layer into the beginning of the stromal tissues at the centre, whereas they did not exceed penetration of the epithelium at the periphery. Figures (7-3G and H) and (7-3K and L) show the central and peripheral views of the H&E stained corneal sections from the IXN and IN-72h-X groups, respectively, where the corneal tissues appear free from any fungal hyphae.

Figure 7-4 shows the percentage of the total corneal depth penetrated by the *Fusarium* hyphae at the central and peripheral regions for all inoculated groups. The percentage of the total corneal depth penetrated by the *Fusarium* hyphae by day 7 was 100% in both central and peripheral regions for the un-treated I group. The treated IN and IX-48h-N groups had a great reduction in the hyphal penetration depth.
at the centre, with no hyphal penetration within stromal tissues at the periphery. The treated IXN and IN-72h-X groups had no fungal hyphae both at the centre and periphery. Analysis of variance showed that there are significant differences in the percentage of the corneal depth penetrated by the fungal hyphae at both the central and peripheral regions between the different inoculated groups ($P < 0.001$). Post hoc tests (Tukey) showed significant differences in the percentage of the penetration depth of fungal hyphae at both the central and peripheral corneal regions between the un-treated group (I) and each of the treated groups ($P < 0.001$).
Figure 7-1: The percentage of the *Fusarium* hyphal volume for the study groups.

Figure 7-1A shows the mean percentage of the total volume occupied by the fungal hyphae within the anterior 100 µm of stromal tissues for the all groups on each day.
post-inoculation. The un-treated group (I) demonstrated significantly greater *Fusarium* hyphal volume on day 7 compared to the treated groups (IN, IXN, IX-48h-N and IN-72h-X). *P* value is derived from *post hoc* tests (Tukey) for ANOVA for day 7 data. Figure 7-1B shows an expanded view of the percentage of the total volume occupied by the fungal hyphae within the corneal tissues for the treated groups only. The treated IN and IX-48h-N groups show increase in the *Fusarium* hyphal volume on day 7 compared to the treated IXN and IN-72h-X groups which almost show nothing of the hyphal volume. The error bars represent ±1 standard error. C: Control; I: Inoculated only; IN: Inoculated, treated with natamycin at 24h post-inoculation; IXN: Inoculated, treated with PACK-CXL and natamycin at 24h post-inoculation; IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, then treated with natamycin at 48h post-inoculation; IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation, treated with PACK-CXL at 72h post-inoculation.
Confocal images of inoculated corneas show the extent of the branching green fluorescent fungal hyphal growth within the anterior central corneal region on day 7 following various regimens of natamycin/PACK-CXL treatment. Each image on the left (A, C, E, G and I) is a projection of a tiled z-stack of confocal images (x20 magnification). The projection of one z-stack of each tiled z-stack image (indicated by a yellow square) is shown on the right (B, D, F, H and J). I: Inoculated only cornea; IN: Inoculated, treated with natamycin at 24h post-inoculation; IXN: Inoculated, treated with PACK-CXL and natamycin at 24h post-inoculation; IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, treated with natamycin at 48h post-inoculation; IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation, treated with PACK-CXL at 72h post-inoculation.
Figure 7-3: H&E stained corneal sections from the different study groups.

Central and peripheral regions of the H&E stained corneal sections at x10 magnification from different study groups on day 7. Blue arrows indicate the *Fusarium*
hyphae which could be seen in the treated corneas. C: Control; I: Inoculated only; IN: Inoculated, treated with natamycin at 24h post-inoculation; IXN: Inoculated, treated with PACK-CXL and natamycin at 24h post-inoculation; IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, then treated with natamycin at 48h post-inoculation; IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation, treated with PACK-CXL at 72h post-inoculation.
Figure 7-4: The percentage of the total corneal depth penetrated by the *Fusarium* hyphae for the inoculated groups.

The graph shows the *Fusarium* hyphal penetration depth at the central and peripheral regions of the corneas from the inoculated groups on day 7. The inset graph shows an expanded view of the hyphal penetration depth of the corneas from the treated groups only. I: Inoculated only group; IN: Inoculated, treated with natamycin at 24h post-inoculation; IXN: Inoculated, treated with PACK-CXL and natamycin at 24h post-inoculation; IX-48h-N: Inoculated, treated with PACK-CXL at 24h post-inoculation, then treated with natamycin at 48h post-inoculation; IN-72h-X: Inoculated, treated with natamycin at 24h post-inoculation, treated with PACK-CXL at 72h post-inoculation.
7.5 Discussion

The present study shows that the monotherapy using natamycin has a beneficial impact in the management of fungal infection caused by *Fusarium*, but dual therapy of natamycin and PACK-CXL shows better efficacy by comparison. The combined therapy of natamycin and PACK-CXL is highly effective in reducing the volume of *Fusarium* hyphae, arresting the hyphal penetration into corneal tissue and suppressing the progression of the *Fusarium* infection. The early initiation of concurrent treatment of natamycin and PACK-CXL treatment has a considerably higher impact in controlling the progression of fungal infection and completely eradicating the *Fusarium* agents, when compared to the late intervention with either natamycin or PACK-CXL treatment. Delaying the natamycin treatment reduces treatment efficacy than does delaying the PACK-CXL treatment.

In this study, applying a monotherapy of natamycin in the IN group or a dual therapy of PACK-CXL/natamycin with a late intervention of natamycin in the IX-48h-N group resulted in presence of few fungal hyphae in the corneal tissues by day 7. Scratching the corneal surface pre-inoculation and/or debriding the epithelial layer pre-PACK-CXL may have contributed to the presence of more fungal hyphae in the central region of the corneas compared to the periphery in the IN and IX-48h-N groups. In these groups the volume of the residual fungal hyphae is not high, and may be suppressed and/or eradicated if the antifungal therapy were to be continued. This highlights the benefit of prompt intervention with both PACK-CXL and antifungal agents in the management of fungal infection. Conversely, it has been reported in an animal study that PACK-CXL procedure has a negative effect in cases of infectious keratitis by reducing the corneal
permeability to the antimicrobial agents (Tschopp et al., 2012). This might go some way to explain the reduced effect of combined PACK-CXL and natamycin in the treated group of the present study in which a late intervention of natamycin was initiated 24h post-PACK-CXL procedure (IX-48h-N). Nevertheless, another clinical study demonstrated that corneal permeability to antimicrobial medications is not significantly altered post-corneal cross-linking (Tappeiner et al., 2015).

Success of the dual therapy of natamycin and PACK-CXL in the management of *Fusarium* infection in this study is possibly mediated through the synergetic effect of the antifungal natamycin and the antimicrobial activity of combined riboflavin/UV-A light against fungus. It is likely that the PACK-CXL treatment supressed the growth of the *Fusarium* hyphae at the central region of the corneas, which was exposed to the UV-A light; whereas the natamycin further suppressed the growth of the residual *Fusarium* hyphae which survived the PACK-CXL treatment at the centre. Furthermore, the natamycin would suppress the growth of the fungal hyphae at the periphery of the corneas: these hyphae are protected from UV-A exposure due to the narrow, highly collimated beam produced by the clinical UV-A light emitter (cross-linker). Although the concentration of *Fusarium* spores present in the culture medium could not be calculated for treated groups, due to the opacity and dappling of the medium caused by the antibiotic, the flow of the residual natamycin into the culture medium after each administration would possibly affect the *Fusarium* sporulation and reduced the accumulation of spores in the culture medium where corneas were incubated overnight. In turn this may have reduced the potential for re-invasion of the corneal
tissues by germination of surviving spores and contributed to the success of the dual natamycin and PACK-CXL treatment in controlling the *Fusarium* infection.

It has been proposed that deep corneal infiltrates in cases of infectious keratitis might not be affected by PACK-CXL procedure because the penetration of UV-A light is limited to a depth of 200-300 µm within the corneal stroma (Wollensak et al., 2004, Kohlhaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016). This suggests that PACK-CXL procedure alone would be an ineffective therapy in the management of deep corneal infections, and should be supplemented by the use of antimicrobial medications. It has also been proposed that when the antifungal agents, such as amphotericin B, are used to treat fungal keratitis, the antifungals interact with the membrane sterols of the fungus and form channels in the fungal membrane. If this is the case then application of the PACK-CXL procedure after pre-treatment with an antifungal should be more effective in clearing the infection as the channels would allow the photosensitizer riboflavin to enter the fungal cells and destroy them (Baginski et al., 2006). In an *in vitro* study carried out by Sauer et al., it has been demonstrated that there is greater antifungal effect of combined amphotericin B and riboflavin/UV-A light against *Fusarium* sp, *Candida albicans*, and *Aspergillus fumigatus* compared to the use of amphotericin B alone (Sauer et al., 2010). The beneficial therapeutic effect of combined antifungal agents and PACK-CXL has also been demonstrated by a clinical study in case of fungal keratitis caused by *Aspergillus* spp (Anwar et al., 2011). In contrast, another clinical study found that in cases of fungal keratitis there is no significant advantage of using the combined PACK-CXL over using natamycin alone (Vajpayee et al., 2015).
Natamycin is a member of the polyene antibiotic family, but acts via a novel mechanism of action which is different from those of other polyene antibiotics (Te Welscher et al., 2008). It has been shown that the mode of action of natamycin is not by the formation of pores in the plasma membrane of fungi (Te Welscher et al., 2008). Instead, natamycin binds to ergosterol in the plasma membrane and forms a complex of natamycin-ergosterol, which inhibits transport of proteins across the plasma membrane. This results in blocking the growth of fungi and causing cell death (Te Welscher et al., 2008, Te Welscher et al., 2012). However, since the natamycin blocks the transport function of fungal membrane, this could inhibit the entry of riboflavin into the fungal cells. The exact mechanism action of the natamycin/PACK-CXL on fungal growth and sporulation is still not fully understood and requires further investigation.

In cases of suspected fungal keratitis, concurrent antifungal and antibacterial medication should be started until the microbiology confirms a fungal infection (Sara et al., 2016). The monotherapy using natamycin, the first line in the management of fungal keratitis, is recommended in the early stage of the infection until the fungal species has been identified. Once the identity of the fungus has been confirmed further antifungal agents can be used in cases of severe or unresponsive infection; voriconazole should be used for filamentous species or amphotericin B for yeasts (Sara et al., 2016). Identification of the underlying causative micro-organism plays a critical role in selection of the appropriate antimicrobial therapy for corneal infections and avoiding the irreversible damage to the eye. However, the identification of the underlying pathogen may result in delayed treatment. In addition, some organisms are
refractory to the conventional antimicrobial agents. Hence, PACK-CXL could be an effective combination therapy that is able to manage the corneal infections, particularly in those infections where identifying the micro-organism is difficult and/or time-consuming, or if it has become drug resistant.

The results obtained from present study indicate that the dual natamycin and PACK-CXL therapy can be considered as an effective treatment in the management of *Fusarium* keratitis. In this study *Fusarium oxysporum* was known to be the causative organism in the infections, which is not the case in the majority of clinical presentations of infectious keratitis. Furthermore, natamycin was used owing to its efficacy against the *Fusarium spp*. In cases of infectious keratitis where the underlying causative pathogen(s) is unknown, PACK-CXL treatment could be used as an initial intervention until the pathogen(s) has been identified and appropriate antimicrobial therapy administered.

### 7.6 Conclusion

The early use of combined natamycin and PACK-CXL treatment has a significant impact on suppression of both *Fusarium* hyphal volume and corneal tissue invasion, which makes it highly effective in the management of *Fusarium* infections. Delay in initiation of natamycin therapy could result in a reduction in the efficacy of the combined PACK-CXL/natamycin treatment in the management of *Fusarium* keratitis.
Chapter 8

Summary, Limitations and Future Work

8.1 Summary

The efficacy of the PACK-CXL procedure in controlling corneal infections in humans is still unclear for two main reasons: (1) the majority of the in vivo studies use animal models (2) the human clinical trials have produced variable results due to various confounding factors. Comparisons and interpretations of the mixed findings obtained in the clinical studies are difficult because of a lack of standardisation of many factors, such as the PACK-CXL treatment regimes, pathogen identification, severity of infection, late clinical presentations, diagnosis method, duration of antimicrobial therapy, the definition of infection resolution, and outcome assessments. Moreover, application of the corneal cross-linking procedure in the clinical trials must be done with caution to protect the patients from any potential risks or undesirable side effects.

This project established a novel ex vivo human corneal fungal infection model using eye-banked human corneas to investigate the efficacy of the combined riboflavin/UV-A light in the management of corneal infections caused by Fusarium oxysporum. The studies aimed to evaluate the antimicrobial effect of PACK-CXL treatment based on assessment of the Fusarium hyphal volume, the depth of hyphal penetration and the Fusarium sporulation, using two main techniques: confocal microscopy and histological staining. The key findings from the studies included in this thesis are:
8.1.1 Establishing the Corneal Infection Model

An *ex vivo* human corneal *Fusarium* infection model was successfully developed and established by means of utilising donor *post-mortem* human corneas and a recombinant strain of *F. oxysporum* expressing GFP: the GFP-expressing strain of the fungus permits the tracking of hyphal growth within the corneal tissue.

8.1.2 PACK-CXL Treatment Regime

With the model established, the PACK-CXL procedure was evaluated in chapters 2 and 3 as a primary, early intervention technique for the management of *Fusarium* infection using the standard PACK-CXL protocol, with an early intervention (at 24h post-inoculation) and a total of 2 days of incubation time post-inoculation. The results showed that the combined isotonic riboflavin/UV-A light appears to be a valuable approach to supress the infecting *F. oxysporum* growth and sporulation, and reduces the fungal load and depth of hyphal penetration into the central corneal tissues directly below the UV-A treatment beam, and consequently slowing the progression of fungal infection.

8.1.3 Trans-Epithelial PACK-CXL

In standard PACK-CXL the corneal epithelium is removed thus potentially exposing the underlying stroma to greater fungal hyphal invasion, especially in well-established (late stage) infections. In chapter 4, the substitution of isotonic riboflavin with trans-epithelial riboflavin was trialled to try to negate this effect. In addition, to mimic later disease presentation the corneas were treated at 48h post-inoculation (cf. 24h) and the post-inoculation incubation time extended to 3 days (cf. 2 days). Comparison of
the standard PACK-CXL and trans-epithelial PACK-CXL regimes showed that whilst the trans-epithelial PACK-CXL was superior in controlling the fungal infection neither treatment alone was effective in fully controlling the infection.

8.1.4 Pre-Treatment with PACK-CXL

The effect of the stiffened biomechanical properties of the corneal collagen produced by pre-treating ex vivo human corneas with PACK-CXL and the contributions of epithelium-on (intact) and epithelium-off (debrided) protocols of PACK-CXL were evaluated in resisting the spread of Fusarium infection in chapter 5. Both pre-treatment regimes failed to prevent or fully control F. oxysporum infection; however, the epithelium-on (plus trans-epithelial riboflavin) pre-treatment reduced the depth to which the hyphae penetrated the corneal tissue.

8.1.5 Effects Multiple PACK-CXL Treatments

The impact of treatment timing and repetition of the standard PACK-CXL in the management of Fusarium infection was investigated in chapter 6 with early (24h post-inoculation), late (48h or 72h post-inoculation) and repeated (24h and 36h post-inoculation) treatments and prolonged incubation time (6 days) post-inoculation of corneas. The early and repeated PACK-CXL treatments are effective in suppressing Fusarium hyphal growth and sporulation, and thus, reduce the pathogen load. Single or multiple treatments with the standard PACK-CXL protocol appear ineffective in reducing the depth of hyphal penetration into the corneal stroma and controlling progression of the infection.
8.1.6 PACK-CXL as an Adjuvant to Antibiotic Therapy

The effect of the PACK-CXL procedure as an adjuvant to antifungal (natamycin) therapy was evaluated in the management of *Fusarium* infections in chapter 7. This showed that the concurrent use of PACK-CXL and natamycin greatly reduced hyphal volume and penetration within the corneal tissues and controlled progression of the infection.

Overall, the project shows that combined riboflavin/UV-A light-induced corneal cross-linking technique holds promise in the management of infectious keratitis. The *in vitro* results suggest that the PACK-CXL therapy may contribute in managing a universally threatening ocular challenge.

8.2 Discussion

The human fungal pathogen *Fusarium oxysporum* was selected for this project due to its aggressive destruction of the cornea, and its regularity and frequency of appearance as a clinical pathogen (Thomas, 1994, Gopinathan et al., 2002, Bharathi et al., 2003, Marangon et al., 2004, Kawakami et al., 2015). In the early experiments (chapters 2 and 3) the *Fusarium* infection was at an early stage and the PACK-CXL procedure showed beneficial effect as a primary therapy in decreasing *Fusarium* load and penetration within central corneal tissues. These observations are consistent with a previous published case report which showed that the PACK-CXL procedure was an effective first-line therapy in the treatment of early fungal keratitis (Tabibian et al., 2014). The previous report used an accelerated PACK-CXL treatment while the standard PACK-CXL treatment was used in the studies described in chapters 2 and 3. Both accelerated and standard PACK-CXL treatments are likely to have similar effects
eliminating the microbes (Richoz et al., 2014), just as they have similar effects in corneal cross-linking (Tomita et al., 2014). The observations in chapters 2 and 3 are also in agreement with previous studies that examined the effect of combined riboflavin/UV-A light in the management of fungal keratitis clinically and suggested that this combination has positive antimicrobial effects, which assist in inhibiting the growth of fungus and managing the infection, although here antifungal agents were not used in contrast to other studies (Iseli et al., 2008, Anwar et al., 2011, Li et al., 2013, Hafez, 2014).

The *Fusarium* infection developed in a later study (chapter 4) was in a relatively late stage compared to that seen in the previous two studies. Both riboflavin formulations (isotonic and trans-epithelial riboflavin) and both PACK-CXL approaches (epithelium-off and -on) showed a beneficial effect in decreasing the infection load with a significant advantage of the trans-epithelial PACK-CXL over the standard PACK-CXL procedure in suppressing the progression of fungal infection further in the corneal tissue. Some studies showed that epithelium-on approach of corneal cross-linking with trans-epithelial riboflavin had similar effectiveness in stiffening the cornea compared to epithelium-off CXL approach (Filippello et al., 2012, Magli et al., 2013). In contrast, other studies reported less effect for epithelium-on CXL (Wollensak and Iomdina, 2009a, Bottós et al., 2011, Kocak et al., 2014, Aldahlawi et al., 2016). For treatment of infectious keratitis with PACK-CXL procedure, epithelium-on PACK-CXL was used by some studies (Anwar et al., 2011, Rosetta et al., 2013, Shetty et al., 2014). It is noteworthy that epithelium-off approach of PACK-CXL would contribute in removing a quantity of the infectious agents during the standard PACK-CXL procedure. However, intact epithelium in the trans-epithelial PACK-CXL procedure would assist in decreasing
the invasion of the deep stromal tissues by *Fusarium* hyphae. The trans-epithelial riboflavin formulation which was used in study described in chapter 4 contains Benzalkonium Chloride (BAC) (Raiskup et al., 2012), which has a toxic effect on corneal epithelium (Debbasch et al., 2000, Cha et al., 2004). This toxic effect of the trans-epithelial riboflavin along with the protective effect of epithelium-on approach of PACK-CXL may enhance the inhibitory effect of trans-epithelial PACK-CXL on fungi. Trans-epithelial riboflavin and epithelium-on PACK-CXL was used in a previous published study and showed a beneficial therapeutic effect in the management of fungal keratitis (Hafez, 2014). However, the efficacy of PACK-CXL treatment described in chapter 4 was less effective in controlling the *Fusarium* infection compared to that seen in the previous two chapters. This difference in the effect of PACK-CXL treatment in the management of *Fusarium* infections may be due to the stage of fungal infection at which the PACK-CXL was performed in the different studies. The effect of PACK-CXL treatment is limited to a depth of 200-300 µm from the corneal surface (Wollensak et al., 2004, Kohlihaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016), which means that the PACK-CXL treatment might have no beneficial effect on deeply penetrating fungi. The limited efficacy of PACK-CXL treatment is documented also by previous studies which reported that the PACK-CXL procedure is not effective in the management of moderate (Vajpayee et al., 2015), or deep fungal keratitis (Shetty et al., 2014, Uddaraju et al., 2015).

The infection model developed in this project presents the worst case scenario of *Fusarium* keratitis that would be considered hard to deal with. This serious form of *Fusarium* infection was a result of several factors. The number of spores used to
inoculate the corneas was far larger than would be seen in clinical cases: infection in patients can result from just a single spore. Therefore, the *Fusarium* infection developed at 24h, 48h and 72h post-inoculation and pre-PACK-CXL was often far greater than what would be observed at same time scale post-infection in patients. In addition, the infection was more severe and widespread because of the large scratch used to inoculate the corneas which is likely to lead to exaggerated levels of localised infections when compared to normal corneal infection process where much smaller breach in the cornea is enough to initiate the infection. Scratching the corneal surface and/or debriding the epithelium before PACK-CXL procedure also possibly contributed to the presence of more intensive fungal infection in the central region of the corneas compared to the periphery. The diameter of the corneal area irradiated by UV-A light was approximately 9 mm at the central region, which means that the *Fusarium* agents at the periphery of the cornea were not directly affected by the treatment. Consequently, the surviving hyphae within the stromal periphery could invade the central region post-PACK-CXL. Furthermore, the treated corneas were continually prone to attacks by the surviving fungal spores residing in the corneal culture medium, when the corneas were incubated overnight; this is very likely to have re-infected the corneas post-PACK-CXL treatment even if the treatment was successful at controlling the infection. All these factors may contribute to progression of the infection to a further advanced stage, and hence, reduced the efficacy of PACK-CXL treatment in the management of *Fusarium* infections. Moreover, the *ex vivo* human corneas used were from elderly donors, due to the lack of availability of younger corneas from the Eye Bank in which natural cross-linking of the corneas has not occurred.
The length of time that the corneas were stored in organ culture prior to use in this project was between 8 and 197 days (mean ± SD, 107 ± 54 days). There was considerable depletion in the populations of cells in the epithelium and stromal layers of the corneal buttons. This is probably due to the prolonged duration of the storage of corneas. In addition, considerable variation in stromal keratocyte population was seen between the corneas within and between the study groups. Stromal keratocytes may be beneficial in cases of fungal infection as they may play a role in arresting the corneal melt in the later stages of microbial keratitis (Chidambaram et al., 2017). Therefore, a reduction in the population of keratocytes prior to conducting the inoculation and treatment processes may slow corneal repair and affect the impact of PACK-CXL treatment in the management of fungal infections.

On the other hand, the ex vivo human corneal model of Fusarium infection which was established in this project had some biological limitations which possibly contributed in reducing the efficacy of PACK-CXL procedure as a monotherapy in controlling the Fusarium infections in vitro. The biological limitations of the ex vivo human corneal model include the lack of a tear production and drainage system to mimic the in vivo human lacrimal system. This permits accumulation of large numbers of Fusarium spores on and around the ex vivo cornea. The tear production and drainage system are a fundamental factor in washing the corneal surface, removing the fungal agents, and protecting in vivo treated corneas from being continually re-infected post-PACK-CXL treatment. The ex vivo human corneal model also lacks a wiping system to mimic the in vivo human eye lids movement. In vivo, the action of blinking is likely to reduce the spore load on the corneal surface, in contrast to the static ex vivo model used in this project. The wiping system would also play an important role in wiping the corneal
surface, which in turn, assists in further removing of the residual germinating *Fusarium* spores away from the corneal surface. By its nature, the *ex vivo* model system is lacking in host immune surveillance and response. The cells of the innate immune system, such as macrophages, can assist in recognizing microbial organisms and products and trigger host antimicrobial immune responses. It has been demonstrated that macrophages play a vital role in host resistance to fungal keratitis (Hu et al., 2009). Macrophage depletion leads to impaired immune response against fungal keratitis, which results in aggravating fungal infection and causing corneal perforation (Hu et al., 2009).

The short-comings described above no doubt contributed to a moderation in the potential efficacy of PACK-CXL treatments in controlling the *Fusarium* infections. These short-comings along with the previously mentioned issues ought to be taken into consideration when the effect of PACK-CXL treatment is evaluated. It is likely that the PACK-CXL treatment would be more effective in clinical conditions than has been found with the *ex vivo* model in this project. Improving the current *ex vivo* human corneal model of *Fusarium* infection by introducing the required systems to mimics *in vivo* human *Fusarium* keratitis more closely would help to address these short-comings. This could be achieved by introducing the following modifications. Using a pump-based system to automatically drip drops of corneal culture medium (or artificial tears) over the corneal surfaces. The dripped liquid would wash the corneas, remove the fungal agents, and then, would be discharged by using a flow system. This may assist in reducing the accumulation of spores in the immediate environment of the corneas thus reducing re-infection by germinating spores. Introduction of artificial lid
wipers, which automatically wipe the corneal surfaces and assist in further removing the residual fungal agents, would again remove spores. Mimicking the immune responses by introducing cells to the corneal environment, such as macrophages, to increase resistance against *Fusarium* growth and sporulation, and therefore suppress the spread of the fungal infection and enhance the therapeutic antimicrobial effect of PACK-CXL procedure. Reducing the concentration of *Fusarium* spores used for inoculation of the corneas along with longer incubation periods would allow the fungal hyphae to grow and mature prior to the PACK-CXL treatment. The mature slower growing hyphae may be more difficult to eradicate, and it is thought that they have a larger surface area over which to secrete digestive enzymes, and likely produce more proteases. The action of the proteases would change the corneal collagen structure, and thus, change the effectiveness of the PACK-CXL treatment as a disorientated matrix may reduce or block UV-A penetration. This approach may assist to further mimic the *in vivo* fungal keratitis, as the infection in patients can result from a single germinated spore, and may take a long time to receive treatment.

The beneficial effect of the PACK-CXL procedure in suppressing *Fusarium* growth, sporulation and penetration within corneal tissues, which was shown in the first chapters, is likely to be due to the antimicrobial action of the combined riboflavin/UV-A light which is mediated by inactivation of ribonucleic acids of organisms (Tsugita et al., 1965, Cadet, 1999, Goodrich, 1999, Corbin, 2002, Goodrich, 2011), and perhaps also by increasing the corneal stiffness and reducing enzymatic degradation (Wollensak et al., 2003b, Spoerl et al., 2004, Kohlhaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2013c, Beshtawi et al., 2014, Beshtawi et al., 2016). The
significant reduction in the number of *Fusarium* spores present in the culture medium after the PACK-CXL treatment is an indication that the direct antifungal effect of PACK-CXL is perhaps the major component in reducing corneal infection by *Fusarium*. The work described in chapter 5 showed that increasing the stromal tissue stiffness was insufficient for controlling subsequent development of *Fusarium* infection. This further supports the hypothesis that the antifungal activity of combined riboflavin/UV-A light is the greater contributory factor in the management of *Fusarium* infections.

The work described in chapter 6 showed that the early intervention and repetition of PACK-CXL procedure has an additional positive impact in reducing the load of the infection and suppressing its progression further. These observations are consistent with the results from previous published studies (Khan et al., 2011, Makdoumi et al., 2012, Saglik et al., 2013). However, the corneas used in the experiments described in chapter 6 were incubated for a longer time period (3-5 days) after PACK-CXL procedure then fixed, unlike the corneas in the previous experiments where that were incubated for one day only post-PACK-CXL treatment. The prolonged incubation of corneas after PACK-CXL procedure showed that the beneficial effect of PACK-CXL treatment in reducing the *Fusarium* infection load disappeared over time. There was no control on progression of the *Fusarium* infection as the hyphae had penetrated the whole thickness of all infected corneas. Again this highlighted that the effect of PACK-CXL treatment was due to the direct elimination of pathogen by the combined riboflavin/UV-A light. Penetration of the whole corneal thickness by *Fusarium* hyphae means the possibility of corneal perforation/melting. This observation is consistent with some clinical studies which failed to demonstrate a valuable impact of the PACK-
CXL treatment in controlling advanced fungal keratitis and reported the incidences of corneal perforation (Escarião et al., 2013, Uddaraju et al., 2015).

The beneficial effect of the dual therapy of PACK-CXL and natamycin in controlling *Fusarium* infection, which was shown in the work described in chapter 7, is probably due to the concurrent antimicrobial activity of combined riboflavin/UV-A light plus the effect of natamycin against *Fusarium* agents. Unlike the 200-300 µm penetration depth of PACK-CXL effect (Wollensak et al., 2004, Kohlhaas et al., 2006, Beshtawi et al., 2013a, Beshtawi et al., 2013b, Beshtawi et al., 2014, Beshtawi et al., 2016) at the central region of the cornea, natamycin was effective in both the central and peripheral regions of the cornea along with affecting the *Fusarium* sporulation and reducing the number of fungal spores in the culture medium. The dual therapy of PACK-CXL with antifungal agents showed confounding results in the management of fungal keratitis in a previous study (Vajpayee et al., 2015), and consistent results in others (Sauer et al., 2010, Anwar et al., 2011).

The reasons for the different effects of PACK-CXL procedure in the management of fungal keratitis between the different studies, including those in this project are still not fully understood. Nevertheless, the timing of the PACK-CXL procedure is likely to be a very important factor for the variation in the efficacy of PACK-CXL treatment. The delay in PACK-CXL procedure allows the infection to progress and the infectious agents to spread and penetrate into the deep stromal layers which in turn result in a reduction in the efficacy of the PACK-CXL treatment. In addition, different causative pathogens among the clinical studies could be a reason for the variation in the effect
of PACK-CXL treatment in treating corneal infections as various organisms may have different responses to the PACK-CXL procedure.

### 8.3 Future Work

The beneficial antimicrobial effects of the PACK-CXL and combined PACK-CXL/natamycin procedures shown by this and other studies support the need for further investigation of these potential treatments. Future laboratory-based work on human corneas can be conducted under controlled conditions using the *ex vivo* human corneal model of *Fusarium* infection established in this project. The *ex vivo* studies on human corneas could also be expanded in the future to include investigations the effect of PACK-CXL procedure in the management of corneal infections caused by other human pathogens.

Future expansion of the scope of this work could include the following:

1. Investigating the efficacy of the PACK-CXL procedure in the management of different kind of fungal infections, and addressing the relationship between the antimicrobial impact of PACK-CXL treatment and the response of different fungal genera and species.

2. Investigating the therapeutic antimicrobial impact of the PACK-CXL procedure as an adjuvant to appropriate antifungal agents in the management of other corneal fungal infections.

3. Investigating the antimicrobial activity of PACK-CXL technique against a variety of human pathogenic microorganisms, and assessing the efficacy of PACK-CXL
therapy in controlling infectious keratitis caused by, for example, bacteria or *Acanthamoeba* *spp*. Although UV-A induced antimicrobial action is broad spectrum, its efficacy may vary dependent upon the pathogen type.

4. Utilising the fresh-frozen corneal tissue from this project to investigate the effect of corneal cross-linking procedure on the corneal biomechanical properties. The techniques of choice include Atomic Force Microscopy (AFM), Scanning Acoustic Microscopy (SAM) and Nanoindentation. Since the corneal cross-linking leads to an increase in the stiffness of the corneal stroma, these techniques would assess the corneal biomechanical changes post-corneal cross-linking treatment, and explore the variation between the healthy un-cross-linked and cross-linked corneas on the one hand, and between the infected un-cross-linked and cross-linked corneas on the other. As increasing the rigidity of the corneal stroma following corneal cross-linking treatment leads to enhancement of stromal resistance to degradation by enzymes produced by microbes, such investigations would offer a chance to address the relationship between the increase in biomechanical stiffness of the cornea and the efficacy of PACK-CXL treatment in reducing the penetration of fungal hyphae within the corneal stroma.

5. Evaluating the safety of PACK-CXL treatment in the management of infectious keratitis. Although the studies have confirmed the safety of CXL treatment in the management of keratoconus (Wollensak et al., 2003a, Spoerl et al., 2007, Goldich et al., 2012), the safety profile of the PACK-CXL keratitis treatment needs to be established. It has not yet been demonstrated whether corneal
defects resulting from infectious keratitis allow the same – or greater - depth of UV-A penetration, and consequently, whether the PACK-CXL treatment, using the standard protocol, is a safe procedure in the management of infectious keratitis.

6. Development of new protocols for PACK-CXL procedure and investigating the therapeutic antimicrobial effect of various PACK-CXL protocols in controlling the corneal infections. The corneal cross-linking protocol utilised for treating keratoconus has been directly extrapolated by majority of researchers - including the studies in this project to treat infectious keratitis - where the total UV-A irradiation dose is under the limit known to damage the endothelium. However, some factors are still not clearly defined, including the most beneficial concentration of riboflavin and the optimum period of UV-A exposure required to kill the pathogens. A comparative investigation of the therapeutic antimicrobial effect of accelerated PACK-CXL procedure compared to the standard PACK-CXL procedure could further extend the therapeutic use of PACK-CXL. Furthermore, the antimicrobial effects PACK-CXL using different chromophores, such as Rose Bengal, should be evaluated for their efficacy and safety as photosensitisers.

8.4 Conclusions

This work has shown that in an ex vivo model of Fusarium keratitis PACK-CXL treatment alone has promising but limited capacity as a monotherapy in controlling progression of the infection. The treatment reduces the fungal load and depth of
hyphal penetration of the corneal tissue, particularly in the region directly beneath the UV beam. The dual therapy of PACK-CXL and the antifungal natamycin appears to be a highly effective treatment in the management of *Fusarium* keratitis. In addition, PACK-CXL treatment could be used as an initial therapy in cases of infectious keratitis where the causative pathogen(s) is unknown to reduce the pathogen load within the corneal tissues. Supressing the progression of the infection to some extent until the causative organism is identified and appropriate antimicrobial therapy is administered could mean the difference between success and failure of treatment.
References


keratitis: a 10-year review at a referral eye care center in South India. *Cornea*, 21, 555-559.


Appendices

Appendix A: Fungi

A.1 Overview

Fungi comprise a group of eukaryotic organisms. The majority of fungi reproduce through asexual and/or sexual spores, many of which are adapted for airborne distribution. Consequently, fungal spores are a constant part of the atmosphere both indoors and outdoors (Levetin, 2004, Deacon, 2005a). Usually, filamentous fungal colonies grow from one germinating spore which creates a germ tube (young hypha) that develops and extends behind the tip. During the development of the initial hypha and first-order branches, additional branches are generated behind their tips resulting in a network referred to as a colony, which ultimately, produces a typically circular outline (Figure A-1). Nonetheless, some fungi develop as single-celled yeasts, which reproduce through symmetrical fission (e.g. Schizosaccharomyces sp.) or asymmetrical (e.g. Saccharomyces sp.) budding (Deacon, 2005a).

![Figure A-1: The development stages of a filamentous fungal colony.](image-url)

Illustration of the developmental stages (A-E) of a fungal colony from a single germinating spore. Source: (Deacon, 2005b).
A.2   *Fusarium sp.*

The genus *Fusarium* includes approximately 200 species. *Fusarium* species are widespread environmental moulds that live in the soil and are the causative agent of many plant diseases (Sugiura et al., 1999, Naiker and Odhav, 2004). *Fusarium* species have a temperature preference of between 25 °C and 37 °C (Veglia and Marks, 1987). *Fusarium* colonies are usually white and cottony (Figure A-2), but as the colonies age may tend to get a pink hue (Veglia and Marks, 1987).

![Fusarium spp. colony](image1)

**Figure A-2: Fusarium spp. colony.**

*Fusarium* spp. is grown in agar growth medium for 72 hours at room temperature (27 °C). The *Fusarium* colony looks cottony and white. Adapted from: (Srinivasan, 2004).

*Fusarium oxysporum* is a major pathogenic cause of plant diseases (Dean et al., 2012). It has no sexual stage in its life cycle (Kistler, 1997), and produce three types of asexual spores which are: microconidia, macroconidia, and chlamydospores (Ohara and Tsuge, 2004) (Figure A-3). Microconidia are small oval spores and contain one or two cells.
They are abundant and produced usually on simple phialides under all conditions. Macroconidia are large spores and contain three to five cells. They are curved-pointed at both ends and less abundant than microconidia. Chlamydospores are round spores, contain one or two cells and have thick wall. They are produced in macroconidia or on older mycelium and survive for a very prolonged time (Ohara and Tsuge, 2004, Kang et al., 2014). The stages of infection of plant roots by *F. oxysporum* include spore adhesion, spore germination, root penetration, root cortex invasion and plant vascular tissue colonisation (Di Pietro et al., 2001) (Figures A-4 and A-5).

![Figure A-3: Fusarium oxysporum spores.](image)

The figure shows the three types of spores produced by *Fusarium oxysporum*: microconidia (A), macroconidia (B), and chlamydospores (C). Adapted from: (Wong, 2003).
Figure A-4: *Fusarium oxysporum* infection of tomato roots *in vitro*.

Tomato plant root inoculated with *Fusarium oxysporum* spores (microconidia) expressing green fluorescent protein (GFP), and then, imaged using fluorescence microscopy. Figure A-4A shows the germinated spores attaching to the tomato root at 24 hours post-inoculation. Figure A-4B shows the penetration hyphae growing into the root cortex at 5 days post-inoculation. Adapted from: (Di Pietro et al., 2001).

Figure A-5: Early and advanced stages of *Fusarium oxysporum* infections of tomato roots *in vitro*.

Figure A-5A shows the early stage of colonization of tomato root by *Fusarium oxysporum* expressing GFP using confocal microscopy at 3 days post-inoculation. Figure A-5B shows the advanced stage of tomato root colonization by *Fusarium* hyphae at 4 days post-inoculation. Adapted from: (Lagopodi et al., 2002).
A.3 Fungi as Human Pathogens

Fungi have a great range of activities as human pathogens and the filamentous fungi are the most frequently documented corneal pathogens (Srinivasan, 2004). By means of a flaw in the corneal epithelial layer, the stromal tissue may be invaded by fungi. This is followed by an attack on the corneal stroma, with consequent tissue necrosis, which stimulates a host inflammatory reaction, resulting in keratomycosis (Idiculla et al., 2009, Jaiswal et al., 2015). Usually, fungal keratitis manifests as severe inflammation, formation of an ulcer, as well as hypopyon, with indications of fungal hyphae in the corneal stroma (Thomas and Kaliamurthy, 2013) (Figure A-6). These organisms may also infiltrate an undamaged Descemet’s membrane and permeate the anterior chamber. Thus, eradication of these fungal pathogens is challenging to treat (Idiculla et al., 2009, Jaiswal et al., 2015). *Fusarium* species are considered the leading fungal pathogens that are responsible for destructive fungal keratitis (He et al., 2011). Unfortunately, in more than 6% of instances of keratitis *Fusarium* may perforate the cornea and create infiltrative endophthalmitis, leaving 30% of eyes having phthisis or needing enucleation, and 60% with finger-counting visual acuity or even worse visual impairment (Pflugfelder et al., 1988, Dursun et al., 2003).

**Figure A-6: Human fungal keratitis.**

This figure shows a corneal lesion and stromal infiltrate caused by fungal pathogens. Source: (Mravičić et al., 2012).
In general, the pathogenic mechanisms of fungi in human infections tend to be highly complex (van Burik and Magee, 2001). Un-published laboratory-based observations of *Fusarium* keratitis development in an *ex vivo* corneal model showed the following stages: spore adhesion on the corneal surface; spore germination; hyphal growth and extension, hyphal branching and fusion, hyphal penetration into the corneal tissue, and sporulation (Kurian, 2016) (Figure A-7).

**Figure A-7: Fusarium keratitis**

The figure shows an infected *ex vivo* human cornea with *Fusarium oxysporum* expressing GFP, using bright field and green channels of confocal microscopy. Figure A-7A shows a bright field image of the anterior region of the cornea with surface corneal scratches and *Fusarium* infection which is more intensive along the scratched areas. Figure A-7B shows the severe *Fusarium* infection where the fluorescent green fungal hyphae can be seen within the corneal tissue using the green channel alongside the bright field. Figure A-7C shows the dense *Fusarium* colonies using the green channel only.

*Fusarium* species are capable of producing mycotoxins, such as fusaric acid, moniliformin or fumonisin B1, which are associated with causing *Fusarium* keratitis (Naiker and Odhav, 2004). Production of mycotoxins is one of the most influential
factors in *Fusarium* pathogenicity (Sugiura et al., 1999). The development of fungal keratitis may also depend on the relationship between virulence of the invading fungus and host cell defences (van Burik and Magee, 2001, Wu et al., 2016). It has been shown that corneal epithelium (Zhu et al., 2015, Karthikeyan et al., 2011) and immune system (Jiang et al., 2015, Huang et al., 2014) play roles in the pathogenesis of fungal keratitis. However, knowledge of the pathogenicity associated with human fungal keratitis is still limited (Wu et al., 2016).
Appendix B: A Pilot Study

Is Corneal Cross-linking Effective in Protecting the Cornea From Developing *Fusarium* Keratitis?

**Contributions**

I designed the study in collaboration with my supervisors, conducted the experiments, prepared the corneal buttons for investigation, treated the corneas, completed the experiments, analysed the data, and wrote the manuscript. All this work was achieved with regular discussion, close collaboration and feedback on data analysis and writing from my supervisors: Dr Hema Radhakrishnan, Dr Chantal Hillarby and Dr Susan Shawcross. Prof. Fiona Carley and Mr. Arun Brahma regularly treat patients with infectious keratitis in the Manchester Royal Eye Hospital. Prof. Carly and Mr. Brahma met with the research team on a regular basis to discuss ideas and progress of the research studies.
Abstract Publication

Part of the data of this study was published in an abstract.


Conference Presentation

Part of the data in this study was presented at an international conference.


Acknowledgment

- The Manchester Eye Bank for providing the human corneal buttons for this study.

- Prof. Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain) for providing the *Fusarium* strain.

- Dr David Caballero-Lima (Manchester Fungal Infection Group, Faculty of Biology, Medicine and Health, University of Manchester, UK) for working on confocal microscopy and Imaris software.
B.1 Abstract

**Aims:** To evaluate the effect of pre-treating *ex vivo* human corneas with corneal cross-linking in terms of the protection from developing subsequent *Fusarium* infection.

**Methods:** Four *ex vivo* human corneas were assigned into one study group: cross-linked at 24h pre-inoculation, inoculated then fixed at 48h post-inoculation (XI); The CXL procedure was performed following the standard protocol for all XI corneas 24h pre-inoculation, and then, they were inoculated with *Fusarium oxysporum* spores. The corneas were imaged at 48h post-inoculation, and the *Fusarium* hyphal volume was calculated. The corneas were fixed at 48h post-inoculation, sectioned, haematoxylin and eosin (H&E) stained, and imaged. The *Fusarium* hyphal penetration depth within the corneal tissue was measured. The results of the *Fusarium* hyphal volume and penetration depth within the XI group were compared with the inoculated only then fixed at 48h post-inoculation (I) and inoculated, cross-linked at 24h post-inoculation then fixed at 48h post-inoculation (IX) groups from previous study in chapters 2 and 3.

**Results:** The XI group had a significantly greater volume of *Fusarium* hyphae compared to the I group (*P*=0.008) and IX group (*P*=0.001). The XI group had a significantly lower penetration depth by *Fusarium* hyphae at the central corneal area compared to the I group (*P*=0.015), and a significantly higher depth compared to the IX group (*P*=0.039).

**Conclusion:** Increasing the stromal tissue stiffness by pre-treating the cornea with combined UV-A/riboflavin-induced corneal cross-linking seems insufficient for controlling subsequent development of *Fusarium* keratitis although it is effective in decreasing the fungal hyphal penetration depth within corneal tissue.
B.2 Methods

Figure B-1: Main experimental procedures along with timeline.

The flow chart shows the main procedures performed on the different corneal groups from this study and previous study in chapters 2 and 3. I: Inoculated only then fixed at 48h post-inoculation; IX: Inoculated, cross-linked at 24h post-inoculation then fixed at 48h post-inoculation; XI: Cross-linked at 24h pre-inoculation, inoculated then fixed at 48h post-inoculation; CXL: Corneal cross-linking with isotonic riboflavin.
B.3 Results

All XI corneas developed *Fusarium* keratitis, seen as green fluorescent *Fusarium* hyphae using confocal microscopy. Figure B-2 shows the increased progress of *Fusarium* infection at 48h post-inoculation in the XI group (Figure B-2C) compared to the I and IX groups (Figure B-2A and B-2B).

The percentage of the fungal hyphal volume for XI group is shown in Figure B-3. The XI group showed a considerably greater percentage of fungal hyphal volume than I and IX groups. *Fusarium* colony volume data showed a significant difference between XI and I groups (*P* = 0.008) and a significant difference between XI and IX groups (*P* = 0.001) (Figure B-3).

The H&E stained corneal sections of an XI cornea are shown in Figure B-4. This figure shows the central and peripheral corneal regions, where the fungal invasion reached a depth of 162 µm through the central corneal stroma (Figure B-4A), whereas reached a depth of 378 µm in the periphery (Figure B-4B).

The percentages of the total hyphal penetration depth within corneal tissues are shown in Figure B-5. This figure shows that the XI group had higher central *Fusarium* penetration depth compared to the IX group and lower penetration depth compared to the I group, while there is no marked difference with regards to the peripheral infection between all groups. However, there is a variation within the XI group in terms of the central infection versus the peripheral infection. Analysis of variance of *Fusarium* hyphal penetration depth data within the central corneal tissues shows that there is a significant difference between XI and I groups (*P* = 0.015) and a significant difference between XI and IX groups (*P* = 0.039). In terms of the central infection
versus peripheral infection, there is no significant difference within the XI group ($P = 0.071$).

Figure B-2: Confocal microscopy images of I, IX and XI corneas at 48h post-inoculation.

The images show the *Fusarium* colonies (cytoplasmic GFP) for the different inoculated corneas at 48h post-inoculation. Each 2D confocal image is a projection of 100 µm thick z-stack using x20 magnification. There is severe *Fusarium* growth in the XI group (C) compared to the I (A) and IX (B) groups.
The percentage of total *Fusarium* volume within the corneal tissue

- *P* value = 0.008
- *P* value = 0.001

Figure B-3: The total *Fusarium* hyphal volume within the corneal tissues for I, IX and XI groups.

The graph illustrates the percentage of the total volume occupied by the *Fusarium* hyphae within the corneal tissues for the different inoculated groups at 48h post-inoculation. The XI group has significantly greater *Fusarium* hyphal volume compared to the I (*P* = 0.008) and IX (*P* = 0.001) groups. I: Inoculated only then fixed at 48h post-inoculation; IX: Inoculated, cross-linked at 24h post-inoculation then fixed at 48h post-inoculation; XI: Cross-linked at 24h pre-inoculation, inoculated then fixed at 48h post-inoculation.
Figure B-4: H&E stained sections of a cross-linked then inoculated cornea (XI).

H&E stained corneal sections of central (A) and peripheral (B) regions of an XI cornea (x10) at 48h post-inoculation. The figure shows the *Fusarium* invasion to be deeper and more extensive at the periphery of the cornea (B; blue arrow) than at the centre (A; blue arrow). The deepest level of penetration at which fungal hyphae could be seen is 162 µm at the centre and 378 µm at the periphery.
Figure B-5: The total penetration depth by the *Fusarium* hyphae within the corneal tissues for I, IX and XI groups.

The graph shows the percentage of the total stromal depth penetrated by the *Fusarium* hyphae at the central and peripheral corneal regions for the different inoculated groups at 48h post-inoculation. The XI group shows significantly greater *Fusarium* hyphal penetration depth at the central corneal region compared to the IX group (*P* = 0.039), and significantly less *Fusarium* hyphal penetration depth compared to the I group (*P* = 0.015). I: Inoculated only then fixed at 48h post-inoculation; IX: Inoculated, cross-linked at 24h post-inoculation then fixed at 48h post-inoculation; XI: Cross-linked at 24h pre-inoculation, inoculated then fixed at 48h post-inoculation.
Appendix C: List of Publications and Manuscripts to be Submitted for Publication


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Submitted: June 10, 2016
Accepted: October 10, 2016


Purpose. Some previous reports have established the use of photoinactivated chromophore-induced corneal cross-linking (PACK-CXL) in treating fungal keratitis. The results of these case reports have often been conflicting. To systematically study the effect of PACK-CXL in the management of Fusarium keratitis, we have developed an ex vivo model of human corneal infection using eye-banked human corneas.

Methods. Sixteen healthy ex vivo human corneas were divided into four study groups: (1) untreated control, (2) cross-linked, (3) infected with fungal spores, and (4) infected with fungal spores and then cross-linked. All infected corneas were inoculated with Fusarium oxysporum spores. The PACK-CXL procedure was performed 24 hours post inoculation for group 4. For PACK-CXL treatment, the corneas were debrided of epithelium, then 1% (wt/vol) isotonic riboflavin was applied dropwise at 5-minute intervals for 30 minutes and during the course of UV-A cross-linking for another 30 minutes. The corneas were imaged using a confocal microscope at 48 hours post inoculation, and the Fusarium hyphae volume and spore concentration were calculated.

Results. The infected and then cross-linked group had a significantly lower volume of Fusarium hyphae, compared to the infected (P = 0.001) group. In the infected and then cross-linked group there was significant inhibition of Fusarium sporation compared with the infected (P = 0.007) group.

Conclusions. A model of human corneal infection was successfully developed for investigation of the effects of PACK-CXL on fungal keratitis. A treatment regimen of combined UV-A/riboflavin-induced corneal cross-linking appears to be a valuable approach to inhibit the growth and sporation of Fusarium and suppress the progression of fungal keratitis.

Keywords: cornea, keratitis, fusarium, uv cross-linking, riboflavin

Fungal keratitis is a destructive corneal infection with a high level of ocular morbidity,1,2 which is particularly common within the developing world and subtropical areas, in patients with compromised corneal integrity. In the developing world, the annual number of recorded corneal ulcers is swiftly nearing between 1.5 and 2 million, and the actual figure is likely to be more. For most of these infections the final outcome is usually corneal opacity, or possibly even more devastating results such as corneal perforation, endophthalmitis, or phthisis.3 Fungal keratitis is viewed as a key blinding eye disease, in particular in agriculture-based geographic areas that have hot, humid, subtropical, and tropical climates. Fungi have been reported to cause 44% of all central corneal ulcers in South India, 56% in Bangladesh, and 17% in Nepal, in addition to 35% in South Florida and 37.6% in Gambia.4,5

Fungal keratitis has been documented to be caused by over 70 species covering 40 fungal genera.6 The key etiologic agents of fungal keratitis are the filamentous fungi, with Fusarium sp, comprising the most frequently occurring corneal keratitis-associated filamentous fungal genus.7,8 Fusarium sp are the key pathogens in 37% to 62% of instances of fungal keratitis.9,10,11,12 Fungal keratitis appears as ulcerative lesions13 and is usually managed using topical antifungal medications, occasionally integrated with subconjunctival injections, although therapeutic keratoplasty may be necessary for patients whose corneal infection persists.14,15 This type of corneal infection presents a problem for the ophthalmologist as its control is limited by the availability of effective antifungal agents and the level to which they may infiltrate the corneal tissue. In addition, fungal infection is inclined to mimic other kinds of infectious keratitis,16,17 often misdiagnosed or diagnosed only at a very late stage, following the failure of extensive treatments for viral or bacterial keratitis. Unfortunately, this delay in...
diagnosis and treatment can cause an irreparable loss of sight. Additionally, some microorganisms have displayed resistance to antimicrobial medications; therefore, the infections could progress through corneal ulceration leading to corneal melting or perforation, even after provision of the necessary treatment. Consequently, current research is aimed at finding an innovative treatment that actively controls and manages infectious keratitis, particularly in infections in which the microorganism is difficult to identify or those caused by drug-resistant microorganisms.

Ultraviolet (UV) light has been identified as an efficient method for disinfection, particularly of water, and as an alternative to chemical treatment; this has resulted in a large demand for UV treatment globally. Regardless of variations in modes of inactivation, disinfection by UV is effective against a range of pathogens encompassing bacteria, protozoa, and viruses. Riboflavin comprises a natural substance and is part of the vitamin B group, which is readily available in various foods. Photoactivated riboflavin has been utilized to minimize the microbial burden of liquids over many years. A good example is within the sphere of transcription medicine, where concentrations of platelets are regularly treated with combined riboflavin and UV-A light. The mixture of riboflavin and UV-A light results in irreversible damage to the DNA and RNA of pathogenic organisms, preventing genome replication and thus preventing infection.

A UV-induced corneal cross-linking (CXL) is a non-invasive procedure established for the management of keratoconus and corneal ectasia. This technique relies on using the photosensitizer riboflavin and UV-A to create photochemical cross-links within the anterior stromal collagen fibres and thus raise the biochemical and mechanical strength of the cornea, which in keratoconus halts the progression of the corneal thinning.

Photoactivated riboflavin and UV-A light have been trialed as potential adjuvant treatment for the management of corneal infections, particularly for the antibiotic-resistant forms of infectious keratitis. This treatment may also be beneficial where corneal melting occurs. Corneal stiffening resulting from this treatment could reduce melting and reduce or prevent corneal perforation. The term photoactivated chromophore (PACK-CXL) was utilized instead of simply CXL in order to distinguish between using the CXL for treatment of infectious keratitis and for keratoconus.

The aim of this study was to establish an ex vivo human corneal infection model by means of utilizing donor postmortem human corneas, infecting them with a fluorescently labeled Fusarium oxysporum, and then investigating the effect of PACK-CXL treatment in the management of fungal keratitis.

MATERIALS AND METHODS

Ex Vivo Human Cornea

Sixteen healthy postmortem human corneal buttons from nine male (M) and seven female (F) donors were used in this study. The corneas provided by the Manchester Eye Bank (NHS Blood and Transplant, UK) were preoxygenated for research if they were not suitable for transplantation. They were from donors in the age range 58 to 83 years (mean ± SD, 67.5 ± 7.6 years). At the Manchester Eye Bank, the corneas, along with a 3-mm scleral rim, were extracted from eyes less than 24 hours after death and placed into Eagle’s minimal essential medium containing 2% (vol/vol) fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (all Sigma-Aldrich Ltd, Poole, UK) and maintained at 34°C. The corneas used in this research had been released for research because the endothelial cell count was below that required for transplantation or for other reasons that did not directly affect the eye, such as inadequate medical history. The project had full ethical approval from the National Health Research Committee (No. 040811) and adhered to the terms of the Declaration of Helsinki.

The corneas were randomly assigned to one of the four groups with four corneas in each group as follows:

- Group C: Control (not infected, not treated with CXL procedure) (one M and three F: 59–83 years)
- Group X: Crosslinked (not infected, treated with CXL) (three M and one F: 59–71 years)
- Group I: Infected (infected, not treated with PACK-CXL) (two M and two F: 59–76 years)
- Group IX: Infected and then crosslinked (infected and then treated with PACK-CXL) (three M and one E: 58–71 years)

Corneal Tissue Culture

The human corneal buttons were transferred into six-well cell culture plates (Costar, Corning Ltd, Elvex, UK) with the outer surface of the cornea uppermost and submerged in fresh culture medium (Dulbecco’s modified Eagle’s medium [DMEM], 1000 mg/l glucose [Sigma-Aldrich Ltd], supplemented with 10% [vol/vol] FBS [Labtech International Ltd, Uxbridge, UK], 2 mM glutamine, 1% [vol/vol] penicillin with streptomycin [all GE Healthcare Life Sciences, Little Chalfont, UK], and 0.1% [vol/vol] hydrocortisone [JH126, Sigma-Aldrich Ltd]). The corneas were incubated at 34°C in 5% CO2 for a minimum of 72 hours prior to the infection and CXL processes (Fig. 1).

The manipulation of corneal tissue, subsequent infection, and CXL treatment processes were performed in a recirculating Class II Microbiological Safety Cabinet (Labeicare S.C.R, Labcare Systems Ltd, Clevedon, North Somerset, UK).

Fungal Strains

The infecting fungus used was Fusarium oxysporum f. sp. pyrosporii strain 4287 (race 2) expressing a cytosolic green fluorescent protein (GFP). The strain was kindly provided by Antonio Di Pietro (Department of Genetics, University of Cordoba, Cordoba, Spain). The F. oxysporum GFP strain was cultured in Potato Dextrose Broth (PDB) (BD Difco, Oxford, UK) for 5 days at 28°C. On the day of corneal inoculation, the spores (microconidia) were harvested. The fungal spore suspension was filtered using a filter cloth (Miracloth; Millipore Limited, Watford, Hertfordshire, UK), and a spore pellet was collected by centrifugation at 3260g for 5 minutes followed by washing with sterile distilled water and further centrifugation. The spore pellet was then resuspended in 1 mL sterile distilled water, diluted 1/1000 with sterile distilled water, and the concentration of this spore suspension was determined using a Fuchs-Rosenthal hemocytometer (Scientific Laboratory Supplies, Nottingham, UK). The spore concentration was adjusted to 5 × 10⁶ spores/ml using fresh culture medium, and 10 μL of this suspension was used to inoculate each of the scratched corneas as detailed below.

Corneal Infection Process

Seventy-two hours post corneal culturing, two of the human corneas, the 1 and IX, were inoculated with the spores of F. oxysporum expressing cytosolic GFP. The GFP fluorescence facilitated the tracking of the fungal hyphal invasion through the corneal layers.

As an ex vivo model of corneal insult and fungal infection, corneal buttons were mounted upon artificial anterior cham-
Figure 1. The main steps of the methodology. The flow chart shows the main procedures, culturing, scratching, infection, cross-linking, and imaging processes, which were carried out for the different experimental groups, along with the time scale (peach color: procedure; green color: time scale).
FIGURE 2. A human corneal button fixed onto an artificial anterior chamber. The figure shows a human corneal button fixed onto an artificial anterior chamber and fitted with PBS via a tube, valve, and syringe (A) to mimic intraocular pressure, which maintains the shape and stiffness of the corneal surface (B).

FIGURE 3. Confocal microscopy images of infected corneal buttons 48 hours post inoculation following PACK-CXL treatment. Each image is a projection of a z-stack of confocal images (<20x magnification) that is 100 μm thick. The images of the different infected corneas show the extent of the branching hypha growth of the fungus, which is expressing GFP in its cytoplasm at 48 hours post inoculation. Extensive fungal growth is visible in the I (A) group, while there are fewer hyphae evident in the IX group (B).

CXL Procedure
The corneas in the X and IX groups were treated with the corneal cross-linking technique according to a standard CXL protocol.25 The X and IX corneas were subjected to the cross-linking procedure 24 hours post inoculation with spores.

The corneal button was mounted on an artificial anterior chamber as described above. Phosphate-buffered saline (PBS) was infused through a tube, valve, and syringe to fill the interior of the cornea to mimic the in vivo intraocular pressure and maintain corneal stability and shape throughout the CXL procedure (Fig. 2).

Debridement of the corneal epithelium layer was performed to allow adequate riboflavin penetration to the corneal stroma. The central 7- to 9-mm-diameter area of the corneal epithelium was debrided with a single-use surgical blade (size 10; Swann-Morton, Sheffield, England), then wiped using a cotton eye spear (Biber) to remove any remaining epithelial cells. The cornea was then immersed in an air-entrained riboflavin solution (0.1% wt/vol riboflavin, 20% [vol/vol] deionized T300) and exposed to UV light for 5 minutes.

The prepared cornea was irradiated with UV-A light utilizing a medical electrical UV-A light emitter (370 nm, irradiance of 3 mW/cm²; VEGA, C.S.O. srl, Florence, Italy), which delivered a total UV-A irradiation dose of 5.4 J/cm² to the corneal surface over a period of 50 minutes, along with application of further isotonic riboflavin, again at 5-minute intervals. Following this procedure, the cornea was rinsed several times with sterile PBS and placed in a fresh six-well tissue culture plate along with fresh culture medium and incubated at 34°C in 5% CO₂.

Imaging
Twenty-four hours post inoculation, the corneas were inverted and placed on a two-well chamber slide (Thermo Fisher Scientific, Rochester, NY, USA) and imaged using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems Ltd., Buxtehude, Germany) with a 40× objective. At 48 hours post inoculation, the corneas were imaged using a camera (Canon PowerShot SX10, zoom lens ×12, 8.0 megapixels; Canon Ltd., Reigate, Surrey, UK) and a confocal microscope. The confocal microscopy images were analyzed with the SURPAS module of the Imsars vi8.0 software (Bitplane Scientific software module; Bitplane AG, Zurich, Switzerland).

RESULTS
Fusarium keratitis-like infection developed successfully in all inoculated corneas. The effect of PACK-CXL treatment on Fusarium was determined by measuring fungal hyphal volume within the corneal tissues.

Twenty-four hours after infection, all inoculated corneas developed Fusarium infection, whereas the C and X corneas remained intact and uninfected. Forty-eight hours after the initial inoculation, the fungal infection had progressed in the I group, while IX corneas showed less severe infection. Figure 3 shows the reduced progress of Fusarium infection at 48 hours post inoculation in the IX group (Fig. 3B) compared to the I (Fig. 3A) group. The Fusarium infection can be seen as branching, green fluorescent fungal hyphae.

The percentage of the total volume occupied by the fungal hyphae and the quantity of fungal spores in the culture medium from each infected group are shown in Figures 4 and 5, respectively. There is variation in the volume occupied by Fusarium hyphae between the infected groups at 48 hours after inoculation. The I group showed greater amounts of Fusarium hyphae and, consequently, more severe fungal infections compared to the IX group. Analysis of variance (ANOVA) of the Fusarium colony volume data showed a significant difference between the I and IX groups (P < 0.001). These results show that the PACK-CXL treatment controlled the fungal infection in the IX group, where the growth of fungus was inhibited by the PACK-CXL procedure.
Treating Fungal Keratitis With Corneal Cross-Linking

The percentage of total Fusarium volume within corneal tissue

![Graph showing the percentage of total Fusarium volume within corneal tissue. The y-axis ranges from 0 to 30, with increments of 5. The x-axis categorizes the data into I and IX groups. The graph shows that the I group had a higher percentage compared to the IX group.]

**Figure 4.** The percentage of the total volume occupied by the fungal hyphae. The graph shows percentage of the total volume occupied by the fungal hyphae, as visualized by the GFP signal from the fungal hyphae within the corneal tissues for the different infected groups at 48 hours post inoculation. The I group exhibited greater Fusarium hyphal volume compared to that of the IX group.

There was also variation in the quantity of Fusarium spores that were present in the culture medium at 48 hours post inoculation between the infected groups. The IX group showed a considerable decrease in the number of Fusarium spores present compared to the I group. Analysis of variance of the Fusarium spore quantity data shows that there is a significant difference in fungal spore density between the I and IX groups \( (P = 0.007) \). These observations show that the combined riboflavin and UV-A cross-linking treatment had an inhibitory effect on the Fusarium sporulation.

**Discussion**

This study aimed to establish a model of human corneal fungal infection using human donor corneas infected with *E. enzooticus* expressing cytosolic GFP, which permits the tracking of hyphal growth within the corneal tissue. With the model established, the combined UV-A/riboflavin-induced corneal cross-linking as a primary therapeutic technique for the management of fungal keratitis could be evaluated. To our knowledge, this study is the first ex vivo attempt to model the treatment of human fungal keratitis using this PACK-CXL technique.

*Fusarium enzooticus* was selected for this study due to its aggressive destruction of the cornea and its regularity and frequency of appearance as a clinical pathogen. Fungal keratitis caused by *Fusarium spp.* is recognized as one of the most sight-threatening corneal infections.\(^{27,38,39,41-44}\) The control of fungal keratitis presents a challenging problem mostly as a result of delayed diagnosis and limited options for therapy.\(^{27}\) As current antifungal medications display little corneal infiltration and restricted effectiveness, the treatment of ocular diseases caused by fungi is presently unsatisfactory.\(^{1,38}\) In addition, most antifungals utilized in the management of *Fusarium* keratitis require a long course of treatment and frequently fail to preserve vision.\(^{39}\)

Introduction of a therapy based upon the photosensitizer riboflavin and UV-A light was first described by Wellensiek et al.\(^{39,40}\) for the induction of corneal cross-linking for corneal ectasia. Recently, indications for the use of the CXL technique have expanded to include Fuch's corneal dystrophy\(^{38}\) and pseudophakic buphthalmic keratopathy\(^{38}\) as well as infectious keratitis.\(^{38,39,41-45}\) The use of the CXL technique for treatment of corneal ectasia and advanced keratoconus is well established, while the efficacy of the PACK-CXL procedure in the management of infectious keratitis is still subject to appraisal.

In this study, a human ex vivo corneal *Fusarium* keratitis model was established. The results show that there was a significant difference in the volume of invading fungus within the infected corneal tissue between the groups, with the infected corneas treated with PACK-CXL showing a significant decrease in the fungal volume compared to the other experimental group. This is a good indication that the PACK-CXL procedure is effective in suppressing the progression of fungal infection. The favorable outcomes obtained in this study are contradicted by some published studies, which report that the PACK-CXL procedure is not effective in managing fungal keratitis.\(^{39,40,47}\) Nevertheless, these observations are supported by numerous in vivo studies that attempted to examine the influence of combined riboflavin/UV-A in the management of infectious fungal keratitis. It is suggested that this combination has positive antimicrobial effects, which assist in inhibiting the growth of pathogens and managing the infection.\(^{38,48-50}\) The beneficial antimicrobial effect of the PACK-CXL procedure in suppressing infection by *Fusarium* is likely to be attributable to several mechanisms. Inactivation of ribonucleic acids of organisms may occur by the combined UV-A/riboflavin-induced cross-linking that may have a cytotoxic effect on the pathogens.\(^{38,39}\) In addition, it has been shown that the PACK-CXL technique brings about cross-linking in the corneal collagen fibers, thus increasing the strength and simultaneously reducing its penetrability by fungal hyphae. Furthermore, the cross-linked collagen is more resistant to enzymatic digestion by microbial pathogens, which in turn reduces corneal melting.\(^{32,53}\) In this study, the direct antifungal activity of the UV-A/riboflavin combination is perhaps the greater contributory factor. It is also possible that cross-linking might entrap the fungal hyphae within the collagen matrix, thereby reducing the growth rate still further. The proposal that the direct antifungal effect of the UV-A/riboflavin treatment is the major component in reducing corneal infection is supported by the fact that the IX group showed a significant reduction in the number of *Fusarium* spores present after the PACK-CXL.

**Figure 5.** The quantity of *Fusarium* spores in the infected groups. The graph shows the number of *Fusarium* spores per milliliter of corneal culture medium at 48 hours post inoculation for the infected groups. The quantity of *Fusarium* spores of the IX group was significantly less than that of the I group \( (P = 0.007) \).

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treatment compared with the I group. This is indicative of the riboavin/UV-A treatment having an inhibitory effect on Fusarium sporulation. On the other hand, dehiding the epithelial layer post inoculation and prior to the PACK-CXL treatment may have played a role in reducing the infection by removing some of the Fusarium hyphae growing within the corneal epithelial tissue. Nevertheless, the reduction in spore numbers in the culture medium post PACK-CXL treatment lends some support to the hypothesis that the riboavin/UV-A combination has an anti-infective action.

Iseli et al.16 set out the first published clinical trial to investigate the effect of PACK-CXL application on a series of five cases, with infectious melting keratitis caused by pathogens including fungi that showed no response to intensive antibiotic therapies. They found that following PACK-CXL, corneal melting was arrested in all cases, and that there was no need for emergency corneal transplantation as would normally have been the case. Another clinical trial presented results that support the efficacy of the PACK-CXL procedure.40 The study looked at seven eyes with infectious keratitis associated with corneal ulcers and melting, some of which were caused by fungi. After PACK-CXL treatment, it was noted that the corneal infections had been well controlled, in all cases, progression of the corneal melting had stopped and ulcers had healed with no severe consequences, and no further surgery was necessary. In recent studies, the therapeutic efficacy of the PACK-CXL technique was assessed by Hafezi26 on a series of five eyes with resistant fungal corneal ulcers. Even though notable vision improvement was not achieved post PACK-CXL treatment, the findings showed that the PACK-CXL procedure can be effective in the management of resistant corneal ulcers, as all cases showed improvement on infection signs and complete ulcer healing. In addition, Li et al.45 presented the clinical findings in eight patients with fungal keratitis who received PACK-CXL treatment. After the PACK-CXL procedure, resolution of infection and ulcer healing were achieved in all cases, and none required further surgical intervention. Li et al.45 suggested that the PACK-CXL procedure is a viable option for treating fungal keratitis.

However, confounding outcomes were found in a clinical trial conducted by Escurio et al.17 on 11 eyes with bacterial or fungal keratitis that were nonresponsive to the medications administered for at least a period of 1 week. The bacterial keratitis cases showed relief in the symptoms post PACK-CXL treatment, whereas in the fungal keratitis cases no improvement was observed, which indicated that the effectiveness of PACK-CXL procedure is related to the causative agent. This finding is consistent with a study by Vajpayee et al.16 in which 41 cases of fungal keratitis were divided into two groups. The first group was treated with the PACK-CXL technique along with antifungal therapy, while the second group received antifungal medications only. It was observed that infection resolution was achieved in 90% and 85.7% of cases, the recovery time rate was 31 ± 27 and 31 ± 20 days, and the best-corrected vision rate was 1.13 ± 0.55 and 1.25 ± 0.46 (logMAR) in the first and second group, respectively. Moreover, keratoplasty surgery was carried out in two cases from the cross-linked group and in three cases from the control group. Vajpayee et al.16 concluded that there was no beneficial effect of the combined antifungal/PACK-CXL treatment over the antifungal agent alone. Additionally, Uddaraju et al.17 evaluated the efficacy of PACK-CXL treatment in resistant deep stromal keratitis caused by fungi. With 15 cases were included, which showed no response to antifungal medications administered for 2 weeks. The cases were randomly divided into cross-linked and control groups; the cross-linked group was treated with UV-A/riboflavin combination along with the medications, while the control group received antifungal therapy only.

Uddaraju et al.17 concluded that the PACK-CXL procedure is not an effective treatment in the management of advanced fungal keratitis, as the crosslinked group experienced a higher perforation rate than the control group. Notably, the majority of the clinical studies were carried out on resistant forms of corneal infections; the antimicrobial medications had been administered for various durations and mostly in cases in which the infections had failed to resolve. In such cases, the PACK-CXL technique was implemented only after the infections had progressed to late stages and had caused further serious damage to the cornea. Thus, late intervention could have resulted in a great reduction in the effectiveness of PACK-CXL procedure, minimization of the healing rate, or even a treatment failure.

In conclusion, the treatment of Fusarium keratitis with combined UV-A/riboflavin-induced corneal cross-linking is effective in inhibiting Fusarium sporulation and hyphal growth, and thus reduces the intensity of infection.

Acknowledgments

The authors thank the Manchester Eye Bank for providing the human corneal buttons for this study.

Supported by Ministry of Health, Riyadh, Saudi Arabia (JMA), DCL was supported by a grant to David W. Dennis (The University of Manchester, The National Aspergillus Centre, University Hospital of South Manchester, Manchester, UK) and from the Global Action Fund for Fungal Infections (GAFF) to NDR.

Disclosure: J.M. Alshehri, None; D. Caballero-Lima, None; M.C. Hillarby, None; S.G. Shawcross, None; A. Brahuna, None; F. Carley, None; N.D. Read, None; H. Rauthakrishnan, None.

References


Does Corneal Cross-linking Prevent Fusarium Keratitis?

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Commercial Relationships Jawaher Alshehri, None; M Chantal Hillarby, None; Susan Shawcross, None; Arun Brahma, None; Fiona Carley, None; Hema Radhakrishnan, None

Support Supported by Ministry of Health, Riyadh, Saudi Arabia

Abstract

Purpose: To evaluate the effect of the photoactivated chromophore corneal cross-linking (PACK-CXL) procedure as preventative therapy for Fusarium keratitis, using ex vivo human corneas and a histological staining technique.
Methods: Sixteen ex vivo human corneas were randomly divided into four study groups: uninfected, untreated control (C), infected only (I), cross-linked then infected (XI) and infected then cross-linked (IX). All infected corneas were inoculated with Fusarium oxysporum spores. The PACK-CXL technique was performed on the cross-linked then infected group 24h pre-inoculation, and on the infected then cross-linked group 24h post-inoculation. For the PACK-CXL procedure, the corneas were debrided of epithelium then 1% (w/v) isotonic riboflavin was applied dropwise at 5-minute intervals for 30 minutes and during the course of UV-A cross-linking for another 30 minutes. All corneal buttons were PFA-fixed, embedded in OCT, sectioned at 30 μm thickness and haematoxylin and eosin (H&E) stained. The stained sections were imaged using a Zeiss Axiosstar microscope, and the depth of fungal hyphal penetration within the stromal tissue was measured using ImageJ.

Results: The depth of fungal hyphal penetration in the central region of the cornea was significantly less in the IX group than in the I group (p=0.001) and the XI group (p=0.039), and significantly less in the XI group than in the I group (p=0.015). However, there was no significant difference in the peripheral infection between the inoculated groups. Furthermore, in terms of the central versus peripheral infection among each group, there was no significant difference observed within the I and XI groups, while the difference was significant in the IX group (p=0.015).

Conclusions: The PACK-CXL therapy seems to be a valuable approach in reducing the depth of Fusarium hyphal penetration in the corneal tissues. However, riboflavin-activated PACK-CXL was not effective as a preventative measure for Fusarium keratitis.

This is an abstract that was submitted for the 2017 ARVO Annual Meeting, held in Baltimore, MD, May 7-11, 2017.