



**Clinical and laboratory investigation of the biomechanical properties of
the cornea**

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CLINICAL AND LABORATORY INVESTIGATION OF THE BIOMECHANICAL PROPERTIES OF THE
CORNEA



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PhD life is like riding a very long way surrounded by obstacles,

To achieve success, you must keep moving, working hard and be patient !!

*By: Tariq A. Alhamad
2011*

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I wish to express my gratitude to God (Allah) for helping me to complete this thesis and for giving me what I asked of Him.

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Abstract

Understanding the biomechanical properties of the cornea is important in order to develop and improve new reliable standard procedures which can be used effectively to assess corneal behaviour in any disease condition, or before/after any ocular surgery. We believe that the Ocular Response Analyzer (ORA) is the only device that can measure the biomechanical properties of the cornea in vivo. However, it has been used for the first time both in vivo and in vitro. This thesis presents a clinical and laboratory investigation of the biomechanical properties of the cornea before/after LASIK and corneal cross-linking to improve our understanding of the knowledge required in both the laboratory and the clinic. Different machines were used in this project, including an ORA, an Oculus Pentacam, a spectrophotometer and a UV-X Illumination system.

Laser in situ keratomileusis (LASIK) is, at present, one of the most well-known operations used to correct refractive errors; however, ocular problems arising from corneal thinning have been reported in some previous studies. Therefore, I looked at the effects of surgery on the central/peripheral thickness and the anterior/posterior curvature, and determined to what extent they affect the biomechanical properties of the cornea.

During the past decade, much research has focused on improving and developing a new operation called corneal collagen cross-linking with riboflavin and UVA, which is used to stop the progression of keratectasia in the cornea (which occurs in keratoconus and sometimes follows refractive surgery). In the next phase, a range of experiments were conducted on cross-linking to determine to what extent this operation affects the molecular structure and biomechanical properties of the cornea.

This thesis has shown for the first time that it is possible to obtain ORA signals in vitro and this opened up the possibility of examining whole eyes as well as excised corneas. It is also confirmed that the values of CH do not represent only a corneal biomechanical property, but rather depend on the presence of the rest of the eye. These in vitro studies have opened up a number of possibilities for future corneal biomechanical studies.

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Chapter1. Introduction

1.1 Structure and anatomy of the human eye

Vision is a complex process which depends on many processes that must work together. The human eye consists of very precise parts, and each part has a different function; this section starts by looking at the external parts and continues with the internal parts of the eye.

The major constituents of the eye are shown in Figure 1.1. The cornea is one of the principal optical components (Maurice 1984); it constitutes the outer part of the front of the eye, and provides 40D or more of the eye's optical power. It is surrounded by fluid anteriorly (tear film) and posteriorly (aqueous humour), and is composed of clear transparent tissue. There are no blood vessels in the cornea; however, it is extremely sensitive because there are many nerve endings (Forrester et al. 2002). The corneal thickness at the apex is around 0.52mm and it increases peripherally to around 0.67mm. The diameter of the cornea is typically 11.7mm horizontally and 10.6mm vertically; i.e. the horizontal diameter is greater than the vertical diameter (Hogan et al. 1971). It comprises five layers of corneal tissue: epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium (Davson 1984).

The rest of the outer eye, constituting approximately 85% of the external surface of the eye, is the fibrous sclera (the white of the eye) (Oyster 1999; Saude 1993). It is a dense and tough structure which provides protection for the inner contents of the eye. Six muscles are attached to the sclera and control the eye's movement; also, the optic

nerve connects to the sclera at the back of the eye (Carpenter 1977). Children have a thin and bluish sclera, whereas in older people it tends to become yellowish because of the deposition of fat (Saude 1993). The sclera can be divided into three layers: the episcleral tissue, the substantia propria and the lamina fusca. Each of these layers has a special and unique structure (Duke-Elder et al. 1968).

The area which is approximately 1.5-2.0mm wide, marking the transition between the cornea and the sclera, is called the limbus (Hogan et al. 1971). It is an important area because it is related to some intraocular surgery such as for cataracts and glaucoma.

The region behind the back of the cornea and in front of the iris is called the anterior chamber. The anterior chamber is occupied by a watery fluid known as the aqueous humour (Hogan et al. 1971), which is produced by the ciliary body and contributes to the eye shape. The posterior chamber, located in a space between the lens and the iris, is divided many times by the zonular system.

One of the principal parts of the eye, which is located behind the iris and in front of the vitreous body and has 15.0 dioptries of the eye's refractive power, is the crystalline lens (Maisel 1985). It is attached to the ciliary body by means of zonular fibres. Many different layers are involved in the construction of the lens: the capsule; the epithelial layer; and the stroma, composed of lens fibres.

The vitreous is a thick transparent substance which occupies the area between the lens and the retina. It is composed mainly of water (> 98%), comprises about 2/3 of the eye's volume and gives it its shape (Forrester et al. 2002).

The retina is multilayered sensory tissue and is the most accessible part of the central nervous system. The retina contains many cells and tissue layers, a pigment epithelium, photoreceptors (which capture light rays and convert them into electrical impulses), an external limiting membrane, an outer nuclear layer, an outer plexiform layer, an inner nuclear layer, an inner plexiform layer, a ganglion cell layer, a nerve fibre layer and an internal limiting membrane (Davson 1990).

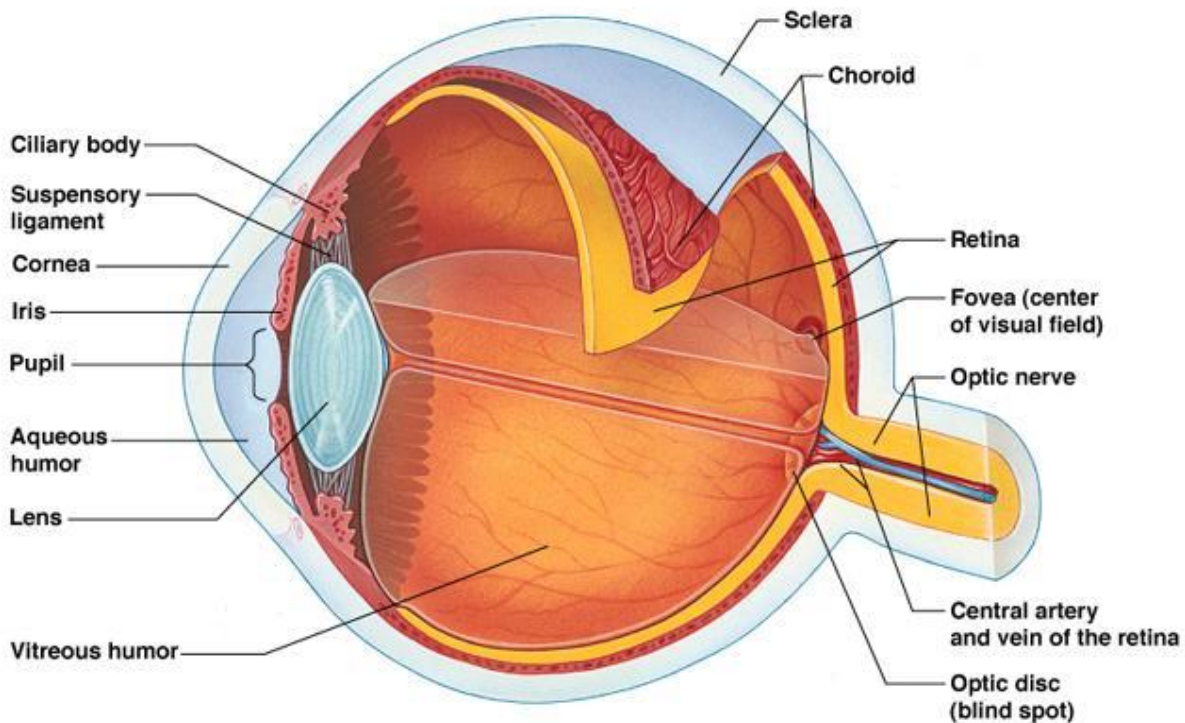


Figure 1.1: The major components of the human eye.

<http://t3.gstatic.com/images?q=tbn:ANd9GcTFU6C49FsMS3bNiKyk20XQGYS8C-I1-oh92rvqyus7ubciiBQh7w> [accessed: 21 September 2011]

The six extra-ocular muscles control eye movement. The extra-ocular muscles (EOMs) are divided into two kinds: four rectus muscles which control the eye's movement from left to right and up and down, and two oblique muscles which move the eye and rotate the eyes inward and outward (Carpenter 1977) (Figure 1.2).

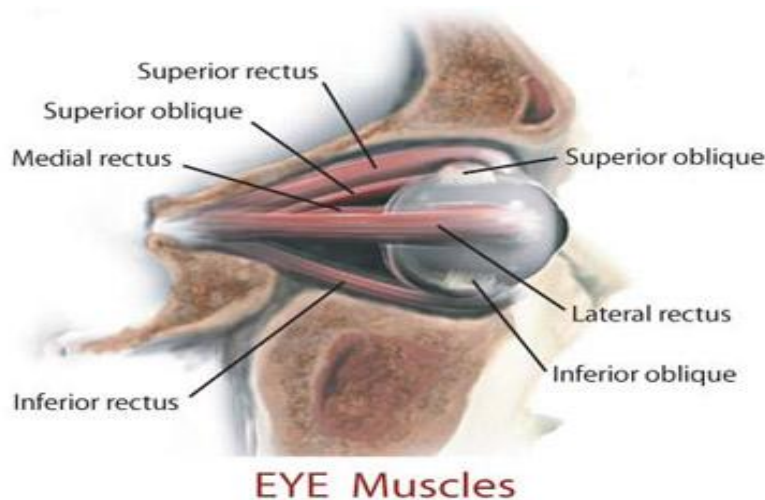


Figure 1.2: The Six Muscles of the human eye. From Healthy eyes for life: http://www.healthyeyesforlife.org/wp-content/uploads/2011/03/Image-2-Eye_muscles_anatomy_names.jpg [accessed: 21 September 2011].

The last part that we will mention here is the conjunctiva. It is the transparent membrane which covers the outer surface of the eye, starting from the outer corneal edge, passing to the visible part of the sclera and finishing inside the eyelids (Maurice 1984).

1.2 General structure and function of the cornea

The cornea is the foremost part of the outer layer of the eye and has a differentiated transparent structure. As the window on the front of the eye, transparency is one of the most important features of the cornea (Maurice 1957). Maurice suggested that the cornea scattered less than 1% of incident light. It is also a powerful lens and provides the eye with 40D of its total refractive power (Oyster 1999). Because of the lack of a blood supply, oxygen cannot be delivered to the cornea by the red corpuscles. Therefore, the anterior precorneal tear film absorbs oxygen from the external environment and supplies it to the cornea. Furthermore, the aqueous humour supports the eye with essential nutrients. The cornea and its tear film protect the eye from germs, dust and other harmful substances. As the cornea represents the outermost lens of the eye, one of its most important functions is to focus light on the retina. One of the most important benefits of the cornea is that it protects the lens and the retina from harmful rays, such as ultraviolet (UV) wavelengths in sunlight, and prevents injury to the eye. Blurred vision, tearing, sensitivity to light and redness are signs resulting from deep scratches penetrating the cornea. In addition, there are some diseases and disorders that affect the cornea such as corneal infections, allergies, dry eye, keratitis, keratoconus and corneal ectasia.

The corneal structure and function depend on several factors, the most important of which are: the collagen fibril density and number; the collagen fibril diameter; the refractive index of the cornea; the corneal thickness; and the collagen orientation in the cornea (Maurice 1970).

1.3 Structure of the corneal layers

The main layers of the cornea are shown in Figure 1.3 and each is described below.

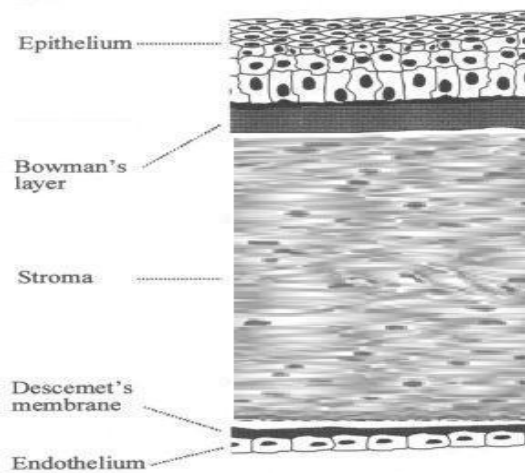


Figure 1.3: The structure of human cornea (Cowell et al.1999).

- 1- Epithelium: The epithelium is the outer and most anterior layer of the cornea. It is a cellular region composed of several layers of squamous cells. It is about 50um thick (10% of total corneal thickness) (Hogan et al. 1971) and is continuous with the bulbar conjunctive which is, at the same time, attached to the anterior surface of the sclera. There are also very strong apoptotic responses by these cells which allow for a balance between natural cell deaths vs. re-epithelisation, and allow the healthy epithelium to maintain a state of homeostasis (Stocum 2006). The average life of an epithelial cell is about ten days; this is the time it takes a

basal cell to replicate and migrate to the superficial layers. Because of this phenomenon, the epithelium often heals rapidly if it is damaged by a foreign body or after some refractive surgeries (Oyster 1999). Due to the fact that there is no vascular supply to the cornea, the tears act as a natural waste transport system. The tears are instrumental in delivering nutrients to the cornea and removing dead cells and other waste materials. Tears also help to maintain homeostasis in the cornea (Probst 2003).

- 2- Bowman's layer: This layer contains disorganized collagen fibrils (rich in type IV collagen and laminar fibres, it also contains collagen types I, III, V, VII, XII and XVI) (Forrester et al. 1996). Type VII collagen especially provides support for cell adhesion. Bowman's layer also provides structural integrity to the cornea and damage to this layer may alter the shape of the cornea (resulting in refractive changes) (Feder and Rapuano 2006).
- 3- Stroma: The stroma is the thickest layer in the cornea, making up around 90% of total corneal thickness, around 0.5mm. (Maurice 1984). Its composition is made up of 78% water, 15% collagen, 5% other proteins, glycosaminoglycan (0.7% keratan sulphates, 0.3% chondroitin sulphates) and 1% salts (Davson 1984).

Structurally, the stroma consists of many layers or lamellae (Fig 1.4), each of which is parallel to the tissue surface. These lamellae are composed of parallel collagen fibrils embedded in a hydrated matrix containing proteoglycans, proteins to which the glycosaminoglycans keratan sulphate and chondroitin sulphate are attached Posterior

lamellae run continuously from limbus to limbus (Davson 1984) and consist of flattened bundles of collagen fibrils running in a parallel manner (Gipson 1994). There are between 200 and 300 lamellae in the centre of the human cornea and around 500 near the limbus (Maurice 1957; Radner et al. 1998). Successive lamellae run at different angles in order to confer the required radial strength to the cornea. At the centre of the human cornea, the lamellae have a preferential vertical and horizontal organisation (Aghamohammadzadeh et al. 2004). This preferred orientation is more prevalent in the posterior than in the anterior (Abahussin et al. 2009). These authors suggested that the collagen arrangement in the anterior third of the cornea may relate to corneal biomechanics, as the collagen in this part of the cornea is more isotropic (uniformity in all orientations). Radiating out from the centre of the eye, the lamellae appear to be stacked in a less 'crossed' manner until the outer boundary of the cornea/sclera join where there appears to be little to no crossing. These outer collagenous lamellae provide the cornea with structural integrity and are believed to be involved in maintaining the cornea's shape (Boote et al. 2006). Although corneal collagen has an organized arrangement in the stroma, Hayes et al. (2007) have shown that there is a remarkable difference in the collagen arrangement between mammals. Furthermore, the collagen fibril orientation can be affected; as an example of this, Kamma-Lorger et al. (2009) have shown that the orientation of the collagen fibrils is significantly changed in trephine-wounded corneas.

The stroma also contains keratocytes (flattened fibroblasts) which are situated between the lamellae and connected by interconnecting processes. These cells

produce collagen and proteoglycans, the major components of the stromal extracellular matrix (Figure 1.4).

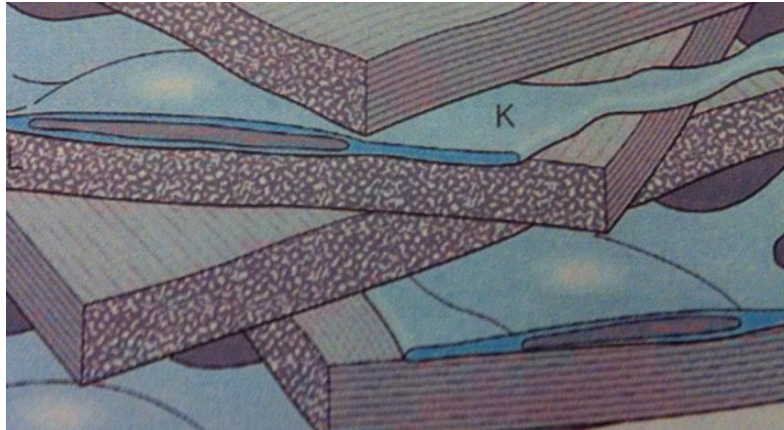


Figure 1.4: The arrangement of lamellae (L) and the keratocytes (K) in the cornea (Forrester et al. 2002)

- 4- Descemet's membrane: This layer is composed of types IV and VII collagen, laminin and fibronectin (which have a role in attaching cells to extra cellular matrices). Type VIII anchorage fibrils are found predominantly in the latter (Snell and Lamp 1998). Descemet's membrane also provides structural integrity to the cornea and maintains the intraocular pressure.
- 5- Endothelium: The posterior layer of the cornea is the endothelium. It is similar to the epithelium except it is only one cell layer thick; it is bathed on the posterior surface by the aqueous humour. Both the epithelium and, more so, the endothelium have a function in maintaining correct tissue hydration. The endothelial cells are rich in mitochondria (Ramey 2007). Any damage to these

cells, either through injury or the natural ageing process, affects the amount of fluid retained in the cornea (Twersky 1975). There are a number of pathologies which can cause disruption in this natural system. As the endothelial layer does not have any regenerative properties, any damage to this cell layer will result in a loss of active cells and will have a direct impact on the transport of fluid

1.4 Stromal collagen

Collagen is the main component of connective tissues; however, there are some differences in the collagen properties in the body. For example, if we compare the cornea, sclera and skin, it can be clearly distinguished that the collagen matrix in the cornea is transparent, compared with others. Collagen has many types, some of them being almost completely triple helical (Figure 1.5) such as types I, II, III, V and VI, whereas collagen type IV has numerous small non-collagenous domains between relatively short collagenous domains (Friend et al. 1994). Collagen makes up about 71% of the dry weight of the cornea (Friend et al. 1994), with mostly types I and VI collagen (type 1 being in a fibrillar form) but with some types III, V and XII; collagen types V and VI represent 10% and 25% respectively of the total content of collagen (Friend et al. 1994) (Figure 1.6).

Corneal collagen fibrils are hybrids of type I and type V. As in most type I fibrils they consist of collagen triple-helical molecules which probably aggregate into microfibrils which then form the fibrils themselves (Meek and Boote 2004). Corneal collagen fibrils are 25-30nm in diameter and the small size is thought to be regulated by

the proteoglycans (Freund et al. 1986). Stromal collagen differs from the other connective tissues, such as the collagen fibrils in the sclera which have a non-uniform diameter from 25 to 230nm. (Komai et al.1991). The narrow, uniform collagen fibrils give the cornea its clarity, because they run parallel to each other. The spacing of the fibrils is another essential factor in maintaining the transparent nature of the cornea (Maurice 1957). When the distance between the collagen fibrils or between the lamellae increases the cornea loses some of its transparency (Edelhauser 1994).

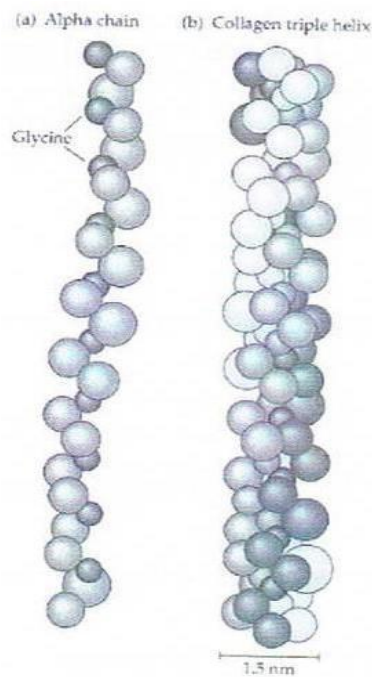


Figure 1.5: The structure of the collagen triple helix (Oyster 1999)

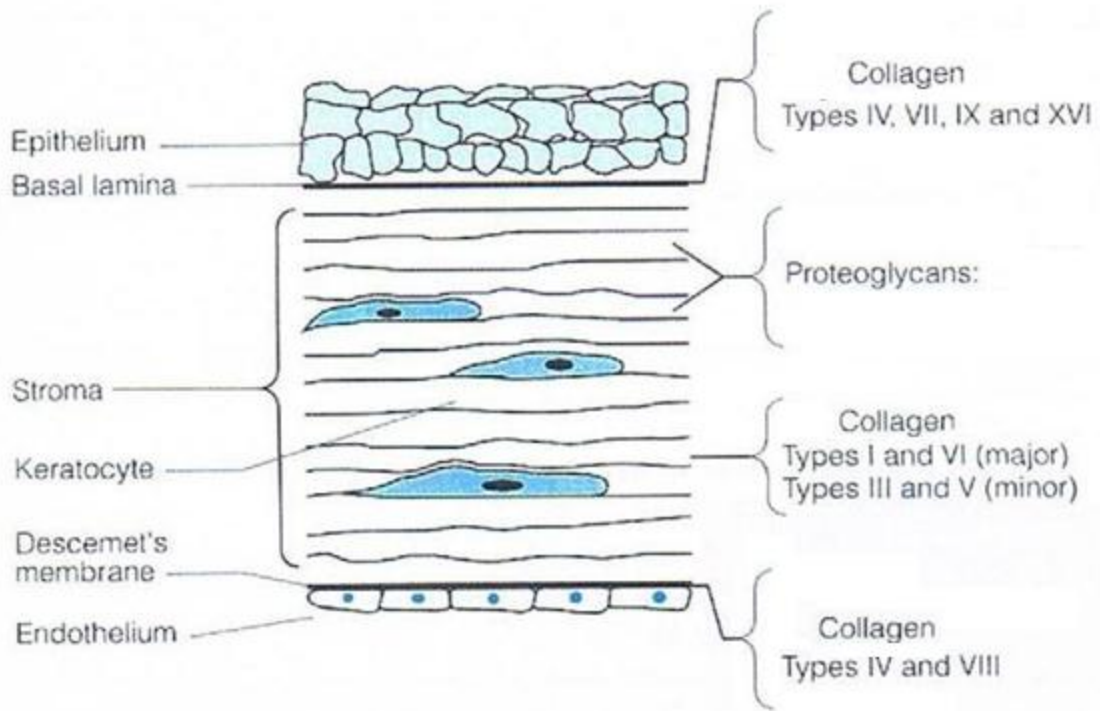


Figure 1.6: The types of collagen in the cornea (Forrester et al. 2002)

1.5 Proteoglycans (PG) and Glycosaminoglycans (GAGs)

1.5.1 Glycosaminoglycans (GAGs)

Sulphated carbohydrate polymers are what make up GAGs. These polymers are formed from 40-100 disaccharide units connected by molecular bonds (Scott 1992); these often contain uronic acid or hexosamines.

GAGs are strongly hydrophilic in nature. Therefore when they are in water-based solutions, they have an extended structure. This happens because of their considerable sulphation; this is more noticeable when they connect to core proteins covalently. In the corneal stroma, the two main GAGs are KS (keratan sulphate) and CS (chondroitin sulphate). These are responsible for the unique function of proteoglycan. GAGs differ in their sugar residues and linkages, and in their possible positions and numbers of sulphation sites.

Normally, the sulphation of GAGs in CS chains is consistent; there is one sulphate for each disaccharide along the chain. There are many water molecules in the molecular domain of GAGs. When they are in aqueous solution, these take up a very large; this is basically an aqueous gel which can withstand forces of compression (Kreis and Vale 1993).

1.5.2 Proteoglycans (PGs)

Proteoglycans (PGs) are hybrid molecules. They have a protein core which has a covalent bond to one or more chains of polysaccharide GAG (Figure 1.7). Stromal proteoglycans come in two main forms or classes; one form has keratan sulphate (KS) side chains, the other form has chondroitin/dermatan (CS/DS) sulphate side chains (Iozzo 1999). The core protein structure of proteoglycans is different to that of glycoproteins. In proteoglycans, the sugar chain is longer, has less variation, and does not branch. Also, it is more acidic as it has high level of sulphur, and is strongly negative charged.

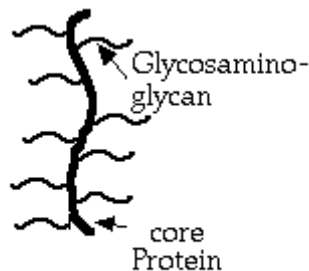


Figure 1.7: Proteoglycans (PG) with attached Glycosaminoglycans (GAGs) (Oyster 1999)

Proteoglycans are widely distributed; they are in intracellular vesicles, on the surfaces of cells and also in the extracellular matrix. The core protein of proteoglycans has a molecular size in the range of 36 to 40 kDa, and its homology has some amino acid sequences (Blochberger et al 1992).

This protein core is found in the keratocytes in the form of ribosomes bound by a membrane. Using particular tetrasaccharide sequences, GAGs are connected to the

protein core; then, proteoglycans are secreted into the extracellular matrix. It is the GAG and the protein component of the molecule which determine the biological actions of proteoglycans.

Proteoglycan binding sites have been found in bovine corneal stromal collagen fibrils, at positions a, c, d and e (Meek et al. 1986).

In the corneal stroma, there are just three different protein cores with KS GAGs; their corresponding proteoglycans are lumican (Blochberger et al. 1992), keratocan (Corpuz et al. 1996), and mimecan (Funderburgh et al. 1997). In the CS/DS fraction, the main stromal PG is Decorin.

- **Lumican**

Lumican is a KS proteoglycan; it shares 50% of its genetic homology with fibromodulin (Grover et al. 2000), it is also has its importance in corneal transparency (Chakravarti et al. 1998). Although lumican is mainly expressed in the cornea, it also has functions in the lungs (Ying et al. 1997), kidneys (Schaefer et al. 2000), muscles and cartilaginous tissue (Saamanen et al. 2001). Generally, lumican is normally expressed by stromal keratocytes. However, soon after corneal damage, it may be briefly expressed by the corneal epithelium (Saika et al. 2000).

The KS chains in corneal lumican have high sulphate content; this indicates a key role in producing and maintaining corneal transparency (Hayashida et al. 2006).

- **Keratocan**

Corneal keratocytes are primarily responsible for expressing keratocan. Thus their physiological distribution is very limited in comparison with other PGs that contain KS, e.g. lumican. Most keratocan is found in the cornea and sclera, much less so in non-corneal tissue, e.g. cartilage, ligament and skin, when it is mainly a non-sulphated glycoprotein. It has been suggested that keratocan and lumican have very similar three-dimensional structures (Dunlevy et al. 1998).

Many believe that keratocan, along with lumican, plays a part in controlling collagen fibrillogenesis and corneal transparency (Carlson et al. 2005).

- **Mimecan**

It was from bovine bone that osteoglycin, now called mimecan, and was first isolated. Subsequently, as a minor KSPG, it was isolated from the bovine cornea and cloned. It is a 25kDa corneal PG and is the product of a gene (Funderburgh et al. 1997). Although mimecan has KS GAGs in the corneas of humans, bovines and chicks, this is not the case for murine corneas (Funderburgh et al. 1997). In many tissues, mimecan is found as a non-sulphated glycoprotein. While it is expressed as the chick cornea develops, it cannot be identified in hen corneas; this is perhaps because the level is too low (Dunlevy et al. 2000). A comparable process can be observed in keratocytes. However, in the stroma, the glycosylation of decorin, keratocan and lumican is tissue-specific. In this process, they are converted from non-sulphated matrix glycoproteins to a particular class of proteoglycans.

- **Decorin**

It has been suggested that decorin is a bidentate ligand which is connected to two adjacent and parallel collagen molecules. In this scenario, collagen fibrils assist in stabilizing the fibrils to enable fibrillogenesis (Scott 1996). Decorin may also play a part in regulating the thickness of collagen fibrils (Vogel et al. 1984).

Decorin was first found in cartilage and bone (Rosenberg et al. 1985; Fisher et al. 1989). Later it was found in many connective tissues; these include tendons, skin, the sclera and cornea.

1.6 Corneal transparency

The light scattering in the cornea can be assessed by using a confocal microscope; however, spectrophotometry is regarded as the most common and most frequently used method to determine corneal transparency. Optical coherence tomography can also be used to measure central corneal transmission; however, it cannot be measure the peripheral region of the cornea. Beems and Best (1990) and Best (1988) have shown that the human cornea is 94% transparent by using a blue-green argon laser; however, the large contribution from scattered light can affect the measurements as the detector is mounted within the anterior chamber.

To date, there is no agreement on an explanation for the transparency of the cornea, although there are a number of models that have been used to explain this property. The thickness of the cornea, the structure of the stroma, the refractive index / orientation / spacing of the collagen, keratocytes and the hydration level in the cornea are considered to be the main factors that contribute to light scattering (Freund et al., 1986). Meek et al. (2003) summarised several factors that can alter the transparency of the cornea: changes to the fibrils' diameters, increases in fibrils density, refractive index changes in the stromal components and increases in corneal thickness.

In 1973, Farrell et al. found an inverse relationship between corneal thickness and transmission (when corneal thickness increases, transmission decreases). The same relationship was found by Boote et al. (2003), between transmission and the number density of fibrils within the stroma (when the density number increases, transmission decreases).

Because the collagen fibril density is not equal between the central and peripheral corneal regions, Dutch et al. (2008) investigated the corneal transmission up to 3mm from the corneal centre and showed that the reduction in transparency in the peripheral region of the cornea is due to differences in fibril radii and possibly in the refractive index ratio between the fibril and interfibrillar substances..

On the other hand, Freund et al. (1995) consider that fluid retention, which causes swelling in the cornea (oedema) (Fig. 1.8a), is an influential factor in the loss of transparency of the cornea (Fig. 1.8b). It has also been demonstrated clinically that corneal swelling causes blurred vision.

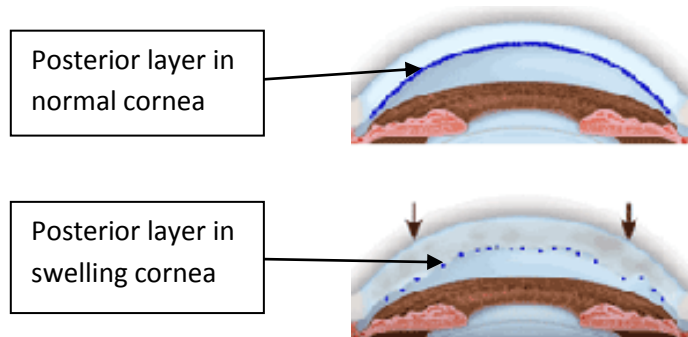


Figure 1.8a: Fluid retention causes swelling in the cornea:
<http://www.harvardeye.com/procedures/images/DSEKAnimation.gif> [accessed: 17 October 2011]

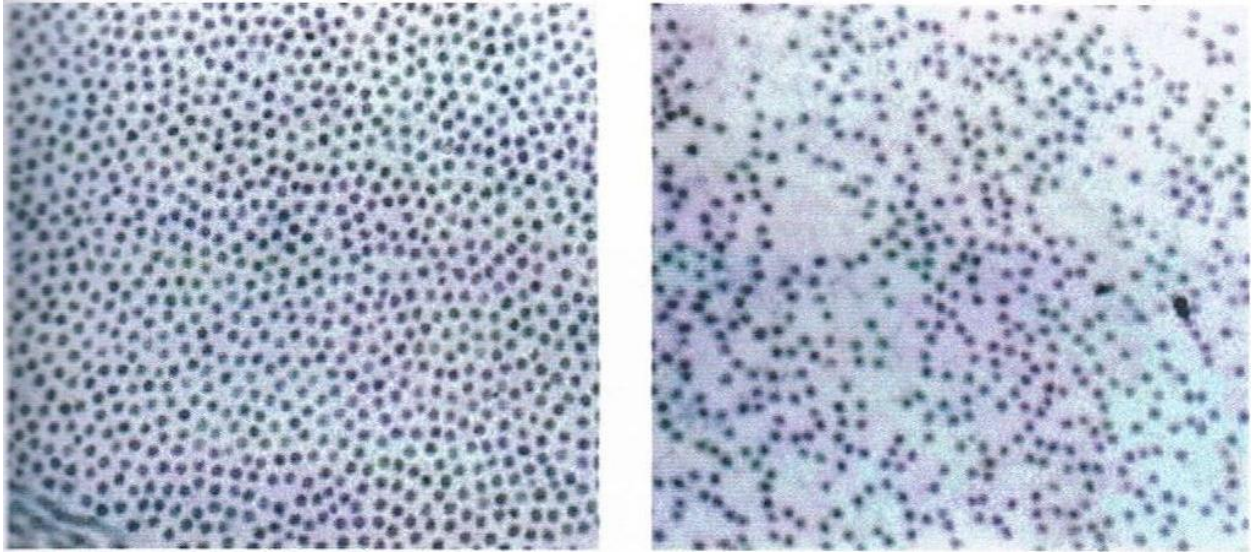


Figure 1.8b: Pictures of normal (left) and swollen (right) human corneal stromal collagen fibril distribution by using transmission electron microscope (Smolin and Thoft 1994)

Mechanical forces can also affect transparency; thus, when applying force to the cornea, transparency properties will be affected until this force is removed (Maurice 1951).

When Maurice sectioned the cornea, he found that the refractive index increased in the deeper layers; however, the refractive index can be estimated by using X-ray diffraction (Leonard and Meek 1997). Meek et al. (2003) have demonstrated the effect on transparency of a change in the ratio of the refractive indices of the collagen fibrils and that of the GAG-rich matrix between them, the greater the mismatch, the lower the transparency.

Moreover, because of scattering in the ultraviolet, the cornea has low transmission in this region. In 1984, Lerman reported that in the case of ageing corneas, the UV

transmission decreased. Later on, however, Best (1988) and Beems and Best (1990) showed that with increasing age, there is no decrease in visible transmission. In the same context, when the cornea is not under pressure, Kostyuk et al. found the transmission of the cornea is decreased by about 5% (Kostyuk et al. 2002).

1.7 Biomechanical properties of the cornea

Research on corneal biomechanics began in the 1960s (Bryant et al. 1996; Kampmeier et al. 2000; Fung 1981; Andreassen et al. 1980). It is important to investigate the biomechanical properties of the cornea in order to understand the whole ocular behaviour.

Nowadays, measurement of the biomechanical properties of the cornea is becoming one of the fundamental factors in refractive surgery as such measurement can detect some corneal disorders. Therefore, researchers have started to find solutions to measure the biomechanical properties of the cornea in vivo. By biomechanical properties we mean the response of the cornea to any stress or strain applied which changes some corneal properties. Previously, researchers had difficulty in finding a device to measure the normal biomechanical properties of the cornea in vivo and to compare those with ex vivo results. As a result of the viscoelastic nature of the cornea (Buzard 1992), any load applied to the cornea will change its biomechanical properties.

First of all, we must understand the impact of each layer on the biomechanical properties of the human cornea. Boote et al. (2005) and Ethier et al. (2004) believe that the stromal layer has a strong influence on the biomechanical properties of the human cornea. However, corneal biomechanics can be affected by an increase in lamella interweaving (Maurice and Monroe 1990). Corneal biomechanics are found to be related to the content and distribution of fibrils which are the major components of the stromal layer in the cornea (Kokott 1938; Boote et al. 2005). The mechanical stiffness

of the stromal layer is higher than the endothelium and epithelium layers and that is because both the endothelium and epithelium are essentially cellular structures (Patel et al. 2004). Moreover, Descemet's membrane has low mechanical stiffness and it is not certain whether or not this layer plays a role, even a very small one in corneal biomechanics (Beuerman and Pedroza 1996; Danielsen 2004). As with Descemet's membrane, in 1992, Seiler et al. reported that the biomechanical property of Bowman's layer is negligible.

There are some general composite microstructure factors that influence the biomechanical properties of the cornea:

- 1- The cornea has hyper-elastic behaviour because the stromal layer is predominant in the cornea (90% of the overall corneal thickness) and has a high density of collagen fibrils which give this layer hyperelasticity and reduce the mechanical stiffness.
- 2- The variation of the corneal thickness between the central and the peripheral regions suggests that mechanical stiffness increases away from the central region.
- 3- Wearing contact lenses for long periods causes corneal swelling which causes the space between the collagen fibrils to increase and then influences corneal biomechanics.

- 4- When the lamella interweaving increases, the biomechanical properties between the anterior and posterior of the stroma become unstable (Hennighausen et al. 1998).
- 5- Some corneal disorders such as keratoconus influence corneal biomechanics (Nash et al. 1982).

As mentioned previously, there are some microstructure factors that affect the biomechanical properties of the cornea; also, any alteration to the cornea could affect the corneal biomechanics, e.g. after a LASIK procedure (Djotyan et al. 2001).

Nowadays, the biomechanical properties of the cornea can be measured in the clinic and in the laboratory. So far, there is just one method to measure corneal biomechanics clinically. In 2005, at the European Society of Cataract and Refractive Surgeons' annual meeting (Lisbon, Portugal), the ocular response analyzer (ORA) was introduced by Reichert ophthalmic instruments. This machine can measure some biomechanical properties of the cornea by measuring corneal hysteresis (CH) and corneal resistant factor (CRF) in addition to the accurate measurement of IOP and corneal thickness. Corneal hysteresis measures a viscoelastic property of the cornea by measuring the difference between two different inward and outward pressures and corneal resistance factor is a measure of the overall rigidity of the cornea.

On the other hand, there are two main techniques for taking this measurement in the lab. The first technique is called inflation testing (Figure 1.9) and the second one is

called strip extensometry testing. However, as will be shown later, the ocular response analyzer can be used in laboratory measurements as well.

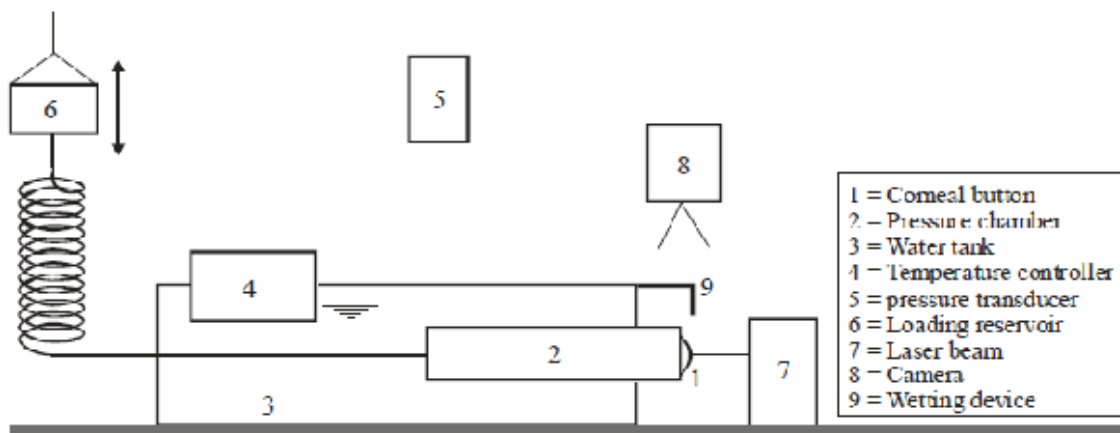


Figure 1.9: components of the inflation test (Elsheikh A 2010)

The biomechanics of the cornea are related to many factors. To prove this, Boyce et al. (2008) have shown that the cornea becomes stiffer when the intraocular pressure is increased; in contrast it decreases as IOP decreases. Moreover, they reported that when strain is applied to the cornea, the peripheral region of the cornea has the most deformation. In 1999, Meek et al. reviewed the biomechanical properties of the tissue affected by the collagen organization, and found that this helps to preserve the shape of the cornea. Interestingly, Meek suggested that the performance of corneal biomechanics is inconsistent because of the regional variation in the orientation of the collagen. In 1997, Shin et al. demonstrated that the strain distribution on the excised human cornea varies across the cornea. Moreover, the biomechanical properties of the

human cornea are disparate between two eyes from the same person (Li and Tighe 2006).

Finally, the biomechanical properties of the cornea are associated with age, due to the fact that corneal tissue becomes stiffer as age increases; as a result, the IOP reading increases (Elsheikh et al. 2007, 2008). IOP measurements are closely connected to corneal biomechanics and, for this reason, in recent years much effort has been devoted to trying to find a method to measure IOP that is not influenced by the biomechanical properties of the cornea.

1.8 General corneal abnormalities

1.8.1 Normal corneal parameters

There are some important corneal parameters which must be known before any test or experimental work can be performed in the human cornea. The mean corneal curvature for infants has been shown by Dubbelman et al. (2002) to be $7.87\text{mm} \pm 0.21\text{mm}$. However, the corneal curvature for adults is $7.95\text{mm} \pm 0.27\text{mm}$. On the other hand, Zadnik et al. (2003) reported that corneal power did not change with age; they found at 6 years corneal power was 43.76D and at 14 years it was 43.37D. Normal corneal thickness in human is around 520 microns and the normal intraocular pressure (IOP) range is between 10 to 22mm. Hg. Normal corneal hysteresis (CH) is 11.19 mmHg (range: 7.24 - 15.80 mmHg) and the normal corneal resistance factor (CRF) is 10.46 mmHg (range: 5.37 - 15.74 mmHg) (Luce 2005).

1.8.2 Refractive errors

Normal vision which has a normal refractive condition is called emmetropia. In this condition, parallel rays of light are brought to a focus on the retina when the lens is in a relaxed accommodative state and visual acuity is 6/6 (at 6 meters) or better. In addition, near vision (at 40cm.) is normal and the lens can accommodate to provide good vision.

In contrast, if there is any abnormality in the refractive condition, the term ametropia describes this situation (Figure 1.10). In this condition, parallel rays of light are not focussed onto the retina when the lens is in a relaxed accommodative state,

therefore vision becomes unclear. Ametropia can be divided into three main categories which are myopia, hyperopia and astigmatism. Myopia is the situation where rays of light are focused in front of the retina and are corrected by a minus lens. Hyperopia is the situation where rays of light are focused behind the retina and are corrected by a plus lens. Astigmatism is a difference in the degree of refraction in different meridians where rays of light are dispersed and there is no specific point for the light to be focused at, and this is corrected by a cylindrical lens.

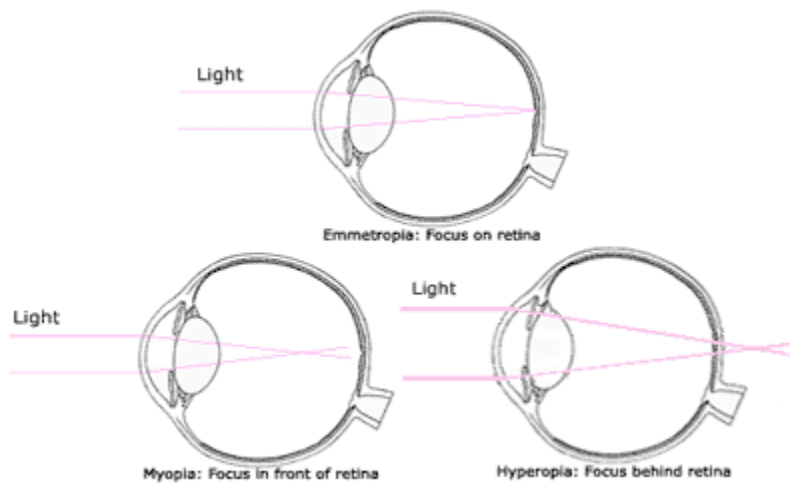


Figure 1.10: Normal and abnormal vision (refractive error). <http://www.psych.ucalgary.ca/PACE/VA-Lab/Marcela/Assets/webreadypics/adjustment.gif> [accessed 21 September 2011]

1.8.3 Keratoconus

The term **keratoconus** can be divided to two parts, kerato meaning cornea and conus meaning conical or cone-shaped, together they mean cornea with a cone shape. Keratoconus is a degenerative non-inflammatory corneal condition which is characterized by thinning of the corneal stroma (Saelens et al. 2008) and an increase in

its curvature to a more conical shape than normal (Krachmer et al. 1984) (Figure 1.11). It affects around 50 to 230 people per 100,000 (Ucakhan et al. 2006). Corneal thinning leads to myopia, irregular astigmatism and protrusion which reduce and affect the quality of vision (Krachmer et al. 1984). One of the most important points about keratoconus patients is that they never become totally blind from this disease (Rabinowitz, 1998). Usually, it is a progressive disorder, affecting both eyes, with perhaps only one eye initially affected (Lee et al., 1995; Rabinowitz et al. 1993).

In 1998, Rabinowitz found some classical histopathological features in keratoconus, i.e. thinning of the corneal stroma, breaks in Bowman's layer, and deposition of iron in the basal layers of the corneal epithelium. There are a number of features noted by Krachmer et al. (1984) in keratoconus, such as the arrangement of fibrils being unregulated in the anterior stroma, in addition to a decrease in the number of collagen lamellae. In 2006, Ucakhan observed that there are some changes in the structure and composition of features of the central part of the keratoconic cornea in all layers.

Until now, the causes of keratoconus have been unknown, however some researchers believe that this disease is common in people who have weakening in the corneal tissue due to an imbalance of enzymes within the cornea (Roy et al. 2007); they also suggest that overexposure to ultraviolet rays from the sun, excessive eye rubbing and chronic eye irritation may lead to keratoconus. Bawazeer et al. (2000) believe that eye rubbing is a major risk for keratoconus development.

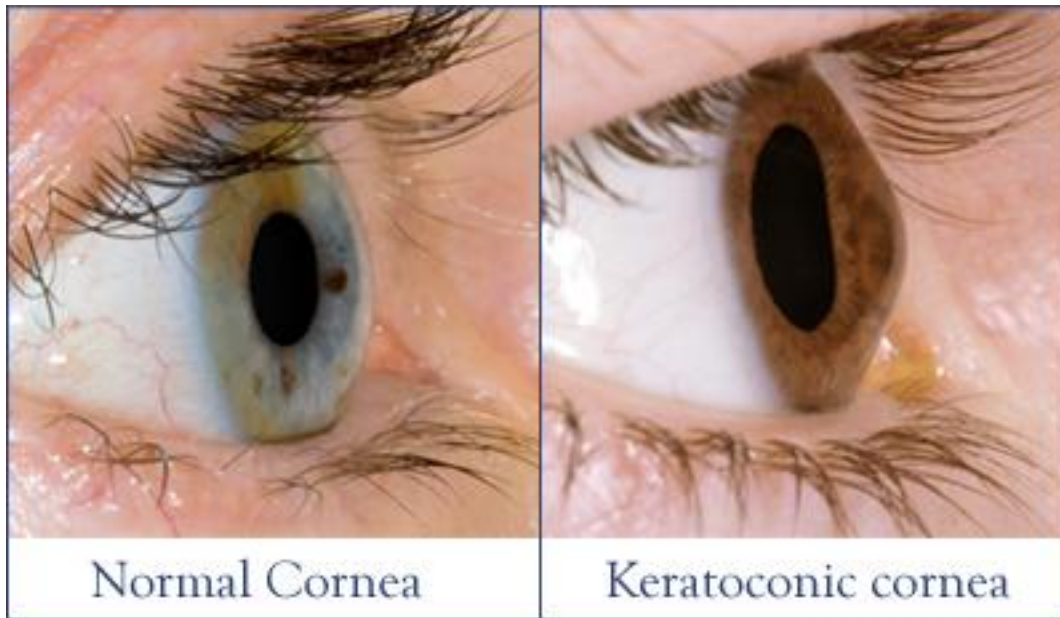


Figure 1.11: Keratoconic cornea: <http://www.eyecarelondon.com/images/img-c3k-eyes.jpg> [accessed 21 September 2011].

1.8.3.1 Signs and Symptoms

The signs and symptoms of keratoconus are extremely variable and depend on the stage of the disease; its early signs and symptoms are unclear and are difficult to investigate because it usually develops so slowly. However, keratoconus can be detected by a specialist when corrected vision for patients does not reach 6/6 (Rabinowitz 1998).

The first major symptoms of keratoconus are blurring and discomfort vision, even with spectacles. Some external signs are presented by Rabinowitz (1998), such as V-shaped conformation of the lower lid produced by the ectatic cornea in downgaze (Munson's sign), stromal thinning and iron ring found when using a slit-lamp, scissoring reflection in retinoscopy examination, and compression of mires inferotemporally (egg-

shaped mires) which is a sign of photokeratoscopy. In an early keratoconus slit-lamp examination, the cornea appears normal; however, slight distortion or steepening in the cornea may be detected by keratometry. In such cases, the specialist will suggest doing central and paracentral corneal topography to detect the disease and confirm the diagnosis (Krachmer et al. 1984). Rabinowitz et al. (1993) believe that early keratoconus can be detected by measuring the anterior corneal topography with several devices and by using pachymetry to confirm corneal thinning in keratoconus patients (Rabinowitz et al. 1993). In the advanced stages of keratoconus, patients may note sudden loss of vision accompanied by pain. This may result in breaks in the Descemet's membrane with stromal imbibitions of aqueous through these breaks – “hydrops”; opacity is noted in the corneal stroma (Rabinowitz 1998).

1.8.3.2 Treatment

There are many various and miscellaneous methods for keratoconus treatment, it all depends on the disease stage. Treatment in the very early stages of keratoconus is possible with **spectacles** and this may give patients good and adequate vision.

However, spectacles are generally considered to be a temporary solution for this disease; therefore, **hard contact lenses** are the best option and the mainstay of keratoconus treatment, and are used in about 90% of patients (Buxton et al. 1984) (Figure 1.12).

Contact lenses are subdivided into two main types, soft contact lenses and rigid gas permeable lenses - “hard contact lenses”. In the initial stages, soft lenses of toric design are possible and are sufficient to provide good vision to the patient; on the other hand, rigid gas-permeable lenses should be used in the advanced stages because soft lenses cannot improve vision significantly (Rabinowitz 1990; Rosenthal et al. 1995; Yeung et al. 1995). Fitting contact lenses to keratoconus patients is a complex and difficult process which requires a specialist’s professional skills. The difficulty lies in keeping the patient comfortable and providing adequate treatment with hard contact lenses while at the same time producing a good visual outcome for the patient (Rabinowitz 1998).



Figure 1.12: Hard contact lens: http://www.eyephysiciansinc.com/images/H-17_Soft_and_Hard_Lens_sm.jpg [accessed 21 September 2011].

In some cases of keratoconus, the use of hard contact lenses becomes inappropriate for several reasons, such as patients being unable to tolerate hard contact lenses or patients being in the very advanced stages of keratoconus when hard contact

lenses become useless. In these cases, **corneal transplant** (penetrating keratoplasty) is the best option for keratoconus patients (Smiddy et al. 1988; Tuft et al. 1994). Because of scarring in the visual axis, between 10 and 20% of patients need a corneal transplant (penetrating keratoplasty), (Saelens et al. 2008) (Figure 1.13).



Figure 1.13: corneal transplantation:
http://119.226.138.13/ypimg/HYTTXNM031609Imagecorneal_transplant.jpg [accessed 21 September 2011]

Intrastromal Corneal Ring implants are another surgical treatment for keratoconus patients. The main idea of this operation is to use two plastic inserts in the cornea to reduce the curvature of the keratoconic corneal tissue and make this tissue flatter (Zare et al. 2007) (Figure 1.14).



Figure 1.14: Corneal ring: <http://www.kestrelophthmics.com/images/intacsBanner.jpg> [accessed 21 September 2011]

Currently, there is a new treatment that is still being developed – ***Corneal Cross-linking with Riboflavin and UVA treatment***. The main idea in this technique is to stop the progression of keratoconus; we will discuss this technique at the end of this chapter.

1.8.4 Keratectasia

In 1998, ectasia after excimer corneal ablation was reported for the first time (Speicher et al. 1998; Seiler et al. 1998), although it had been reported in 1994 after incisional corneal surgery (Wellish et al. 1994). Excimer laser surgery causes weakening of the cornea, leading to keratectasia (Binder et al. 2005; Seiler et al. 1998). Laser in situ keratomileusis (LASIK) is currently considered one of the most prevalent treatments for refractive errors correction. However, It was noted that the cornea was not stable biomechanically which leads to keratectasia (Figure 1.15) (Seiler et al. 1998).

Keratectasia was one of the disturbing results following LASIK when progressive symptoms and bulging occurred on the cornea, leading to visual disturbance similar to keratoconus (Comaish et al. 2002). The signs of keratectasia appear either immediately after LASIK or later, but usually occur during the two years following surgery (Geggel et al. 1999; Rao et al. 2002). After LASIK surgery, there are some changes in the anterior surface of the cornea. However, if the surgery is based on tissue subtraction the posterior corneal surface may also change after LASIK (Baek et al. 2001; Wang et al. 1999). Although there are some disadvantages of LASIK, such as the risk of keratectasia, Amoils believes that LASIK should be successful in a cornea which has a thickness 500µm. or more; he also implies that corneal ectasia and weakening occur even in the case of myopia (Amoils et al. 2000). After several studies, researchers have

found that LASIK possibly affects the tissue and leads to ectasia, which has similar properties to keratoconus (Hjortdal et al. 1995a). Wang et al. (1999) believe that intraocular pressure (IOP) plays a role in the posterior lamellae bulging after LASIK.

Changes in the level of oestrogen may affect LASIK-induced keratectasia, reducing corneal biomechanical stability and leading to keratectasia (Randleman et al. 2008). There are many factors that must be taken into account before LASIK in order to avoid ectasia, such as corneal topography, stromal thickness, age and refractive errors (Randleman et al. 2008). Although there are some solutions to ectasia, such as hard contact lenses, intrastromal corneal rings, PKP and corneal crosslinking with riboflavin and UVA treatment, most researchers suggest that prevention is better than treatment (Comaish et al. 2002). As a result of the corneal thinning which occurs after LASIK, there is a higher probability of the occurrence of keratectasia (Ou et al. 2002) (Figure 1.15).

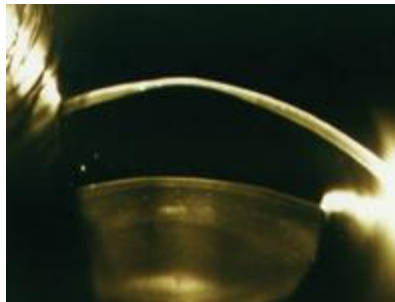


Figure 1.15: Scheimpflug image of post-LASIK corneal ectasia (Lume et al. 2006).

1.8.4.1 Signs & symptoms

In addition to the signs and symptoms of keratoconus, some other signs also appear in keratectasia, such as corneal bulging and thinning, a high amount of astigmatism, advanced corneal scarring and loss of visual acuity (Randleman et al. 2003). The important negative consequences of keratectasia, severe myopia and irregular astigmatism, result in bad spectacle-corrected vision, and reduced corneal thickness which becomes less than 500 μ m, causing corneal weakness due to tissue loss (Randleman et al. 2003; Vinciguerra et al. 2001; Haw et al. 2001, Argento et al. 2001). However, Marinho et al. (2000) have suggested that keratectasia is commonly central and symmetric and does not create irregular astigmatism; therefore, it is unlike keratoconus. Many of these studies support the rigid contact lens as a good choice when keratectasia symptoms do occur (Marcos et al. 2001).

1.8.4.2 Treatment

There is more than one way to treat keratectasia: surgically, such as PKP, intrastromal corneal ring segments and corneal collagen crosslinking (CCL) with riboflavin-ultraviolet type A; and non-surgically, such as with hard contact lenses (rigid gas-permeable "RGP") (Colin et al. 2003a and 2003b). The commencement of surgical treatment is important in cases of failure of non-surgical treatment. Until recently, CCL was considered to be a temporary treatment for keratectasia (Caporossi et al. 2006). Intrastromal corneal ring segments (Intacs) can be used in keratectasia to avoid regression after LASIK (Barbara et al. 2004; Pokroy et al. 2004; Siganos et al. 2002).

Some studies indicate that Intacs are a good option for central ectasia treatment rather than for inferior ectasia (Alio et al. 2005). In contrast, some studies believe that Intacs are not a successful treatment when compared with corneal transplantation (penetrating or lamellar) which produces successful results (Seiler et al. 1998; Amoils et al. 2000; Argento et al. 2001; Rao et al. 2002; Spadea et al. 2002; Rumelt et al. 2001). Before the emergence of CCL with riboflavin and ultraviolet-A, keratoplasty was the first option for keratectasia treatment, but following CCL with riboflavin and ultraviolet-A can stop the progression (Hafezi et al. 2007; Kohlhaas et al. 2005). Some studies have indicated that 30% of keratectasia patients require corneal transplantation for visual rehabilitation; however, Randleman et al. (2003) believe that most keratectasia patients do not need corneal transplantation for visual rehabilitation.

Among the disadvantages of penetrating keratoplasty (PKP) is the probability of graft rejection after surgery (Amoils et al. 2000; Seiler et al. 1998; Argento et al. 2001; Rao et al. 2002).

It is considered that contact lenses (CL) are the best non-surgical option for keratectasia treatment. There are different kinds of contact lenses, soft CL, hard CL, piggyback CL and scleral CL, but hard contact lenses can amend the corneal-ectasia surface and provide good vision. Soft contact lenses, which cannot correct irregular astigmatism, can be used in simple ametropia (Randlemen et al. 2003; Choi et al. 2004; O'Donnell et al. 2004). Collagen crosslinking with riboflavin and UVA can stop the progression of keratectasia by stiffening the corneal stroma (Wollensak et al., 2003a). Choi et al. (2004) reported that hard contact lenses with topographic data and slit-lamp evaluation produce successful results in visual rehabilitation with keratectasia patients.

Despite the advantages of hard contact lenses in keratectasia patients, some patients report pain, therefore the fitting of hard contact lenses needs specialists who are aware of the difficulties (Seiler et al. 2000; Amoils et al. 2000).

1.9 Correction of refractive errors

Refractive error as described previously is an abnormal condition affecting vision.

This abnormality can be treated in two different ways:

- 1- Non-surgical
- 2- Surgical

1.9.1 Non-surgical correction

There are two main methods to treat refractive error in a non-surgical way, with glasses or contact lenses. Glasses are considered to be the first method which treats the refractive error by converting or diverting the light entering the eye. Glasses were invented at least two thousand years ago but, until the present day, the debate is still open about who was the inventor of spectacles and the answer is still not clear, there remaining disagreement over the inventor and developers (Ilardi 2007).

In the sixties, Otto Wichterle invented the first step in the use of contact lenses; since then, scientists have developed lenses that are more comfortable for the patient (Myers 2009). There are two main types of contact lens: soft lens and hard lens. The soft contact lens can treat mild and moderate refractive errors. The hard lens such as rigid gas permeable lenses (RGPs) is usually employed to treat advanced refractive errors and corneal disorders such as keratoconus.

1.9.2 Surgical correction

For a long time researchers have studied the best ways to correct refractive errors. As a consequence, nowadays, there are several methods that can be used to correct refractive errors. Two main optical devices are used, spectacles and contact lenses, though these are considered traditional methods of correction. However, refractive surgery is now an alternative approach to correct refractive errors, rather than the traditional methods. It is important to know that the main aim of all these methods is to focus the rays of light on the retina, and thus get the best vision (Bansal et al. 2001).

The procedure of Radial Keratotomy, one of the oldest surgical methods to correct refractive errors, works to flatten the cornea by using radial incisions to alter the curvature of the cornea (Rowsey et al. 1982; Fyodorov et al. 1979). Luttrull et al. (1982) and Larson et al. (1983) believe that even if this procedure does give good results, as confirmed by Waring (1987), it leads to weakness in the eye and instability of vision. In 1967, Barraquer started to work on the cornea by adding and removing tissues in the stroma to change the curvature; this procedure is called Lamellar Refractive Surgery. It was noted that all previous refractive surgeries caused irregular astigmatism and regression to the patient (Nordan et al. 1986; Goosey et al. 1990). In 1988, Ruiz and Rowsey developed other procedures to reduce the side effects of the previous procedures by using the corneal bed to shape the cornea, which is called the “in situ keratomileusis” technique. In the Automated Lamellar Keratoplasty technique, the myopic patient’s cornea is flattened to correct its refractive errors (Ruiz et al. 1988). In 1983, Trokel started to use an excimer laser to change corneal curvature. Nowadays, photorefractive keratectomy (PRK) and laser-assisted in situ keratomileusis (LASIK) are

the most common surgeries to correct refractive errors and utilise an excimer laser to alter corneal curvature (Bansal et al., 2001).

Following initial positive results, the excimer laser became the preferred option for clinical use rather than other lasers that were used for curving the cornea such as the carbon dioxide laser, hydrogen fluoride laser and dye laser (Srinivasan 1986). The term excimer comes from “excited dimer” (Bansal et al., 2001). Buratto and Pallikaris were the first to use the excimer laser and the microkeratome together (Buratto et al. 1997); however, Trokel and Srinivasan (1983) were the first to use it for corneal cutting and suggested its use in refractive surgery.

1.9.2.1 Photorefractive keratectomy (PRK)

Following this, Marshall et al. (1986) suggested a new technique of using an excimer laser to correct refractive changes by ablating the corneal surface; this procedure is called photorefractive keratectomy (PRK) (Figure 1.16). Despite the satisfactory results of PRK in low and moderate myopia, it has shown some disadvantages, such as development of haze in the central cornea, delay in wound healing and delay in the stabilization of the refractive result (Gartry et al. 1992).

This technique works by initially removing the epithelium, under topical anaesthetic, in one of several ways. These include the use of a blade, or chemically, e.g. by using alcohol. Following epithelial removal, the central cornea is ablated using a computer-controlled excimer laser to steepen or flatten the cornea. Following the procedure the epithelium layer reforms to cover the area operated upon. This usually takes a few days (Bansal et al., 2001).

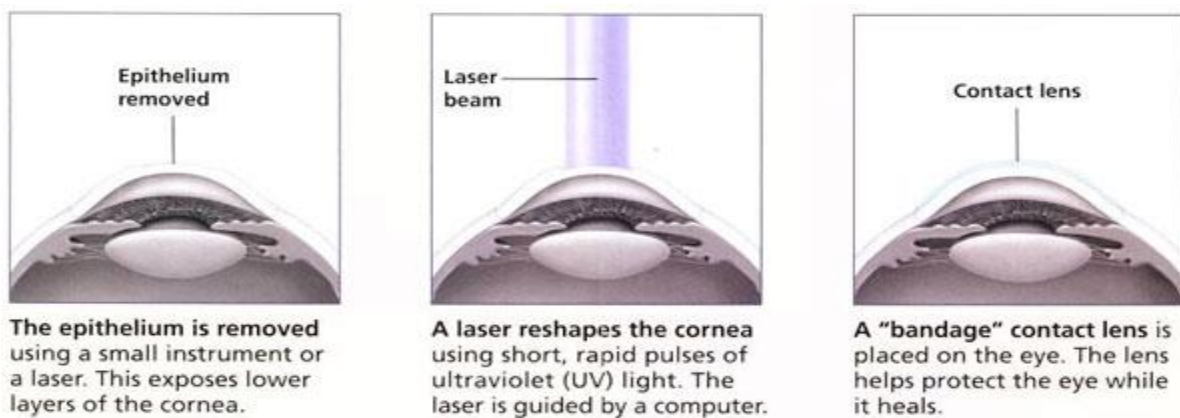


Figure 1.16: Photorefractive Keratectomy (PRK): lasikbiloxi.com/services/prk/ [accessed 21 September 2011].

1.9.2.2 Laser in situ keratomileusis (LASIK)

Subsequently, a new laser technique emerged which depends on ablation of the midstroma of the cornea rather than the surface; this procedure is called laser-assisted intrastromal keratomileusis (LASIK) and was first introduced in Greece, in 1991, by Pallikaris et al. and in Italy in 1992 by Burrato et al. (Figure 1.17). It has many advantages over PRK, including less discomfort, haze and regression, quick visual recovery and little stromal healing which make the refractive results appear quickly (El Maghraby 1995; Gimbel et al. 1998).

In this technique many layers are lifted – the epithelium, Bowman's membrane and anterior stroma – by using a microkeratome to cut a flap in the cornea. Then, stromal ablation is performed using the same procedure as in PRK. Finally, the flap is replaced (Bansal et al. 2001).

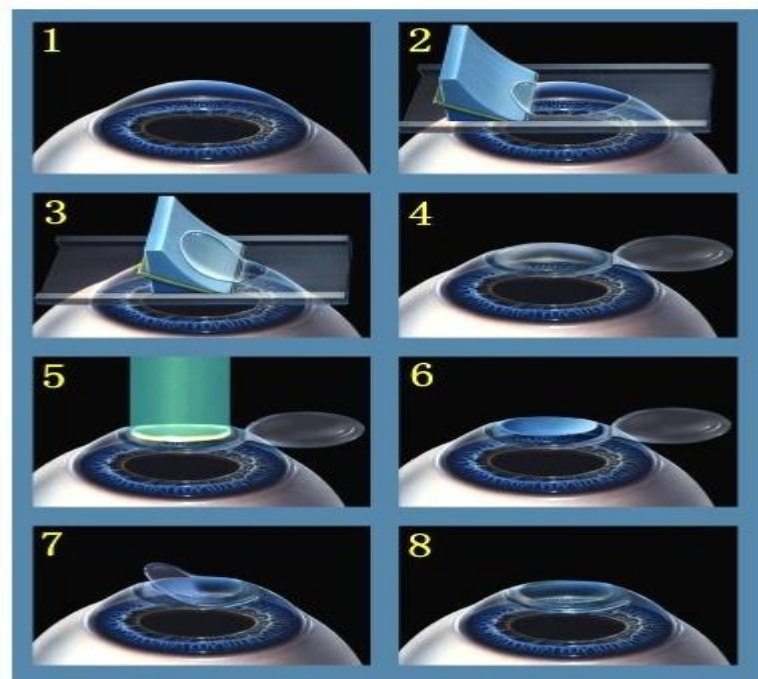


Figure 1.17: The LASIK Procedure: www.epyon-1.com/blog/category/cool-stuff/ [accessed 21 September 2011]

1.9.2.3 Significant rules for LASIK surgery

Several eye examinations must be done before LASIK surgery can be carried out and we can summarize these as:

- Full refraction (visual acuity corrected for distance and near and cycloplegic refraction);
- Corneal examination, including central corneal curvature, tonometry, ultrasonic pachometry and corneal topography (Seiler et al. 1998).

The importance of these examinations is to determine whether the patient has a normal and healthy cornea on which to perform LASIK or not. In addition, they should assess if the corneal thickness and curvature are appropriate for LASIK and make sure that the patient has no signs of keratoconus (Buratto 1997; Singerman 1999; Bores 2001; Probst 2004a and 2004b).

The patient must have had stable refraction for at least one year and must be over 18 years of age. Moreover, the refraction should be between -4.00D and -16.00D in myopia, between +3.00D and +7.00D in hyperopia, and with astigmatism between -2.00D and -7.00D. In addition to that, the central corneal thickness must be more than 500 μ m and the mean value of keratometry must be more than 39.0D and less than 47.00D.

Patients who have ocular diseases – dryness, high level of diabetes (which might affect wound healing) or keratoconus – are not suitable for LASIK surgery.

1.9.2.4 General problems of post-refractive surgery

It is important to know how much LASIK affects the cornea. Occasionally, LASIK surgery results in visual acuity problems and a change in the corneal structure and these are considered the major complications after LASIK.

To avoid mechanical instability after LASIK, the stromal bed should still be at least 250µm thick (Seiler 1998; Hjortdal 1998; Wang 1999). Corneal infection, dislocated flap, under or over correction and epithelial ingrowths are some other post-LASIK complications.

There are not many studies that have examined the factors that influence the biomechanical properties of the cornea after surgery. However, Djotyan et al. (2001) reported that any alteration to the cornea such as LASIK surgery could affect the biomechanical properties of the cornea.

1.10 Corneal collagen crosslinking with riboflavin and Ultraviolet A (UVA)

Corneal Collagen Crosslinking (CXL) with Riboflavin and ultraviolet-A (UVA) was introduced as a new treatment to increase the biomechanical stability of the cornea (Figure 1.18). The UVA light and riboflavin are believed to induce additional crosslinks between or within the collagen fibrils (Spoerl et al. 1999). This method was suggested as a treatment for keratoconus and keratectasia (Wollensak et al. 2003a). Caporossi et al. (2006) mentioned that the cornea after crosslinking is very close to a normal cornea with improvement in best spectacle-corrected visual acuity (BSCVA). The endothelium layer is protected by the riboflavin when the corneal stroma is more than 400um thick (Spoerl et al. 2007). Therefore, if the cornea is too thin and less than the normal thickness, there is a high risk of damage to the endothelium (Hafezi et al. 2007).



Figure 1.18: Corneal Collagen Cross-linking: www.emagin.com.au/cornealx.html [accessed 21 September 2011]

Until recently, corneal ectasia was treated by hard contact lenses; if this was not suitable for the patient then penetrating keratoplasty (PKP) or intrastromal corneal rings were the surgical option; now, corneal collagen crosslinking is considered the new and advanced treatment rather than PKP or corneal rings. Hafezi et al. (2007) believe that corneal collagen crosslinking should be started with all patients, whether hard contact lenses are suitable for them or not. Studies have differed in their opinions over the issue of removing the epithelium.

In 2008, Hayes et al. clarified the importance of epithelial removal before riboflavin-UVA corneal collagen crosslinking, thus making riboflavin penetrate the corneal stroma which increases the efficacy of this therapy (Hayes et al. 2008). This therapy will help in reducing the symptoms of ectasia which has become a problem threatening patients after LASIK (Randleman et al. 2008). In addition to stopping corneal ectasia, Wollensak et al. (2004) believe that corneal collagen crosslinking may treat strong myopia, corneal ulcers and scleral biomechanical weakness. In addition to that, corneal collagen crosslinking could be used to stiffen the cornea before PKP and LASIK which give the procedure more positive results. Despite the promising results for riboflavin-UVA collagen crosslinking so far, we must search for long-term stability indications and contra-indications if this therapy is to be a solution to progressive eye problems. Recently, surgeons have preferred to use corneal collagen crosslinking with riboflavin and UVA for keratoconus and keratectasia patients (Spoerl et al. 1998; Wollensak et al. 2003a).

Braun et al. (2005) believe that keratoconus patients are likely to undergo refractive surgery correction after using crosslinking to reduce cornea elasticity, which

leads to stabilization of the progression of keratoconus. Sandner et al. (2004) expect that CXL will help in the treatment of keratoconus and thus reduce the use of penetrating keratoplasty, particularly after they obtained the result that there were no negative side effects for their patients up to 38 months after crosslinking treatment (Sandner et al. 2004). Usually, crosslinking is suitable for patients with keratoconus or keratectasia whose corneas are over 400um thick. Patients who feel pain during the first 48 hours after surgery, sensitivity to light and blurred vision for several days need drops to reduce the effects of these problems.

1.10.1 Surgical technique

The surgical technique begins with local anaesthesia on the eye using tetracaine 1% and oxybuprocaine 0.4%, removing the central 7-8mm epithelium layer, then applying riboflavin (0.1% solution 10mg. riboflavin-5-phosphate in 10ml. dextran-T-500 20% solution) every 3 minutes for 30 minutes to make sure that the stroma is completely penetrated so as to provide protection for the deeper parts of the eye. After that, ultraviolet-A irradiation is focused on the cornea, riboflavin still being applied on the cornea every 5 minutes during the UVA focusing (Wollensak et al. 2003a). Contact lenses are necessary after treatment, for approximately 3 days until the epithelium heals, followed by application of fluorometholone 0.1% eyedrops for 6 weeks (Hafezi et al. 2007).

Retaining all layers of the cornea in cross-linking, except for epithelial cells (which grow back with around 48 hours) is one of the main advantages of this therapy. However, researchers have started to assess the possible benefits of a different kind of

riboflavin, Ricrolin TE (Riboflavin 0.1% with trometamol and sodium EDTA) and in this thesis we investigate trans-epithelial stromal absorption and determine if the corneal collagen cross-linking could be completed without removing the epithelium or not.

1.11 Aims of this thesis

The hypotheses behind this thesis are:

- that the ocular response analyser can be used both in vivo and in vitro to investigate a number of biomechanical properties of the cornea and how these depend on other factors.
- that crosslinking with UVA/riboflavin may be characterised at the molecular level using Fourier Transform Infrared Spectrometry (FTIR) and may be improved using a new treatment called Ricrolin TE.
- that crosslinking under different conditions can affect the biomechanical properties of the cornea in different ways.

The overall aim of this thesis is to improve understanding of the biomechanical properties of the cornea, and how they change in different conditions. Therefore, I aimed to measure corneal hysteresis and resistance factor in animal and human eyes both in vivo and in vitro and show its dependence on intraocular pressure and other factors. In addition to that, compare the biomechanical properties of normal and post-LASIK corneas. Then, use FTIR to determine the molecular effects of crosslinking with riboflavin/UVA. After that, investigate the penetration of Ricrolin TE into the cornea with and without removal of the epithelium and compare the results with the penetration of conventional riboflavin. Finally, compare the biomechanical effects of UVA/riboflavin crosslinking after conventional riboflavin vs Ricrolin TE and before/after a laser in situ keratomileusis-like flap.

Chapter 2: General Materials and Methods

2.1 Introduction

This thesis includes many different experiments and each experiment has its own materials and methods. This chapter describes only the specialised equipment and its use. Experimental details relevant to specific chapters are described within the chapter itself.

Experiments in this thesis used a healthy volunteer group, a post-LASIK group, an intact human eyes group, an intact animal eyes group, a human corneas group and an animal corneas group. The study protocol, where human and animal tissue was used, was consistent with the tenets of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on the use of animals in ophthalmic research.

The volunteers and patients who participated in clinical experiments were students at Cardiff University, Cardiff, UK. The studies which included patients had local ethical committee approval and informed consent was obtained from each participating subject after full explanation of the experiment's steps and why we were doing this test on them.

The human eyes used in this thesis were obtained from the National Disease Research Interchange, Philadelphia, USA. In addition to that, bovine eyes were

obtained from the abattoir at Cinderford, Gloucestershire within hours of death and were used only if in good condition.

Human corneas with at least 2-mm scleral rims were received from the Manchester Eye Bank, Manchester, UK. These corneas could not be used for any corneal transplantation due to their low count of endothelial cells.

For the statistical analysis, linear regression was used to determine trends in the data. Statistical comparisons of significance were carried out using SPSS (IBM SPSS statistics 16.0, Chicago, IL, USA). Non-overlap of 95% confidence intervals of regression lines was taken as evidence of statistical significance or differences between them. The residuals from the regression analysis were assessed for normal distribution using a Kolmogorov-Smirnov test and indicated that the data met the necessary assumptions for regression analysis. Pearson tests were carried out to confirm the correlation between the datasets. The Pearson correlation coefficient and p-value are indicated for each experiment where appropriate.

2.2 The Ocular Response Analyzer (ORA)

2.2.1 Introduction

The ocular response analyzer (ORA) (Reichert Ophthalmic Instruments, Depew, NY, USA) is a non-contact applanation tonometer that can provide a measure of the corneal compensated intraocular pressure (IOPcc) which is independent of corneal factors that can influence results, and is derived from Goldman tonometry (IOPG) (Luce 2005). In addition, two biomechanical parameters are provided, corneal hysteresis (CH) and corneal resistance factor (CRF) (Figure 2.1).



Figure 2.1: The ocular response analyzer (ORA)

Corneal hysteresis is defined as the difference in IOP recorded during inward (P1) and outward (P2) applanation and is therefore regarded as an indicator of the viscoelastic properties of the cornea – the higher the CH value, the more energy is

absorbed by the system during the deformation process, therefore the more viscoelastic it is (Figure 2.2).

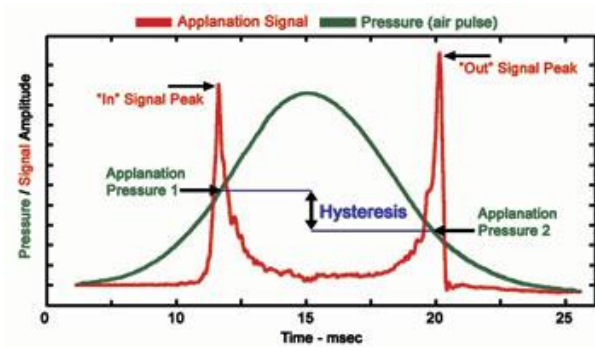


Figure 2.2: ORA signal

Moreover, the ORA has an inbuilt ultrasound pachymeter which measures the central corneal thickness (CCT).

2.2.2 Measurements from whole eyes and excised corneas

The eyes (human and animal) which were used in the ORA experiments were allowed to thaw if they were frozen and then the central corneal thickness (CCT) was measured (using a Pachette 2, Model DGH 550 ultrasonic pachymeter (DGH Technology Inc., Exton, PA, USA)) to ensure that they were in the correct physiological range in all cases and stages. This type of pachymeter takes 25 readings and the average of these with the standard deviation appears on the screen at the end of the measurements. After that, the IOP was measured and adjusted depending on the

experimental protocol; the IOP was adjusted by injecting the eye horizontally from the limbus with 0.9% sodium chloride.

At the end, the eyes were gently clamped using a retort stand and carefully positioned in front of the ORA machine after applying artificial tears on the front surface of the cornea; then, the IOP, CH and CRF were measured (Figure 2.3).



Figure 2.3: A fresh eye during the measurement in front of the ORA

On the other hand, the human corneas (with a rim of sclera) which were used in these experiments were examined and their integrity was checked. If corneas were swollen when they arrived, we removed the swelling using a standard equilibration method (Meek et al. 1991) which reduced the corneal thickness to within the correct

physiological range ($\approx 520 \mu\text{m}$). After the epithelium and endothelium were removed from the corneas by gentle scraping, the corneas were clamped within 12,000 MW cut-off dialysis tubing that was carefully smoothed to remove any air bubbles and to ensure close contact between cornea and tubing. They were then dialysed overnight at 4°C in a solution containing 0.154 m NaCl in 5 mm Hepes, pH 7.4, and 2.75% polyethylene glycol (MW 20,000). The corneas were then removed from the dialysis tubing, carefully centred within an artificial anterior chamber (Fig 2.4) and then clamped gently by the scleral rim. A balanced salt solution was placed within the chamber behind the cornea, and a pressure equivalent to an IOP was maintained by a supporting column of solution; the pressure could be adjusted by varying the height of the supporting column or by increasing the pressure on the supporting column.



Figure 2.4: artificial anterior chamber

A similar procedure was carried out on the animal corneas. When the fresh animal eyes arrived at the laboratory, we excised the cornea from the eye (by using an appropriate scalpel) with part of its sclera to enable us to clamp it during the experiment. If we needed to remove the epithelium or the endothelium, both were removed by using appropriate scalpels.

At the end, both human and animal corneas were gently clamped and placed into the artificial anterior chamber, then using a retort stand we carefully positioned the cornea in front of the ORA machine, and then the IOP, CH and CRF were measured (Figure 2.5).

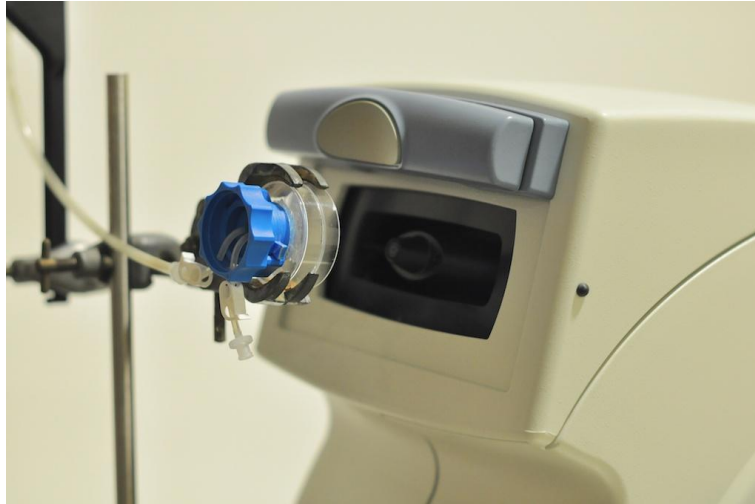


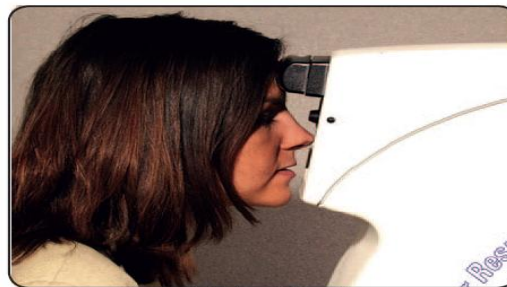
Figure 2.5: cornea in an artificial anterior chamber positioned in front of the ORA using a retort stand

In both intact eyes and corneas, artificial tears were applied to the front of the cornea to keep the front surface of the cornea wet and thus produce a good optically reflecting surface.

2.2.3 Measurements from patients

As mentioned previously, each participating subject signed a consent form and a full explanation was given by the examiner. After we ensured that the patient had come without wearing contact lenses (patients were informed in advance), the patient was

informed that nothing would touch their eyes and there would just be a puff of air coming from the machine and one test was applied to show the patient the expected puff of air. After we adjusted the height of the table and chair, the patient sat in the correct position, as shown in Figure 2.6, with the chin and the nose inside the front surface of the device. We asked him or her to focus on the green fixation light, which was located in the tube between four red dots of light, and not to blink for a few moments.



Correct Patient Positioning



Incorrect Patient Positioning

Figure 2.6: Patient position in front of ORA

Previously, the machine had old software, so we usually took multiple measurements of each eye and then the average was used in the final results.

However, later on, we used the waveform score of the later version (version 2.04) of the ORA software which provides the best signal value (BSV); this was used in the final results.

2.3 The Oculus Pentacam

2.3.1 Introduction

The Oculus Pentacam (Oculus, Inc., Lynnwood, WA) is a non-contact, fast and accurate device to measure certain corneal properties such as corneal curvature and corneal thickness (Figure 2.7).



Figure 2.7: The Oculus Pentacam

An image of the anterior eye segment can be taken in a maximum of 2 seconds. The Pentacam provides a three-dimensional scan of the anterior/posterior corneal topography using a rotating *Scheimpflug* camera. This camera rotates from 0° to 180° and takes two seconds to capture 25 to 50 images of each patient eye. One of the most important features of Pentacam is that it can detect the eye's movement by a second camera during the scan which gives the machine its accuracy; moreover, blinking is also taken into account in the final results. There are many different types of maps that

can be provided by the Pentacam, which include anterior and posterior elevations (Figure 2.8).

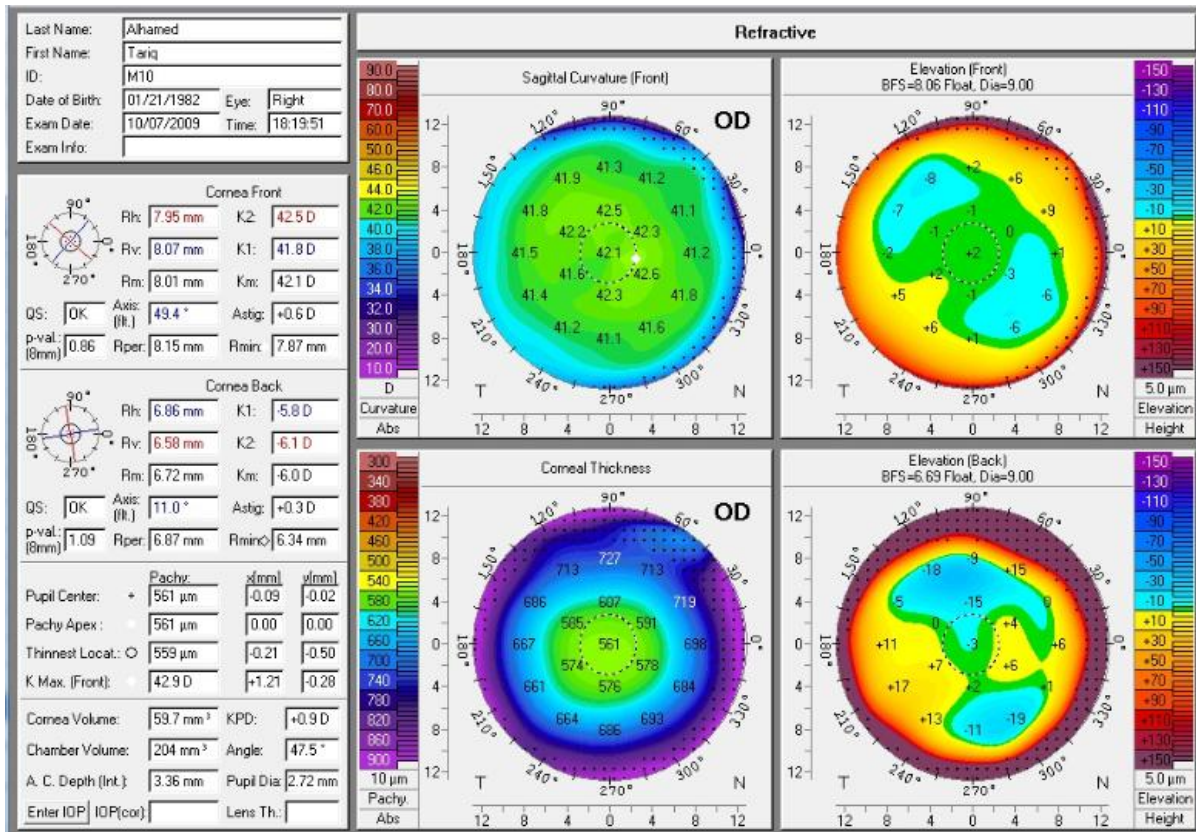


Figure 2.8: Overview map from the Oculus Pentacam. It can be clearly seen on the left side of the map the evaluation of the front and the back of the cornea, also, the corneal pachymetry display on the bottom left side. In addition to this, the four maps show the general situation of the front and the back of the cornea.

2.3.2 Screening process for patients

When the patient had arrived and signed a consent form, we explained the procedure step by step for the convenience of the patient. After that, and before starting the measurements, we ensured that the patient was in the correct position and could focus on the light inside the device and would not blink for 2 seconds until the camera had taken the images. The patients could not wear contact lenses and we also mentioned that nothing would touch their eyes during the test.

2.4 Spectrophotometer

2.4.1 Introduction

Two combined instruments make up the spectrophotometer (Figure 2.9). One of them is for the production of light, which is called a spectrometer, and the other is for measuring the intensity and absorption of light (the detector). In general, therefore, we can define the spectrophotometer as a quantitative device which measures light transmission of different tissues at different wavelengths.



Figure 2.9: The spectrophotometer

A one millimeter beam width exits from a small gated light source in the device, goes through the sample chamber and ends up in the detector.

A specialized sample chamber was made with two polished glass stands on a plastic base and filled with Dow Corning silicon oil 200/5cs by injecting a needle through

a valve in the top of the chamber (Figure 2.10). The silicon oil was used because it has a refractive index very close to the corneal refractive index and so minimizes reflections. The chamber had a valve on the top with a stopper to retain the silicon oil in the chamber and hold the cornea (Figure 2.11).

The chamber was placed about 1.5 cm. away from the light source and measurements were taken.

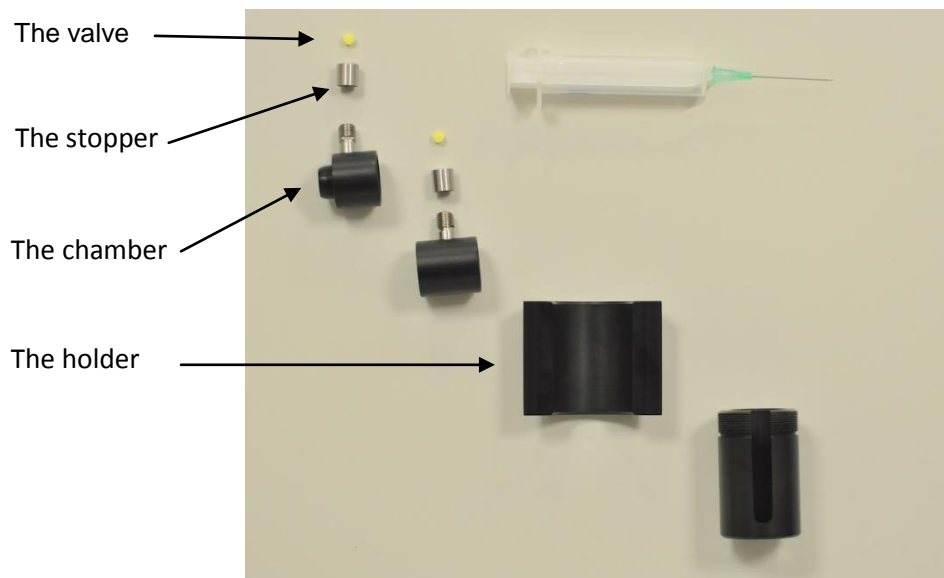


Figure 2.10: A sample chamber to hold the cornea in the spectrophotometer

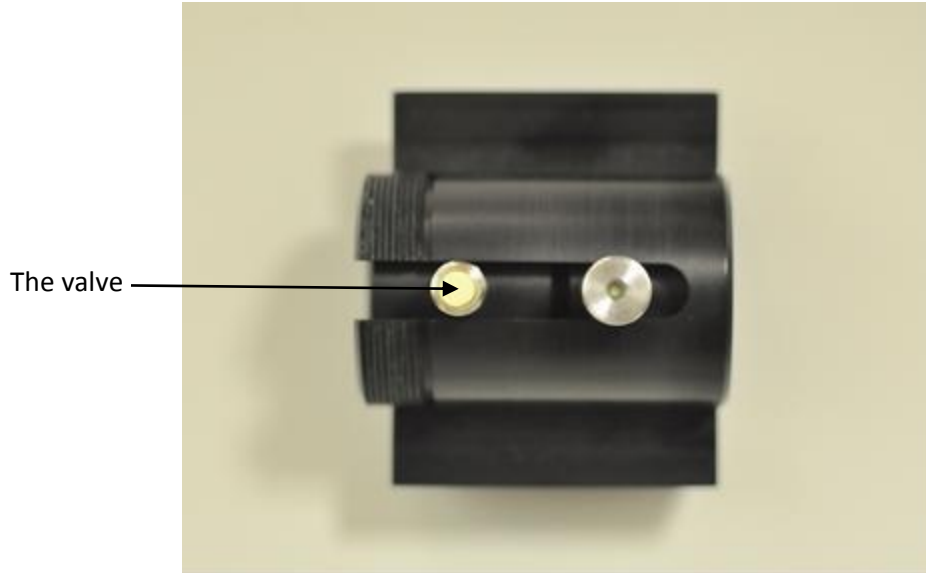


Figure 2.11: The valve on the top of the chamber.

2.4.2 Measurement and preparation of samples

The machine was switched on, left for about 30 mins. to warm up and set to a concentration of 100. After that, blank measurements were obtained by placing the sample chamber in the spectrophotometer filled only with silicon oil (without a tissue). The wavelength was varied from 400nm to 700nm by increasing it by 10nm intervals and the results were recorded at each stage. After achieving blank measurements and writing down all the results, the corneal measurements were obtained. The corneas had been cut from the eyes with their scleral rim in order to be able to adjust their position within the sample chamber. This was necessary to ensure that the cornea was placed in the centre of the chamber and that the beam was going through the centre of the cornea. The chamber was filled with silicon oil by using a syringe injected into the stopper through the valve (Figure 2.12).



Figure 2.12: T syringe inserted through the valve in the chamber

Finally, we placed the sample chamber in front of the light gate and ensured that the anterior of the cornea was placed in front of this gate and then took the measurements (Figure 2.13).

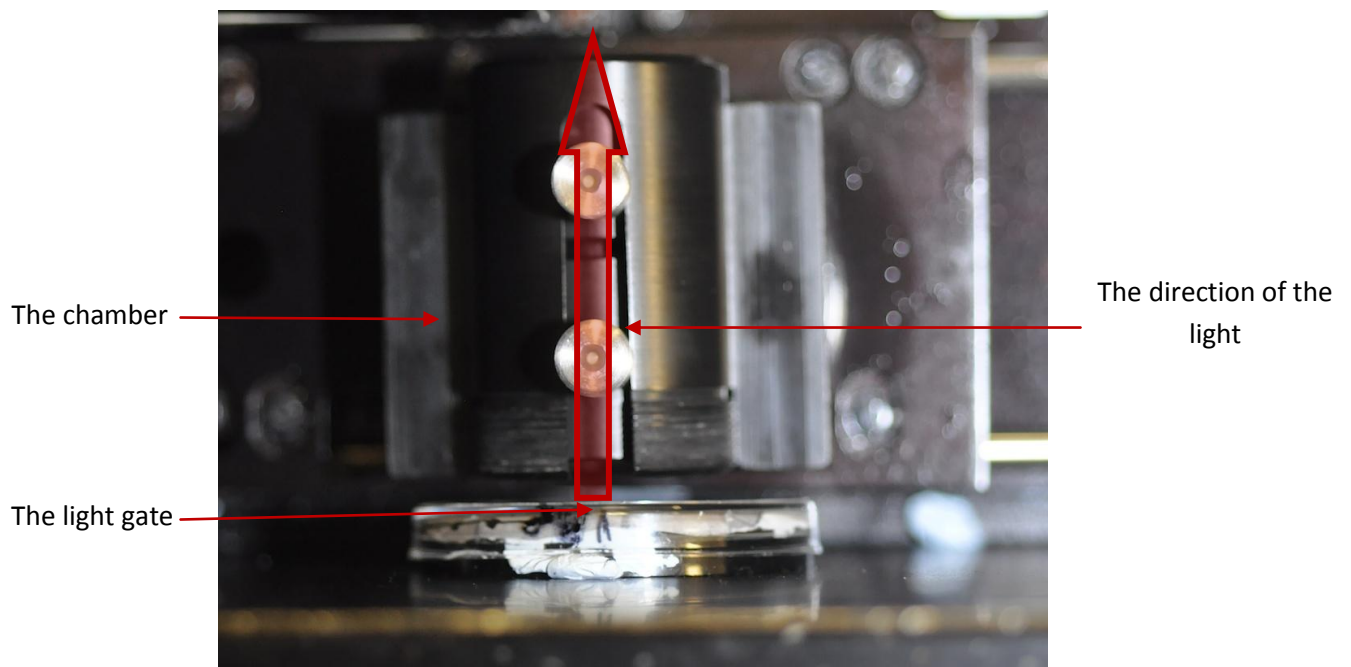


Figure 2.13: The chamber inside the spectrophotometer and front of the lights gate

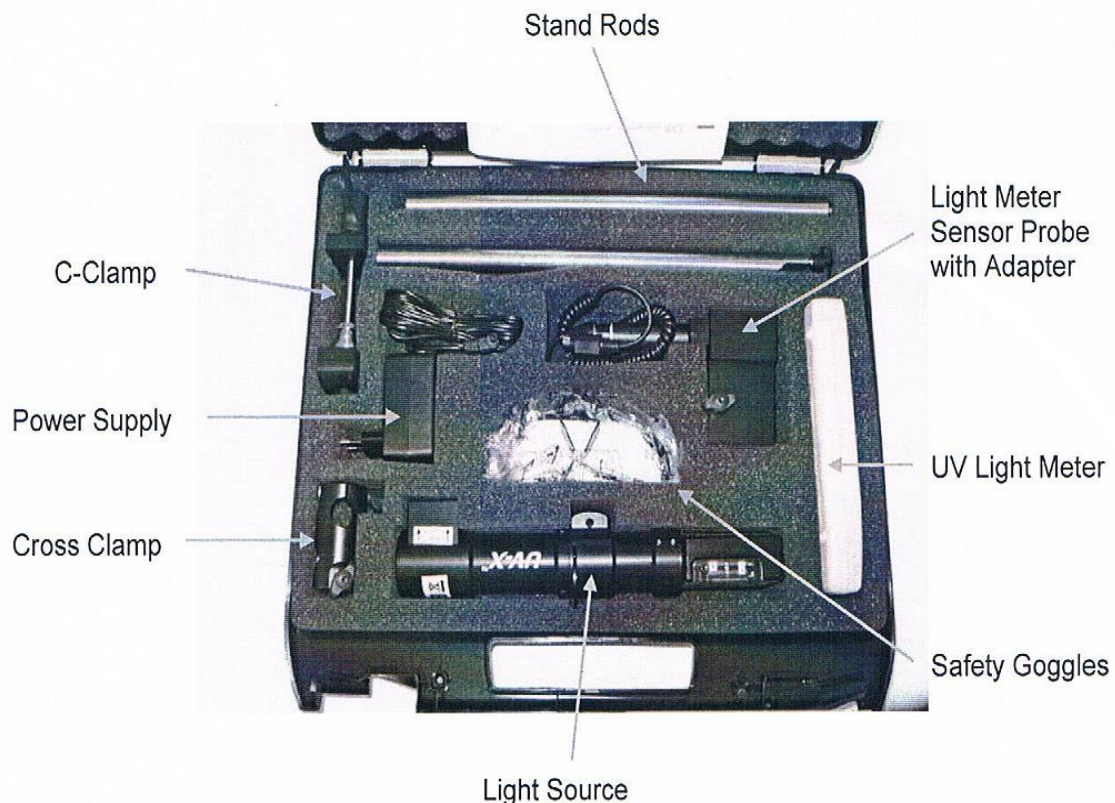
2.4.3 Precautions and advice

- Tissue samples should be cleaned by using, for example, sodium chloride 0.9% before placing them in the chamber.
- Care should be taken when cleaning the chamber; it should be ensured that nothing can scratch the glass.
- Warm up the machine and double check that the machine is working well so as to not to lose tissue samples if it is not working well.
- Before taking measurements, it must be ensured there are no air bubbles in the medium which must be clear enough for the beam to go through it.
- To achieve a satisfactory result, we must ensure that the cornea is placed in the center of the chamber.

2.5 Crosslinking using the UV-X illumination system

2.5.1 Introduction

The UV-X illumination system is a medical electronic device which creates UV-A rays to increase the biomechanical stability of the cornea (Figure 2.14). It emits UV-A during corneal collagen cross-linking surgery and targets a specific area in the treated cornea at a wavelength of 365nm with an internal microprocessor controlling the device. A photo-sensitizer such as riboflavin must be used in this procedure to protect the inner layers of the cornea, particularly the endothelium, to protect the back of the eye, including the retina, and also to increase the cross-linking in the cornea. This procedure is becoming common practice to treat keratoconus patients and the keratectasia which sometimes follows refractive surgery.



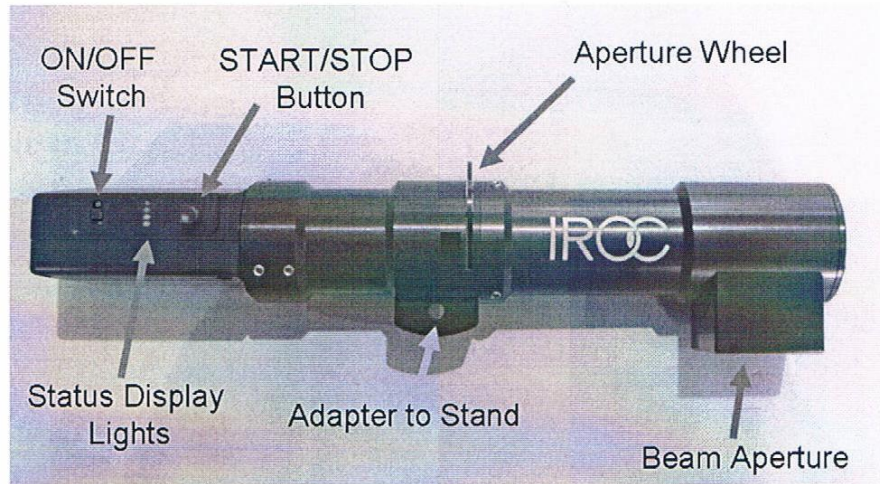


Figure 2.14: The UV-X illumination kit (above) and the assembled UV light source (below)

The distance between the treated cornea and the beam aperture is 50mm (the beam aperture diameter is 25mm) (Figure 2.15). This system has three different sizes of light diameter: S \approx 7.5mm, M \approx 9.5mm and L \approx 11.5mm.

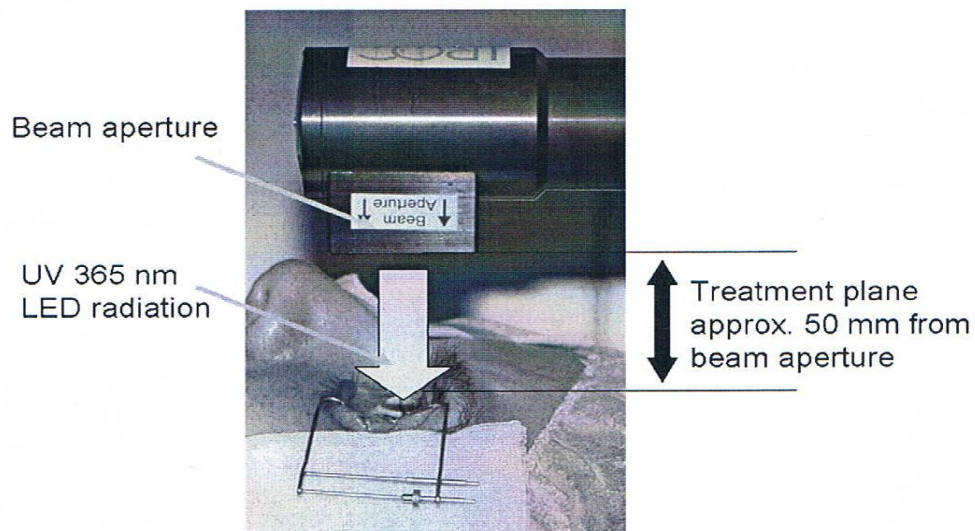


Fig 2.15: UV crosslinking set-up

2.5.2 Preparation of sample

In our studies, we treated samples in vitro only, and either normal riboflavin (vitamin B₂) in dextran, prepared riboflavin (ricrolin®), or ricrolinTE® was used.

When fresh eyes arrived at the laboratory, we measured the corneal thickness to ensure that the corneas were not swollen, and then we checked the integrity of the eyes in general. Before starting the treatment procedure, we had to clarify how we would prepare the riboflavin. In the first studies, we prepared the riboflavin ourselves by mixing 1.25g. of dextran T500 with 3.5ml. of NaCl 0.9% (solution 1). After that, we mixed 10mg. of vitamin B₂ with 2ml. of NaCl 0.9% (solution 2). Then, we mixed 1.5ml. of solution 1 with solution 2 and covered the container (because the solution is very sensitive to the light).

Later on, we used prepared normal riboflavin and riboflavin TE (SOOFT italia, Montegiorgio (FM) Italy) which came ready to use as shown in Figure 2.16.



Figure 2.16: prepared Riboflavin

After preparing everything, we started treating the corneas as described in Chapter 1; each group of corneas was used in different experiments as the protocol of the experiment required. One of the most important features in this system is that the UV emission stops 30 minutes after the beginning of treatment.

2.5.3 Precautions and advice

1) The beam aperture must be free from dust and dirt and nothing must touch it before or after use, and it is recommended to cover it when not in use.

2) The person using this equipment should wear safety glasses during the treatment as this system is a class 3R LED product, which means any direct exposure to the rays could affect the examiner, especially if the site has some reflective materials.

3) The whole of this procedure should be carried out in a good and quiet atmosphere as it requires careful attention before, during and after doing it.

4) The device must be set up on a stable surface to ensure that the system does not move during the procedure.

5) Users should be cautious when using this system, ensuring that the correct dose of photo-sensitizer is applied because, as mentioned previously, any error in the work order could result in harm to the patient or the sample, for example:

The back of the eye specially the retina could be damaged if the photo-sensitizer (riboflavin) is not applied before emitting the UVA.

The endothelium layer could be damaged if the photo-sensitizer (riboflavin) is not sufficient to protect the inner layer of the cornea. Generally, the cornea could be harmed if the UVA level is too high.

Chapter 3: Comparison of factors that influence the measurement of corneal hysteresis in vivo and in vitro.

3.1 Introduction

The Ocular Response Analyzer measures corneal hysteresis and corneal resistance factor (see Section 2.2). Corneal hysteresis is defined as the difference in intraocular pressure recorded during inward (P_1) (P_1 is the inward pressure loading to the front surface of the cornea) and outward (P_2) applanation (P_2 is the outward pressure (unloading) coming back from the cornea) ($CH = P_1 - P_2$) and is therefore regarded as an indicator for the viscoelastic properties of the cornea, the higher the value of CH, the more energy absorbed by the system during the deformation process therefore the more viscoelastic it is. CRF is a new corneal measurement which shows overall resistance (or rigidity) of the cornea.

In recent years there have been numerous reports describing the use of the ORA for measuring corneal biomechanics as a function of corneal or other variables. Some reports indicate that CH is correlated with central corneal thickness (Luce 2005; Shah et al. 2006; Montard et al. 2007; Kamiya et al. 2008), though the others found no strong correlation (Touboul et al. 2008). It has also been correlated with intraocular pressure (IOP) (Liu et al. 2008; Kamiya et al. 2008), though, again, this has been questioned (Touboul et al. 2008). CH has been found to be independent of gender (Montard et al. 2007; Liu et al. 2008; Kamiya et al. 2008). It is also independent of spherical equivalent or whether left or right eyes are examined (Montard et al. 2007; Ehongo et al. 2008). Most studies report that CH shows little or no dependence on age (Kirwan et al. 2006;

Montard et al. 2007; Liu et al. 2008; Kida et al. 2008; Kamiya et al. 2008; Fontes et al. 2008) but others have reported a negative correlation with age (Moreno- Montanes et al. 2008).

There appears to be only weak dependence on corneal curvature, diameter and astigmatism (Montard et al. 2007; Liu et al. 2008).

In the same context, CRF has been investigated in some aspect. Montard et al. (2007) show no correlation between CRF and CCT, gender and age, however, Fontes et al. (2008) did find a correlation between them. The measurement of CRF has been found higher in post-LASIK patients than in keratoconus patients (Ortiz et al. 2007). Kida et al. (2008) suggested that CRF decreases when the age is increasing. Moreover, Franco and Lira (2009) did not find any correlation between CRF and anterior corneal curvature. Although there is a strong correlation between CH, CRF and CCT, only CH correlated with high myopia; Shen et al. (2008) show how CH significantly decreased with high myopic patients.

Since we investigate the factors that affect the measurement of some corneal biomechanics properties, we have to mention that the corneal hydration is considered to be a factor that could affect CRF measurement as Lu et al. (2007) observed a significant increasing in CRF value in eyes wearing a contact lenses and kept closed for 3 hours.

The relationship between CH and more conventional biomechanical measurements such as rigidity, stiffness or elasticity has yet to be fully determined. ElSheikh et al. (2008) showed, on isolated corneas, that the value of hysteresis

depends on the rate of pressure application. Glass et al. (2008) developed a model for evaluating viscosity and elasticity to examine the effects that both properties have on hysteresis. They found that low hysteresis can be associated with either higher elasticity or low elasticity, depending on the viscosity. Furthermore, these authors pointed out that viscosity and elasticity affect hysteresis in potentially offsetting ways. This complex relationship probably underlies some apparent contradictions in the literature, for example why hysteresis in keratoconic corneas decreases whereas these corneas are known to have low elasticity (Edmund et al. 1988).

To date, all studies using the ORA have been carried out on corneas in vivo, but natural variability can limit the control available in such studies. In this chapter, therefore, we describe experiments using the ORA both in vivo and in vitro. This has allowed us to more clearly demonstrate the relationship between CH/CRF and IOP, and has given some insight into other factors that can influence CH measurements.

3.2 Materials and Methods

3.2.1 In Vivo

This study included fifty-six eyes of 56 healthy volunteers combined male and female who had no current or history of significant ocular or systemic pathology or any general health problems other than refractive errors. The volunteers were within the age range of 18-70 years, and had a range of refractive errors (spherical equivalent) from -3.75 to 2.25 D.

The measurements of intraocular pressure, corneal hysteresis and corneal resistance factor were performed using the Ocular Response Analyzer (ORA) (Reichert Ophthalmic Instruments, Depew, NY, USA). We used the IOP_{cc} , a cornea-corrected value of IOP produced by the ORA that the manufacturers claim to be less affected by corneal properties than other methods of tonometry, such as Goldmann. Measurements were made four times and the average was used in the final result. The validity of this approach was confirmed on some data sets by repeating the analysis using the waveform score of the later version of the ORA software (version 2.04). Age, gender, central corneal thickness (CCT) and refractive error were also recorded.

3.2.2 In Vitro

Seven intact human eyes from different donors (aged between 65 and 69) were obtained from the National Disease Research Interchange (NDRI), Philadelphia, USA. The eyes were frozen within 6 to 8 hours of death and shipped to the UK. The eyes were allowed to thaw and showed no signs of corneal swelling. The central corneal thickness was measured and was found to be within the physiological range in all cases. The intraocular pressure was adjusted by injecting the eye from the limbus with 0.9% sodium chloride.

To keep the front surface of the cornea wet and thus produce a good optically reflecting surface, artificial tears were applied to the front of the cornea. The eye was gently clamped using a retort stand and carefully positioned in front of the ORA

machine. When the cornea was in the correct position (only at this point was an ORA signal produced), we carried out CH and CRF measurements as described above.

The same procedure was performed using twenty-one human corneas with at least 2mm scleral rims, received from the Manchester Eye Bank, Manchester, UK. These corneas had low endothelial cell counts so were deemed unsuitable for transplantation. On arrival in culture medium, the corneas were found to have swollen by different amounts, with central thicknesses (CCT) in the range 780 - 961µm (measured using a Pachette 2, Model – DGH 550 ultrasonic pachymeter (DGH Technology Inc., Exton, USA)). The corneas were de-swelled using a standard equilibration method (Meek et al. 1991). Corneas were clamped within 12kD cut-off dialysis tubing which was carefully smoothed to remove any air bubbles and to ensure close contact between cornea and tubing. They were then dialysed overnight at 4°C against a solution containing 0.154M NaCl in 5mM HEPES, pH7.4, and 2.75% polyethelene glycol (MW 20,000kD). This procedure produces an osmotic gradient (Hodson et al., 1991) that equilibrates the cornea to normal hydration. This reduced corneal thicknesses to $495\mu\text{m} \pm 13.0$, close to the physiological level ($\approx 520\mu\text{m}$).

Each cornea was then removed from the dialysis tubing, carefully centred within an artificial anterior chamber and then clamped gently by its scleral rim. A balanced salt solution was placed within the chamber behind the cornea, and a pressure equivalent to an IOP was maintained by a supporting column of solution. This pressure could be adjusted by varying the height of the supporting column. For each cornea, CH was measured within the normal range of IOP and at an elevated pressure. Each cornea was mounted in front of the ORA in the same way as the whole eyes, bathed with

artificial tears, and ORA measurements recorded as above. In all cases, ORA data were scrutinised carefully following procedures suggested in the ORA User's Guide (Reichert Ophthalmic Instruments, 2005, ISO-9001/13485) and unsuitable readings were discarded. In the experiments with excised corneas, this meant that some data needed to be excluded, so that we were only able to use data from 17 of the original 21 corneas.

In the second part of this experiment, we measured CH and CFR in 8 whole bovine eyes. The corneas from these same eyes were excised using a medical scalpel, ensuring that this cutting did not affect the structure of the cornea (leaving at least 2mm of scleral rim to adjust these corneas correctly in the artificial anterior chamber to hold them in front of the ORA). Artificial tears were applied during the test to keep the front surface of the cornea wet. Measurements of CH and CRF were then taken from the excised corneas for comparison with the values obtained from the whole eyes.

Linear regression analysis was used to determine trends in the data. Statistical comparisons of the significance were carried out using SPSS. Non overlap of the 95% confidence intervals of regression lines was taken as evidence of statistical significance between them. The residuals from the regression analysis were assessed for normal distributions using the Kolmogorov-Smirnov test, and indicated that the data met the necessary assumptions for regression analysis. Pearson tests were carried out to confirm the correlation between the data sets. The Pearson correlation coefficient and p-value are indicated on each graph.

3.3 Results

3.3.1 In vivo

Figure 3.1a and shows that there is a negative correlation between CH and IOP_{cc} (the Pearson correlation between CH and IOP_{cc} was $r = 0.53$, $p < 0.0001$) and figure 3.1b shows the same negative correlation between CRF and IOP_{cc}, whereas Figure 3.1c indicates no correlation between CH and IOP_g, and Figure 3.1d shows it between CRF and IOP_g.

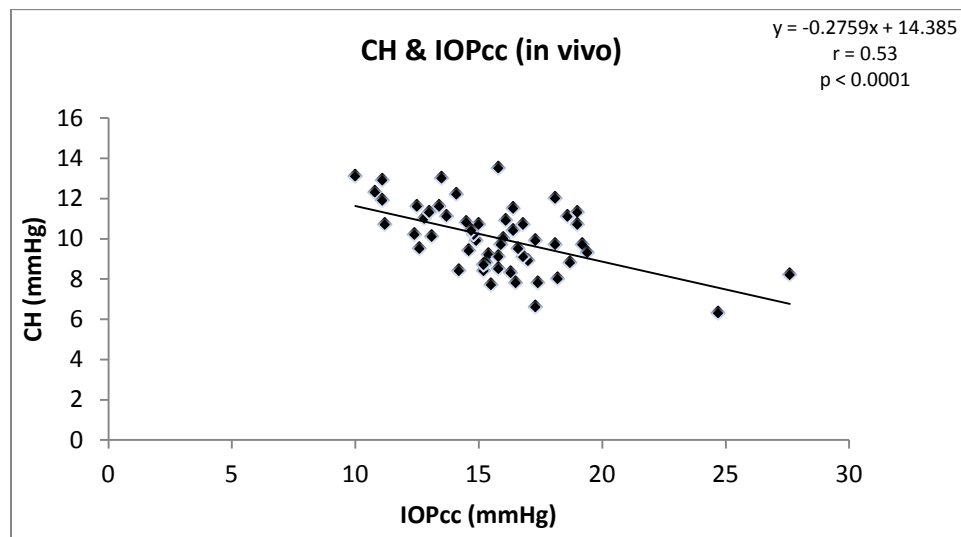


Figure 3.1a: The significant negative correlation between CH and IOP_{cc} in vivo.

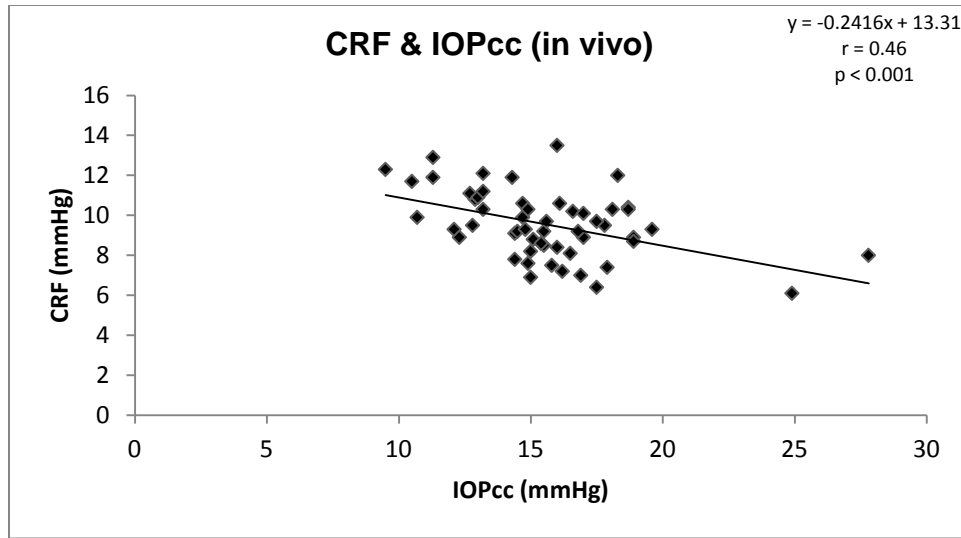


Figure 3.1b: The significant negative correlation between CRF and IOP_{cc} in vivo.

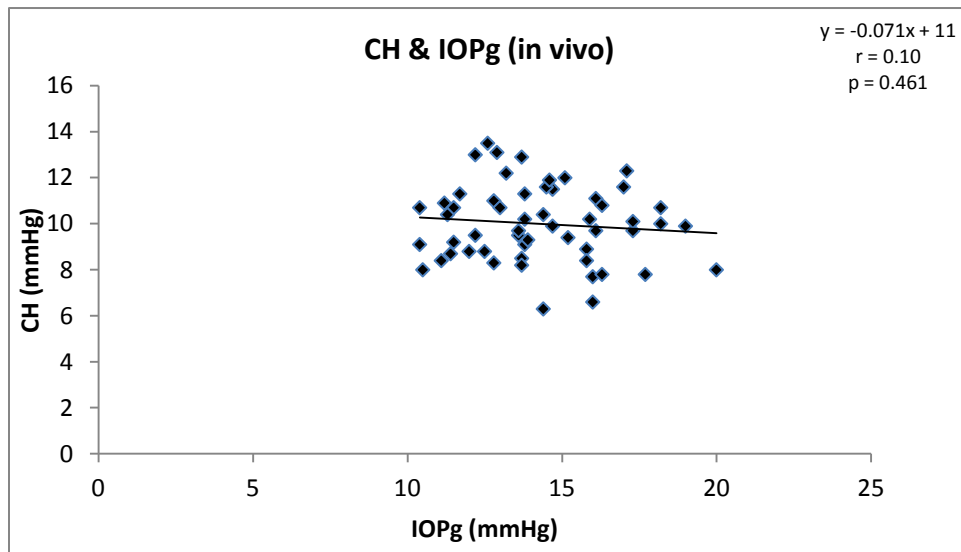


Figure 3.1c: The insignificant correlation between CH and IOP_g in vivo.

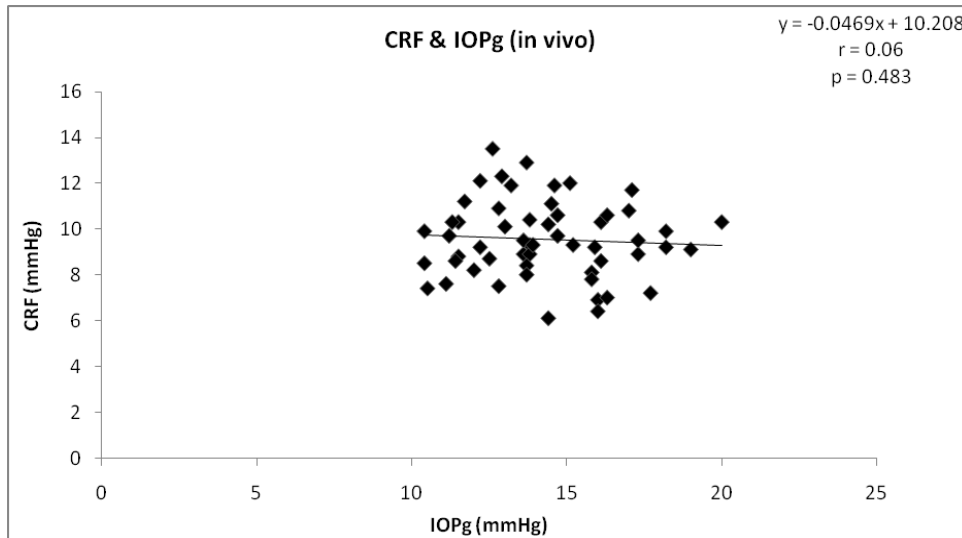
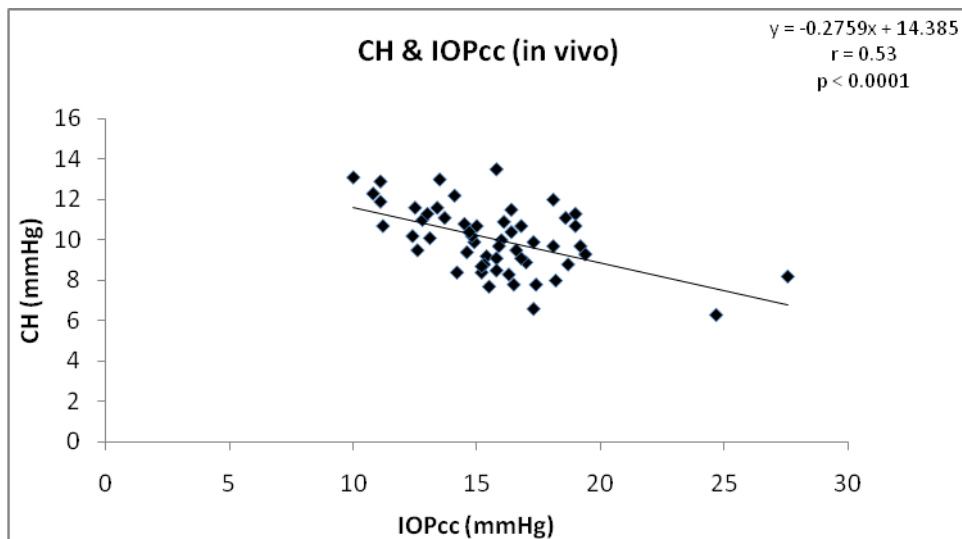
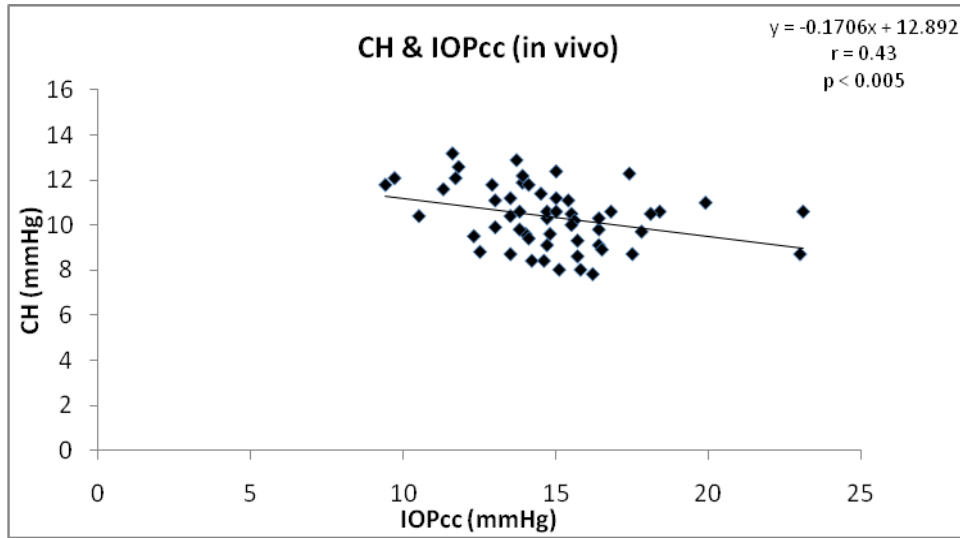


Figure 3.1d: The insignificant correlation between CRF and IOP_g in vivo.

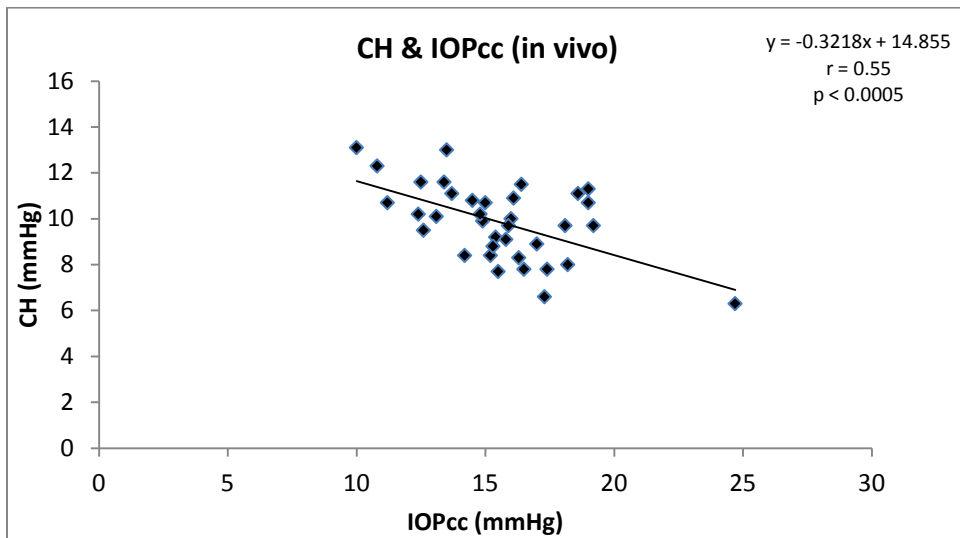
Figure 3.2 shows the effects of left vs right eyes and male vs female eyes as a function of IOP_{cc}. Comparison of confidence intervals in Figure 3.2 showed that there is no statistical difference of this dependency between right and left eyes, or between males and females. Therefore, in all remaining results, data were used from left eyes, and were combined between the males and the females.



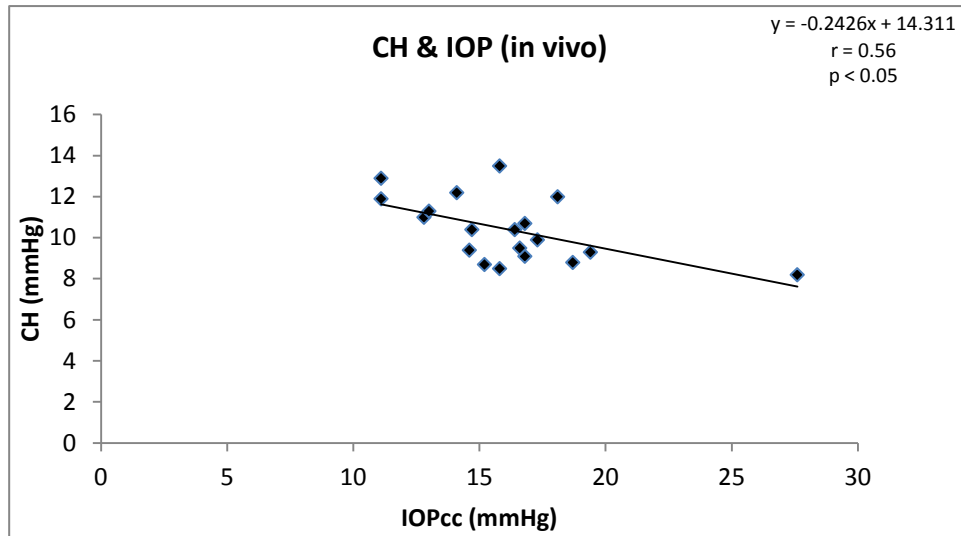
A: The significant negative correlation between CH and IOP_{cc} in Male and Female (left eyes).



B: The significant negative correlation between CH and IOP_{cc} in Male and Female (right eyes).



C: The significant negative correlation between CH and IOP_{cc} in Male (left eyes).



D: The significant negative correlation between CH and IOP_{cc} in Female (left eyes).

Figure 3.2 (a, b, c and d): The effects of left vs right eyes, and male vs female eyes.

Accordingly, Figures 3.3a and 3.3b clearly show that there is no dependence of CH on either the age or the mean spherical equivalent of the subjects. In addition to that, no correlation has been found between CRF with either the age or the mean spherical equivalent (Figures 3.4a and 3.4b).

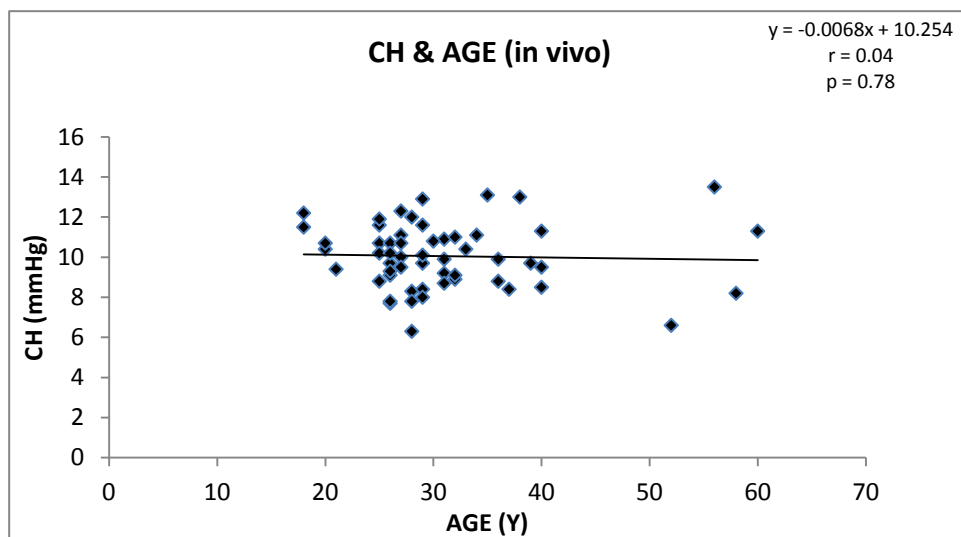


Figure 3.3a: The insignificant correlation between CH and Age.

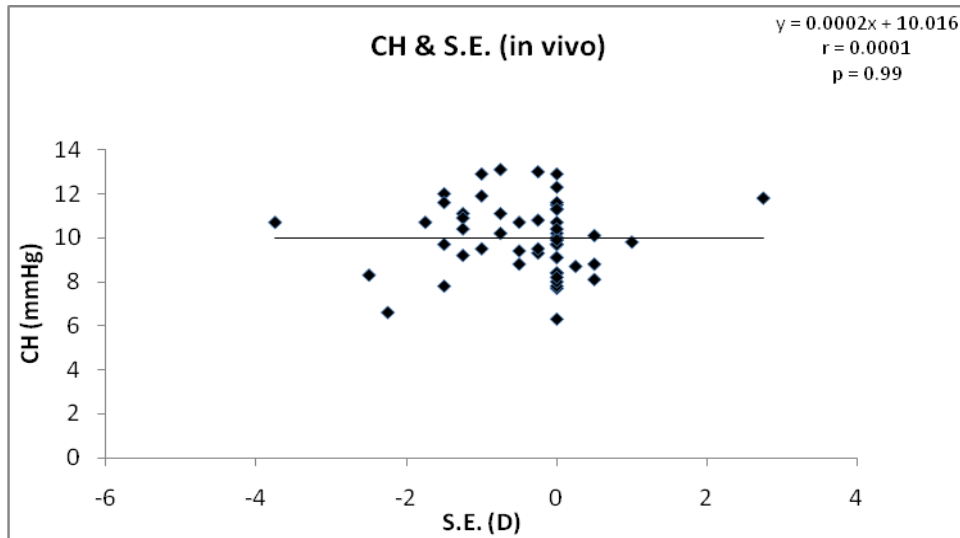


Figure 3.3b: The insignificant correlation between CH & S.E.

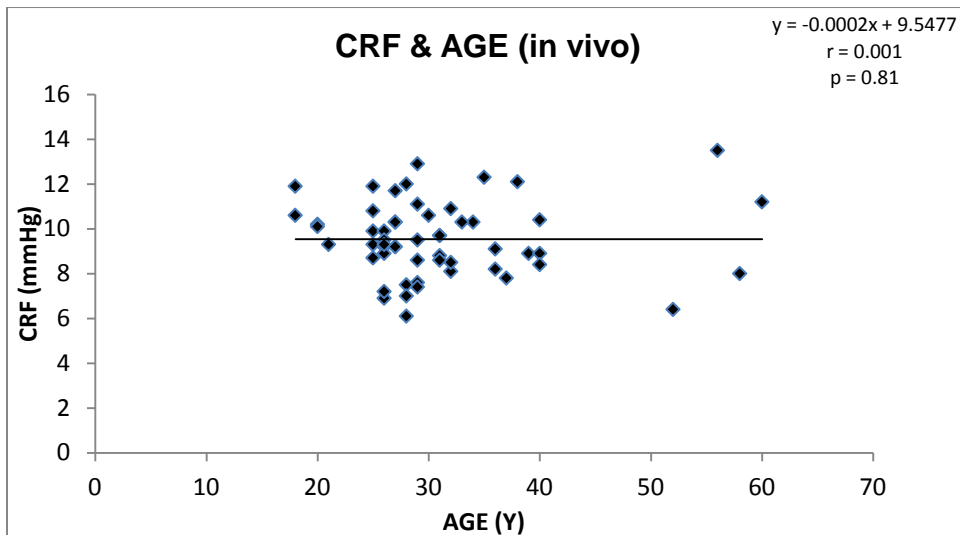


Figure 3.4a: The insignificant correlation between CRF and age.

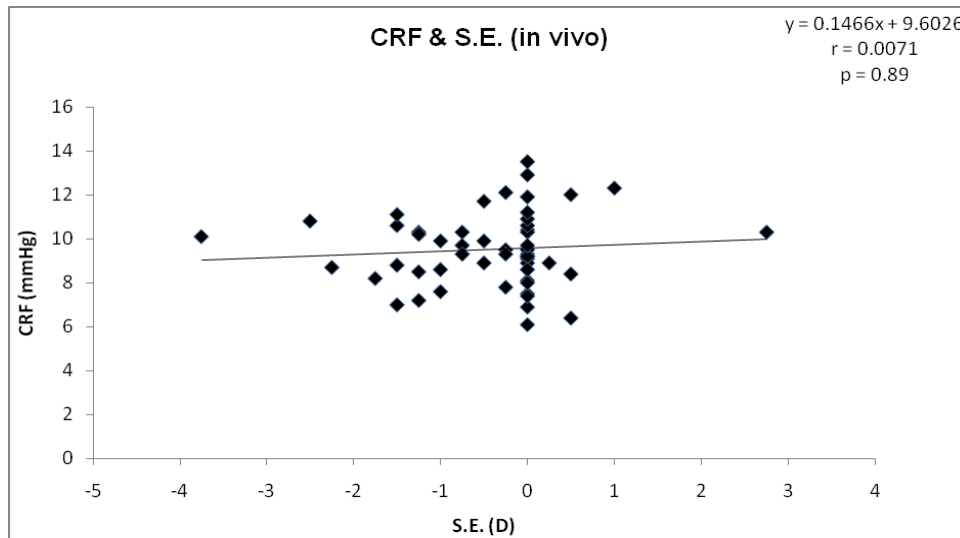


Figure 3.4b: The insignificant correlation between CRF and S.E.

3.3.2 In Vitro

As was the case *in vivo*, the excised human eyes showed a significant negative correlation between CH and IOP_{cc} (Figure 3.5a) and between CRF and IOP_{cc} (Figure 3.5b). As expected, the scatter in the data was much less ($r = 0.74$ compared to $r = 0.53$ *in vivo*). The CH values from excised eyes were similar to those observed *in vivo*, and comparison between the CH- IOP_{cc} dependencies observed *in vivo* and *in vitro* (Figure 3.1a and 3.5a) showed no statistical difference ($p < 0.0001$). Figure 3.6a shows that there is a significant correlation between CH and CCT in the excised eyes ($r=0.99$; $p<0.0001$). In addition, Figure 3.6b shows the same dependence between CRF and CCT.

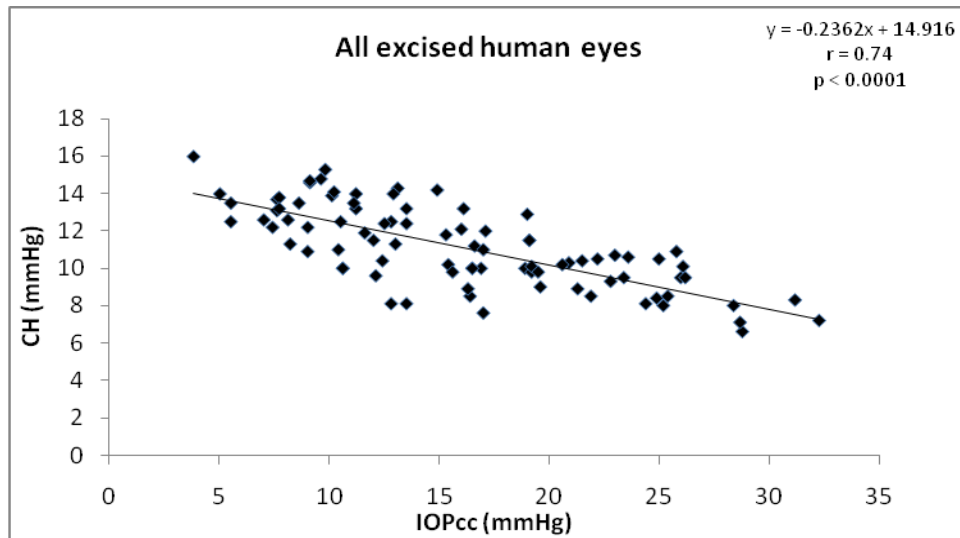


Figure 3.5a: The correlation between CH and IOP_{cc}.

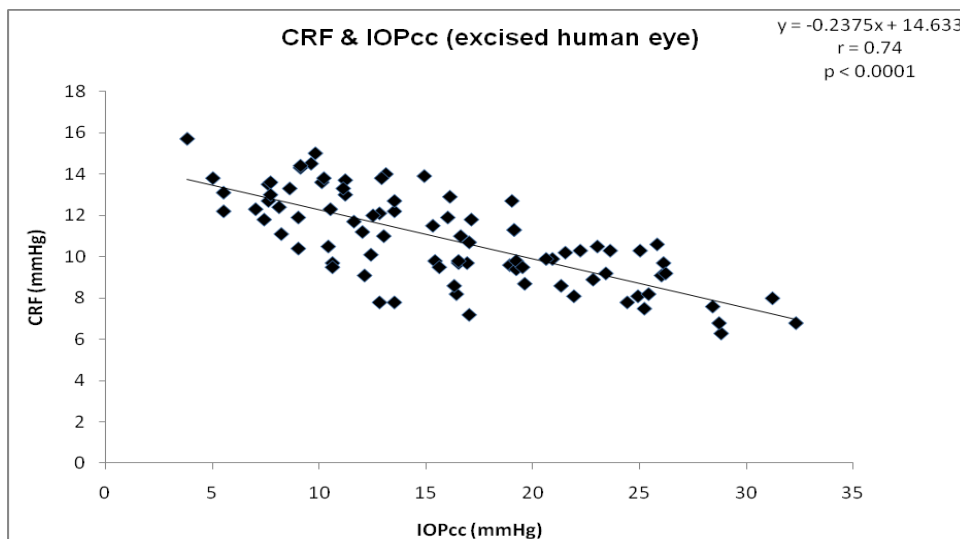


Figure 3.5b: The correlation between CRF and IOP_{cc}.

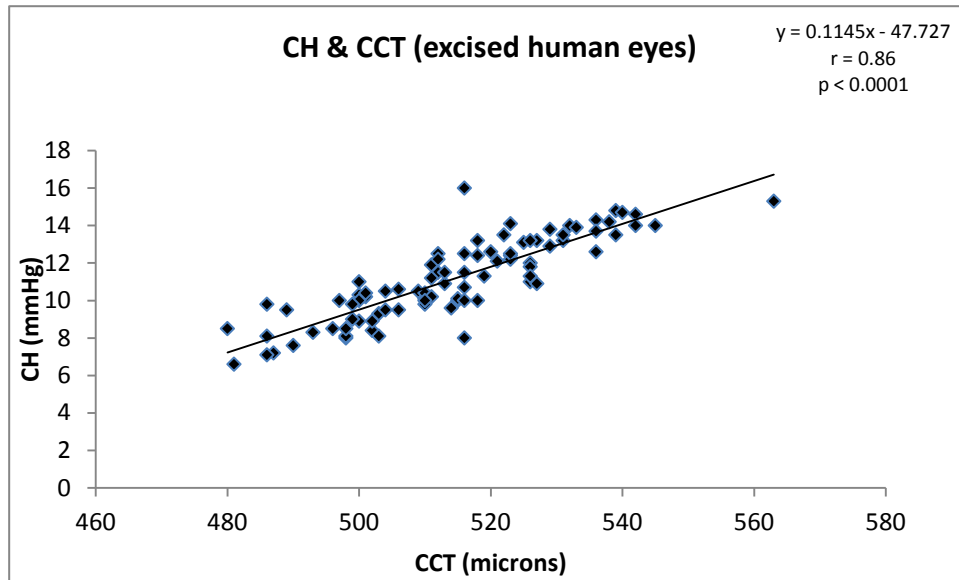


Figure 3.6a: The correlation between CH and CCT

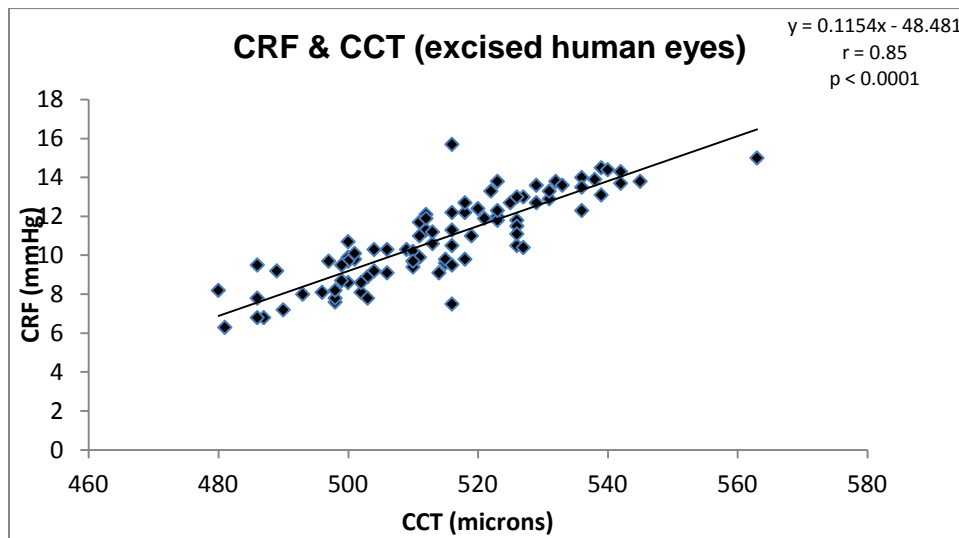
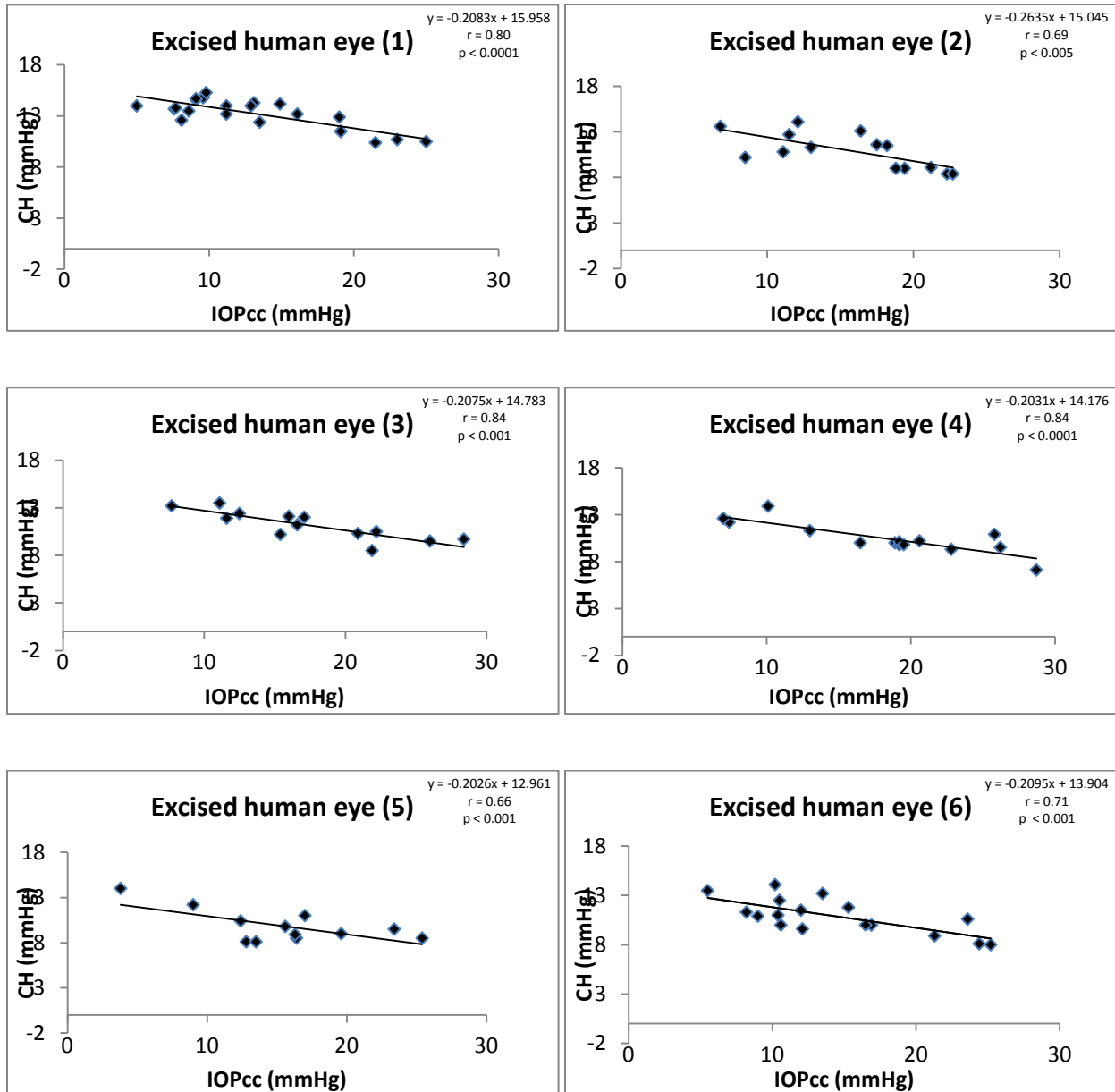


Figure 3.6b: The correlation between CRF and CCT

It is also useful to deconstruct Figure 3.5a in order to examine the response of the individual eyes, as it is not possible to evaluate the response of an individual eye to

a change in IOP in vivo and the results are presented in Figures 3.7 (for the IOP_{cc}). The similarity in the gradients of each of the graphs in Figure 3.7 apparent on visual inspection is confirmed statistically; in each case every graph falls within the 95% confidence limits of every other graph in the figure.



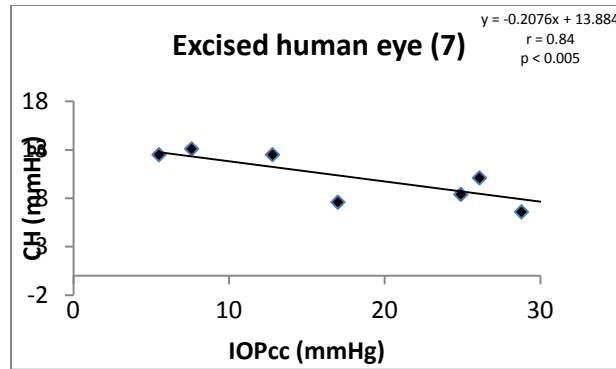


Figure 3.7: The significant (negative) response of the individual excised eyes.

The result from the excised corneas also showed a negative correlation between CH and artificial chamber pressure, presented as the IOP_{cc} reading from the ORA ($r = 0.72$, $p < 0.0001$) (Figure 3.8a) and also a negative correlation between CRF and artificial chamber pressure (figure 3.8b). However, the CH values recorded were lower than those observed over the same IOP_{cc} range *in vivo*. This was not due to the slightly lower than normal thicknesses of the corneas. Although in the *in vitro* experiment there was a positive correlation between CH and CCT ($r = 0.61$, $p < 0.0005$) (Figure 3.9a) and between CRF and CCT (Figure 3.9b), values of hysteresis and corneal resistance were consistently lower than values at the same CCT recorded *in vivo* (Kamiya et al., 2008) and the data show signs of levelling off above about 490 μm .

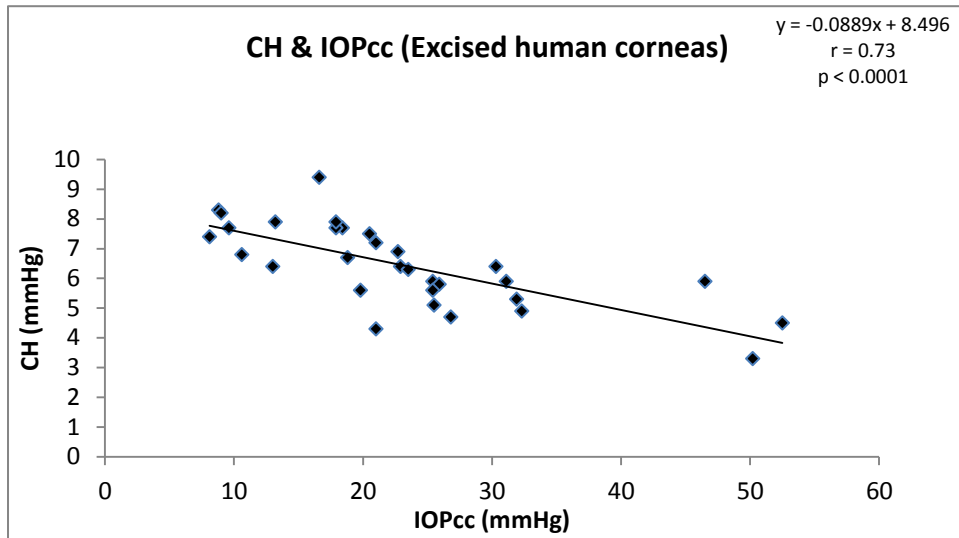


Figure 3.8a: The correlation between CH and IOPcc.

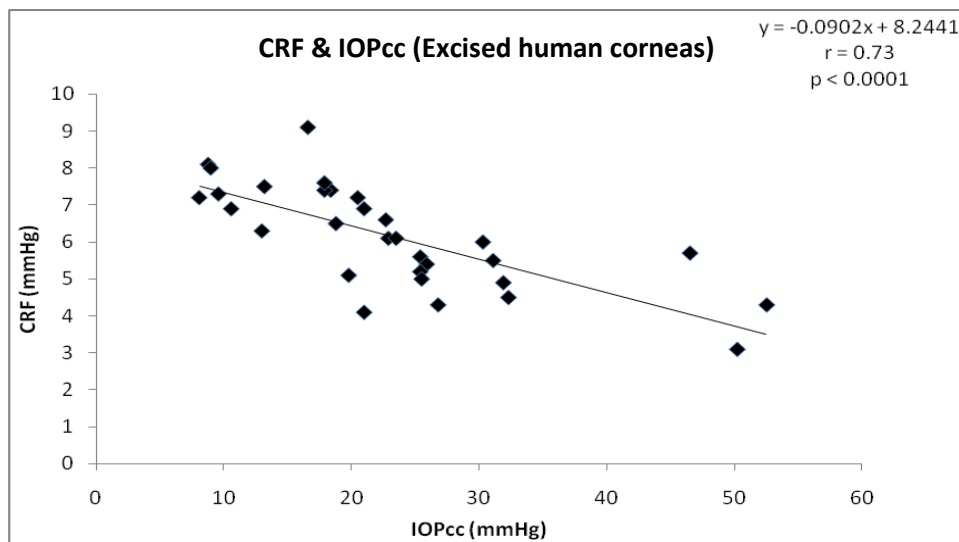


Figure 3.8b: The correlation between CRF and IOPcc.

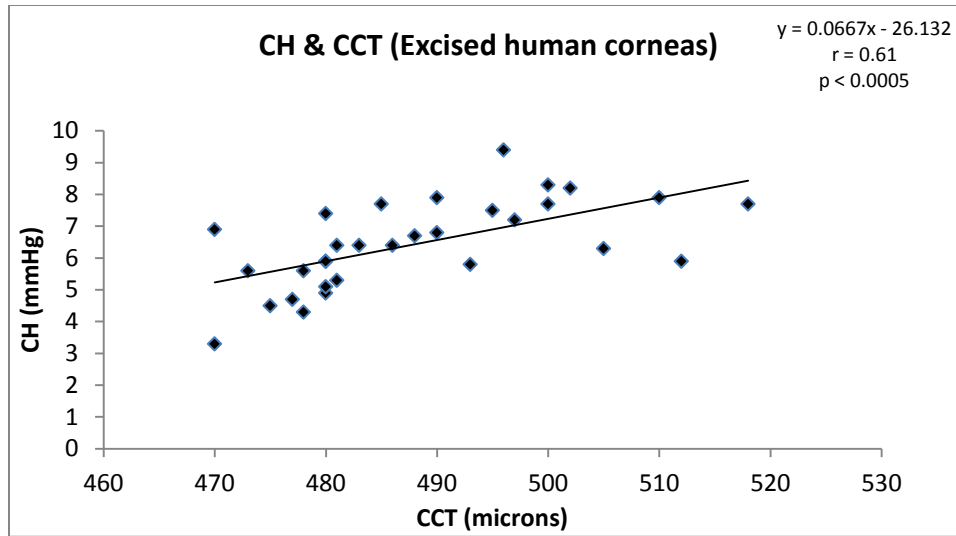


Figure 3.9a: The correlation between CH and CCT.

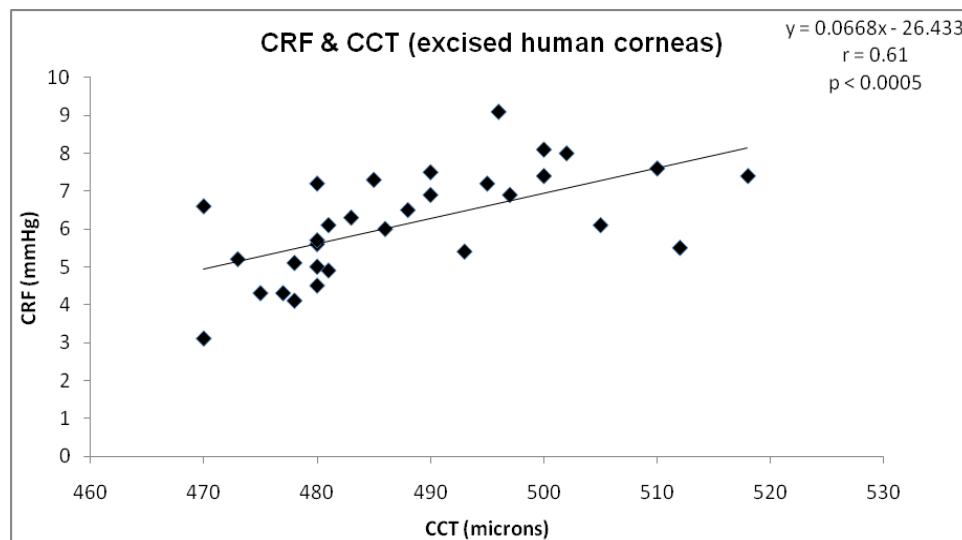


Figure 3.9b: The correlation between CRF and CCT.

In order to further examine the reduction of CH seen between whole human eyes and excised corneas, we examined bovine eyes where we could measure hysteresis in individual corneas before and after excision from the eye. Figure 3.10 clearly shows a significant reduction in CH and CRF after excising the cornea from the whole globe, with

a p value less than 0.001. The mean CH (\pm the standard deviation) for whole eyes and excised corneas were 11.95 ± 2.1 and 6.39 ± 2.6 (respectively), the mean CRFs were 11.6 ± 2.3 and 6.38 ± 2.2 , respectively.

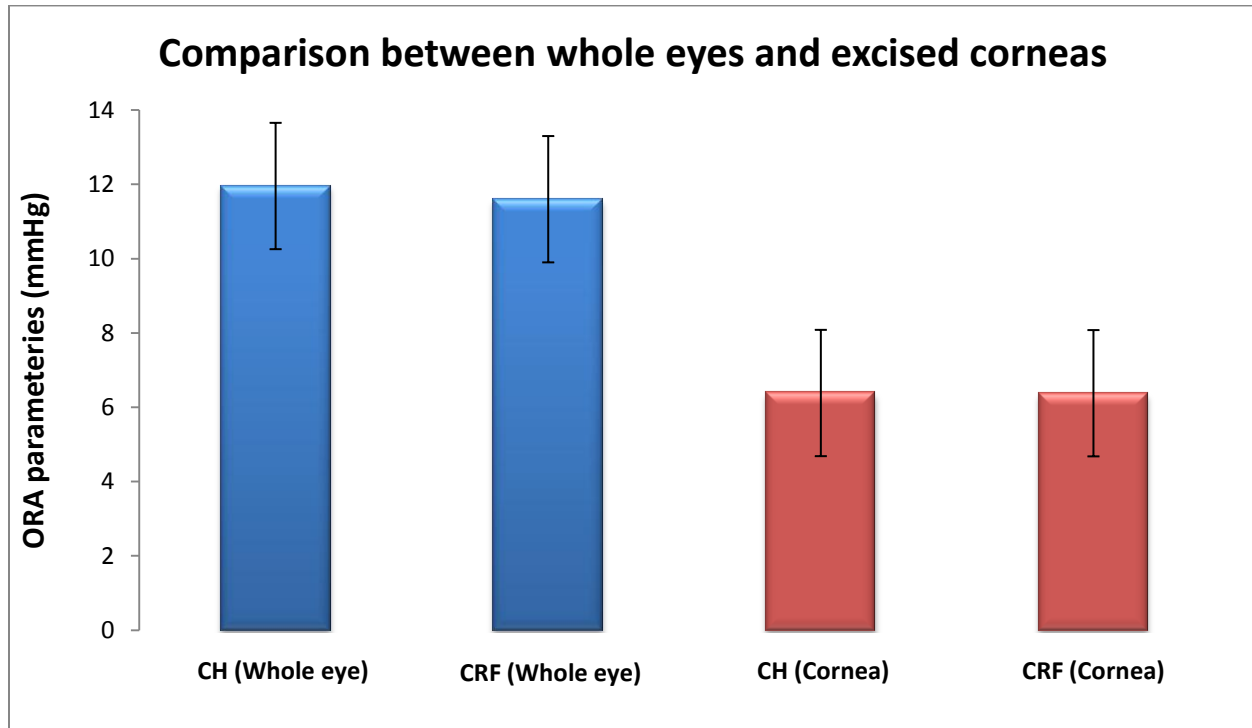


Figure 3.10: The biomechanical properties of the cornea before and after excising the cornea

3.4 Discussion

Our in vivo studies found no correlation between CH and gender, or whether left or right eyes were examined, as a function of IOP. This is in agreement with most of the previous reports cited earlier, although Fontes et al. (2008) did report gender differences. Several publications have indicated no dependence of CH on age (Kirwan et al. 2006; Montard et al. 2007; Liu et al. 2008; Kamiya et al. 2008) though others have shown a negative correlation (Kida et al. 2008; Moreno- Montanes et al. 2008). The

insensitivity of CH to age found in many reports, including ours, is of interest as it is known that older corneas are considerably stiffer than younger ones (EISheikh et al. 2007). This led Kotecha et al. (2006) to suggest that CH values should be normalised to produce what they term the cornea constant factor (CCF), which they found to increase with corneal thickness and decrease with age. The CCF may thus be related directly to corneal stiffness and viscoelasticity, and recently, these correction methods on ORA data have been improved to estimate IOP values that are independent of corneal stiffness (Kotecha et al. 2006; EISheikh et al. 2009).

Our studies also confirm that, in vivo, there is an inverse correlation between CH/CRF and IOP_{cc} , though not between CH/CRF and IOP_G . This is also in accord with previous studies (Kotecha et al. 2006; Liu et al. 2008; Kamiya et al. 2008; Hager et al. 2008). However, Kotecha et al. (2006) pointed out that, although the IOP dependence may be real, it could also be influenced by the instrument measurement. We and others have shown that CH decreases with increasing IOP_{cc} . This is unlikely to be due to the effects of corneal thickness (CCT) changes. Some studies showed only a small increase in CH with increasing CCT (Luce 2005; Shah et al. 2006) whereas others found a more significant correlation (Liu et al. 2008; Kamiya et al. 2008; Fontes et al. 2008; Mangouritsas et al. 2009; Schroeder et al. 2008). The ORA uses a different maximum air pressure (P_{max}) to achieve applanation in different subjects. The pressure at applanation (P_1) depends both on the true IOP and on the resistive properties of the individual eye, which could themselves depend on IOP. Furthermore, the rate of application of the pressure force increases with P_1 , and the authors indicate that this could also affect hysteresis. Understanding the true causes of the effect of IOP on CH is

further confounded by the fact that IOP appears to vary independently of variation in CH or CCT throughout the day (Laiquzzaman et al. 2006; Kida et al. 2008 Gonzalez-Meijome et al. 2008).

Only one study has been reported in which CH values in individual eyes were measured before and after pharmacological reduction of IOP (Kotecha et al. 2006; Kotecha 2007). The study found a weak negative correlation between changes in CH and changes in IOP. Although Luce (2005) stated that CRF is not affected by IOP changing, we found that CRF decreases with increasing IOP.

The present chapter is the first report of the use of the ORA to measure the relationship between CH/CRF and IOP in individual eyes *ex vivo*. This has several advantages, for example it allows much more precise control of the experimental conditions and it allows us to see if post-mortem changes affect the measurements of corneal hysteresis. By changing the IOP within a single eye, inter-sample variability is obviated and, as expected, the data showed less scatter than was obtained from *in vivo* studies. Furthermore, *ex-vivo* studies allowed us to examine CH over a much wider range of IOP.

The results for all seven eyes (Figure 3.6a) were very similar to those from a cohort of patients measured *in vivo* (Figure 1a). This suggests that freezing/thawing and/or any post-mortem changes in the eyes we examined did not affect CH or its dependence on IOP_{cc} . Furthermore, it was interesting that all individual eyes showed the same dependence of CH and CRF on IOP_{cc} (Figure 3.8). It would appear likely,

therefore, that a given cornea within the normal CCT range, and at a given intraocular pressure, has an intrinsic corneal hysteresis.

In vivo, several authors have documented that there is an direct relationship between CH and CCT (Liu et al. 2008; Kamiya et al. 2008; Fontes et al. 2008; Mangouritsas et al. 2009 Schroeder et al. 2008) and also between CRF and CCT (Fontes et al. 2008). Excised eyes show a similar dependence (Figure 3.7a) whereas excised corneas show a weaker correlation (Figure 3.11b). However, comparisons with in vivo results may be misleading. In a clinical CCT measurement, it is difficult to distinguish between the two factors that could affect CCT; thickness related to tissue mass (CCT_{mass}) and thickness related to tissue hydration (CCT_{swelling}). In some clinical studies, thickness variability may be due to the presence of more or less tissue, in others to more or less water. Corneal swelling associated with contact lens wear was found to be unrelated to CH (Liu et al. 2008; Kida et al. 2008). On the other hand, the elevated hydration, and hence increased thickness of the cornea, in Fuch's dystrophy leads to a reduction in CH (Del Buey et al. 2009). This implies either no correlation or a negative correlation between CH and CCT_{swelling} , rather than the positive correlation reported between CH and CCT from studies of the normal population. Following refractive procedures such as LASIK, corneal thickness changes are due mostly to loss of tissue (i.e. a change in CCT_{mass}). This procedure has been found to cause a reduction in CH, though it is interesting that the reduction does not correlate with the amount or percentage of tissue removed (Kirwen and O'Keefe 2008). In our in vitro experiments variation in CCT is likely dominated by tissue hydration (CCT_{swelling}), particularly for the excised corneas, which needed to be deswelled before use. For this

reason, caution is needed when comparing the results to those obtained from in vivo studies of the normal population where changes in both CCT_{mass} and $CCT_{swelling}$ are likely to occur. Nevertheless, our results have shown for the first time that it is possible to obtain meaningful results using the ORA from whole excised eyes, and this opens up the possibility of using the ORA in controlled ex vivo studies that will allow us to better understand the relationship between CH measurements and conventional biomechanical properties such as stiffness and elasticity (Glass et al. 2008).

When excised corneas with scleral rims were examined, great care was needed in the tissue preparation, and poor signals needed to be discarded in accordance with the manufacturer's instructions. Nevertheless, with these provisos, we found that the CH values were reduced (between 8mmHg and 22mmHg, the mean values were: $CH_{in\ vivo} = 10.1 \pm 1.6$; $CH_{whole\ eye} = 11.1 \pm 2.16$; $CH_{excised\ cornea} = 7.2 \pm 1.5$). The inverse relationship with IOP_{cc} was still present, but was also much reduced. The reduction in CH in excised corneas is perhaps not unexpected, as the pressure wave produced by the ORA is transmitted to the inner contents of the eye and also to the sclera, both of which are absent in the artificial chamber. To examine this further we measured CH and CRF in bovine corneas before and after removal from the eye and were able to confirm that the values of these parameters depend on the presence of the rest of the eye.

These results are in agreement with in vivo studies, which have also indicated that the lower the CH, the lower its correlation with IOP_{cc} (Touboul et al. 2008). However, there are a number of experimental factors whose influence on these results needs to be considered. Firstly, there is the possibility of post-mortem changes affecting the in vitro results. The fact that the same results were obtained from whole eyes post-

mortem and eyes measured in vivo, however, suggests that freezing/thawing and/or any post-mortem changes in the eyes we examined did not affect CH or its dependence on IOP_{cc} . This is not surprising as the biomechanical response of the cornea depends almost entirely on the collagenous component, which is highly resistant to proteolysis. Thus, in the absence of any in vivo technique other than the ORA, the body of literature on corneal biomechanics has all been carried out using post-mortem eyes or excised corneas. Secondly is the fact that most excised corneas were originally swollen. There is a significant relaxation of posterior fibres with increasing hydration and this increases the extensibility of swollen corneas (Hjortdal 1995a and 1995b). For this reason, tissues were equilibrated to near-normal hydration prior to CH measurement. Thirdly, it has previously been reported that when a cornea is stressed at physiological pressure as part of an intact globe it is less elastic than a cornea tested by strip extensimetry (Smolek 1994). Although this seems to support our results, it is not possible to make a direct comparison as the behaviour of a stretched corneal strip is likely to be different from the behaviour of a cornea inflated in an artificial chamber, where collagen fibrils have not been severed. Finally, the elastic properties of the cornea have been shown to be influenced by the presence of the limbus when IOP is varied (Asejczyk-Widlicka et al. 2007; Boyce et al. 2008). In our system, a scleral rim was used to clamp the corneas so that the limbus was retained during the measurements, which mimics the situation in vivo.

So why are values of CH from isolated corneas different to those from whole eyes? To answer this one needs to consider what tissue changes might occur during applanation and recovery. Certainly, when the cornea is distorted by inflation, significant

changes in the lamellae are observed (Wu and Yeh 2008) and the same may be the case during applanation (Sporn et al. 2009). Therefore most energy is likely to be stored within the cornea and limbus and this would be the same in vivo as in vitro. The absence of epithelial, endothelial and keratocyte function is not likely to have influenced our measurements; if it did, one would not have expected identical results in vivo, and from excised eyes where cell function was lost. A more likely inference from our results is that the CH value depends to at least some extent, on the presence of the rest of the eye globe, as was suggested by Kucumen et al. (2008). This would explain how phacoemulsification (Kucumen et al. 2008), axial length (Song et al. 2008), primary open angle glaucoma (Schroeder et al. 2008; Shah et al. 2008), high myopia (Shen et al. 2008) and deep sclerectomy (Iordanidou et al. 2010) are all associated with changes in CH even though the cornea is not directly involved.

The reduction in both CH and CRF when the cornea is removed from the eye is almost 50%, calling into question to what extent the ORA is measuring corneal biomechanics, and therefore if the terms corneal hysteresis and *corneal* resistance factor are appropriate. Therefore, until CH and CRF are correlated with more classical concepts such as elasticity, one should perhaps be reminded of the original definition of CH proposed by David Luce: “corneal hysteresis is the output of the ORA under the specific measurement conditions imposed by the ORA” (Dupps 2007). However, our results have shown for the first time that it is possible to obtain meaningful results using the ORA from whole eyes, and this opens up the possibility of using the ORA in controlled ex vivo studies that will allow us to better understand the relationship

between CH measurements and conventional biomechanical properties such as stiffness and elasticity (Glass et al. 2008)

Chapter 4: A determination of the relationship between corneal biomechanical properties, the anterior/posterior corneal curvature and peripheral /central corneal thickness accompanying LASIK surgery

4.1 Introduction

A greater understanding of the biomechanical properties of the cornea and the factors that influence these properties is important for explaining the biomechanical response of the cornea following refractive surgical procedures. This Chapter aims to examine some of these properties in normal and LASIK-treated patients.

The biomechanical properties of a tissue influence its behaviour when it is subjected to stress (Glass et al. 2008). Recently, many studies have investigated the ORA as a device for measuring corneal biomechanical properties in vivo. Some of these studies indicate that CH is higher in normal patients than in keratoconus patients (Shah et al. 2007). Others show that CH is similar between keratoconus and post-refractive surgery patients (Shah et al. 2009). CH has been found to significantly decrease after phototherapeutic keratectomy (PTK) (Kamiya et al. 2009), and after laser in situ keratomileusis (LASIK) (Chen et al. 2008). According to Montard et al. (2007) and Liu et al. (2008), CH is not correlated with anterior corneal curvature.

So far, most studies have concentrated on the factors that influence CH in normal eyes, or after various eye conditions such as keratoconus or refractive surgery. Therefore, we investigated some factors that influence the biomechanical properties of the cornea in vivo (normal) and in vitro and compared them statistically. However, the

relationship between CH and anterior/posterior corneal curvature in LASIK patients has not previously been investigated. Also, there is no study of the correlation of CH with the peripheral corneal thickness before and after LASIK. We believe that all corneal refractive surgery affects corneal stability; therefore in this study we investigated some of the factors that may influence corneal structure and biomechanics. Agarwal (2006) believes that the LASIK flap affects both the anterior and the posterior layers of the cornea, which leads to a weakening in the cornea. Therefore we here investigated for the first time the back surface of the cornea as there might be some unexpected changes to this surface.

4.2 Material and Methods

Fifty-two eyes of 52 healthy volunteers were divided into 2 groups: control group (26 normal eyes) and post-LASIK group (26 eyes). The range of the volunteers' age was between 24 and 43 (y). Apart from refractive errors and moderate dryness, there was no history of ocular or health problems. The informed consent given by participating subjects was consistent with the tenets of the Declaration of Helsinki.

Corneal Measurements:

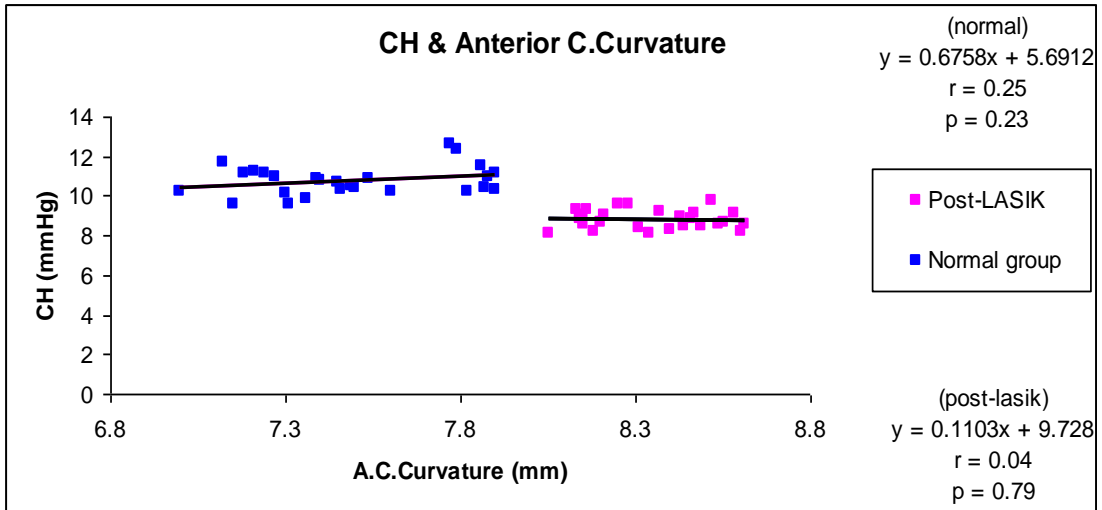
Corneal hysteresis was measured using the Ocular Response Analyzer (ORA). Data were recorded using the waveform score of the later version of the ORA software (version 2.04). The measurements of anterior/posterior corneal curvature and peripheral

corneal thickness were performed using the Oculus Pentacam (Oculus, Inc., Lynnwood, WA) which acquires 25 images in each patient eye. The central/peripheral corneal thicknesses and anterior/posterior corneal curvatures were taken from the acquired image.

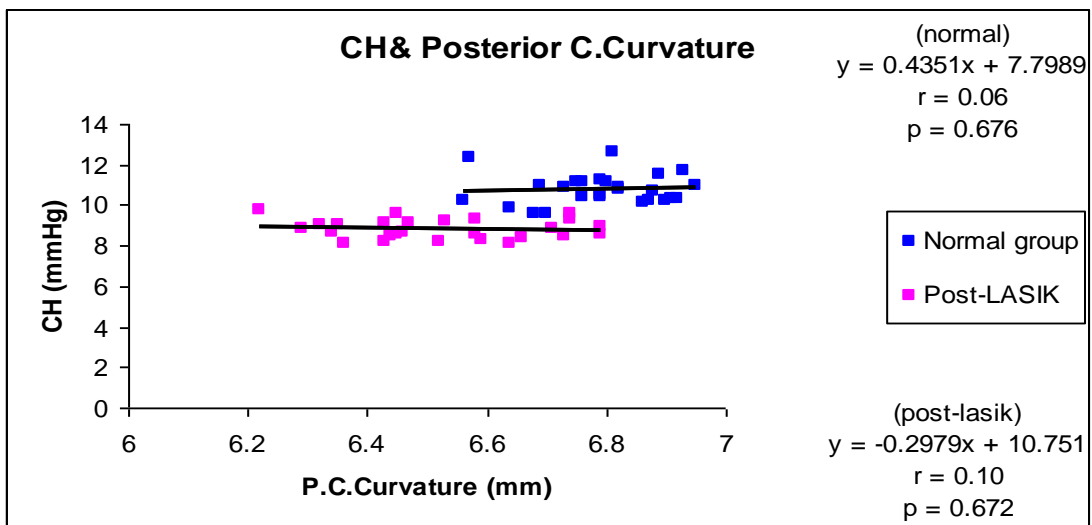
SPSS was used for statistical comparisons of the significance. The Pearson correlation coefficient (r) (which was carried out to confirm the correlation between the data sets) and p -value (which is considered statistically significant if less than 0.05) are indicated on each graph. Non overlap of the 95% confidence intervals of regression lines was taken as evidence of statistically significant difference between them.

4.3 Results

Figure 4.1 shows that there is no statistical correlation between CH and either the anterior or the posterior corneal curvature in both the control and post-LASIK groups. However, the results of this study show that CH was higher in the control group than in the post-LASIK group; the mean value of CH was decreased from 10.76 ± 0.71 mmHg to 8.89 ± 0.44 mmHg (Table 4.1). On average, the anterior corneal curvature in the post-LASIK patients was higher (mean = 8.34 ± 0.17 mm) than the normal patients (mean = 7.49 ± 0.27 mm), however, this difference is not statistically significant (Table 1). Moreover, the results show that there is no statistically significant change in posterior corneal curvature (PCC) between these groups (Table 4.1).



a



b

Figure 4.1: The relationship between CH and anterior (a) and posterior (b) corneal curvature in normal and post-LASIK patients.

	Normal	Post-LASIK	P value
CH (mmHg)(±SD)	10.76 ± 0.71	8.89 ± 0.44	< 0.001
CCT (µm)	550.92 ± 25.65	437.76 ± 25.23	< 0.0001
PCT (µm)	648.65 ± 31.14	595.15 ± 37.92	< 0.005
ACC (mm)	7.49 ± 0.27	8.34 ± 0.17	>0.05
PCC (mm)	6.74 ± 0.12	6.56 ± 0.23	>0.1

Table 4.1: Comparison between normal and post-LASIK patients.

The study showed a significant correlation between CH and central corneal thickness in normal patients and a similar correlation in post-LASIK patients (Figure 4.2). Furthermore, comparison of the normal and post-LASIK data sets in Figure 4.2

shows that dependence of CH on CCT does not change following LASIK surgery (at the 95% confidence level).

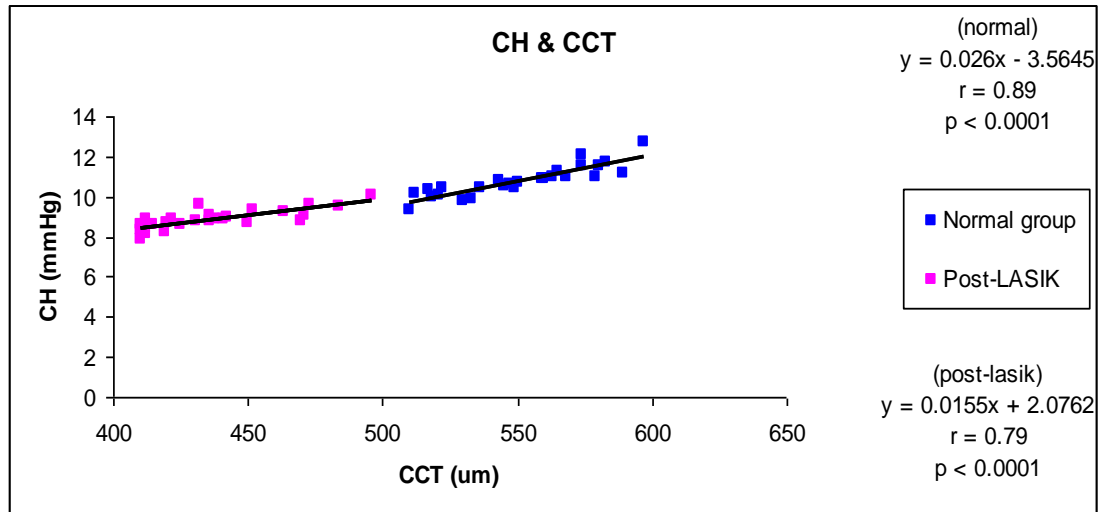


Figure 4.2: The relationship between CH and central corneal thickness (CCT) in normal and post-LASIK patients.

Our data also show a statistically significant correlation between CH and peripheral corneal thickness in both normal and post-LASIK patients (Figure 4.3). As expected, CCT was reduced after LASIK (average reduction 21%) but Table 1 also indicates that peripheral corneal thickness reduces by 8% on average.

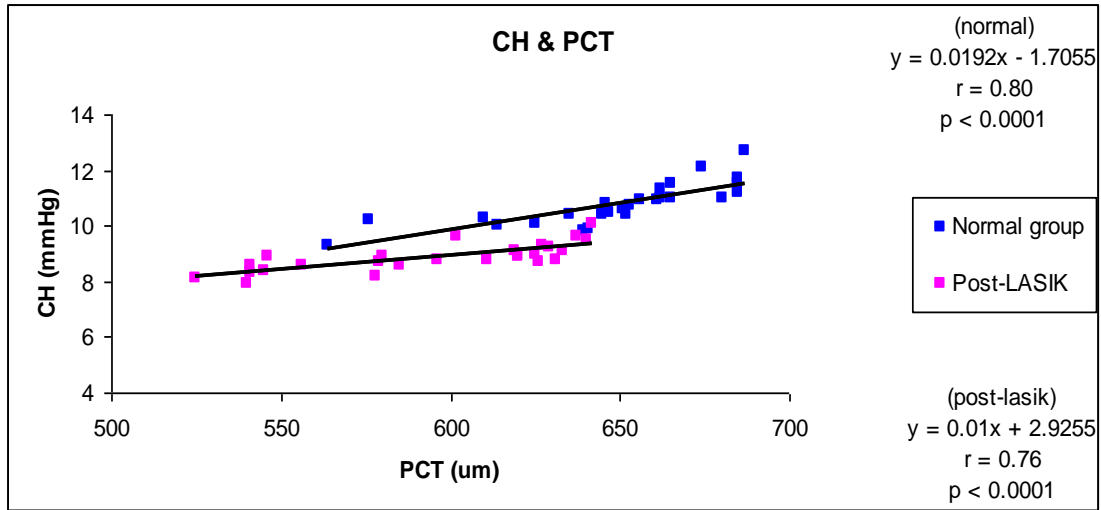


Figure 4.3: The relationship between CH and peripheral corneal thickness (PCT) in normal and post-LASIK patients.

Moreover, we tried to investigate if there is any difference in the ORA signal between normal and post-LASIK patients and we found a significant difference between them as explained in Figures 4.4a and 4.4b.

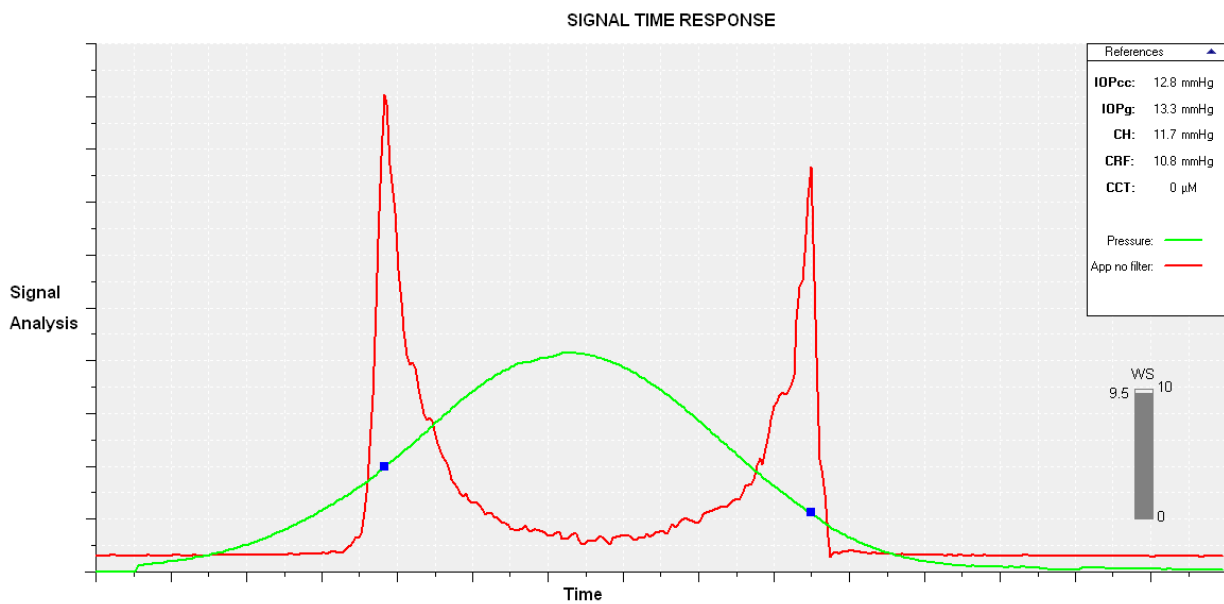


Figure 4.4a: Normal signals obtained using ORA.

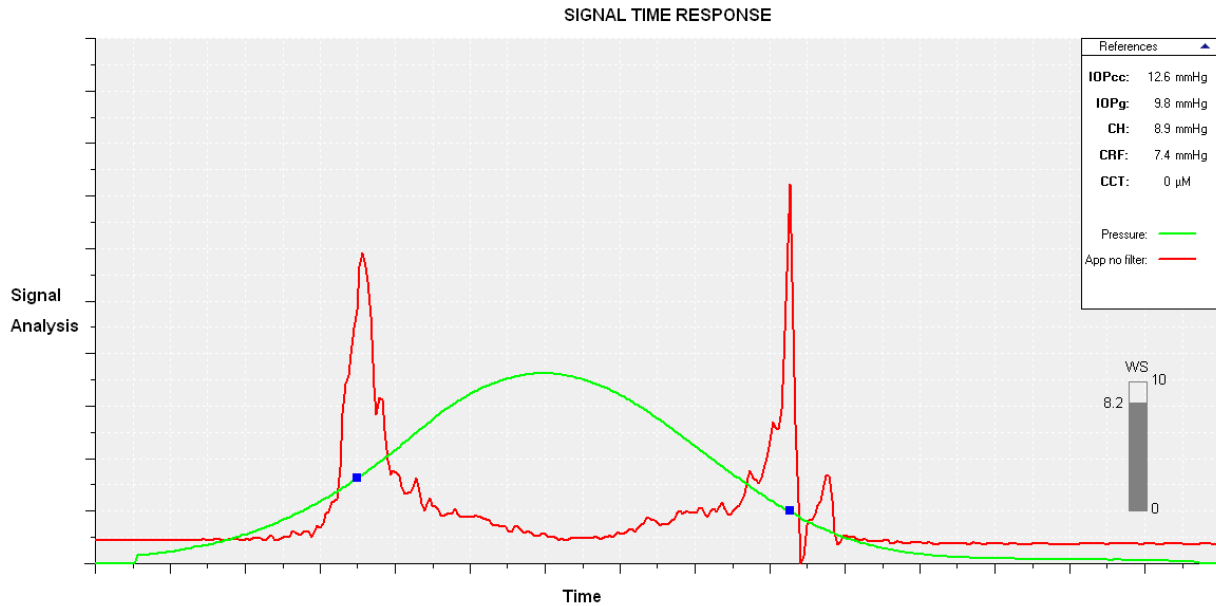


Figure 4.4b: Post-LASIK signals obtained using ORA.

4.4 Discussion

In the results described here, we investigated the relationship between CH, central/peripheral corneal thickness and anterior/posterior corneal curvature in normal and post-LASIK patients. The Oculus Pentacam is a non-contact, fast and accurate device to measure some corneal properties such as corneal curvature and corneal thickness. The Pentacam shows high repeatability (Barkana et al. 2005; Amano et al. 2006). Buehl W et al. (2006) believe that the CCT thickness measured by the Pentacam is dependable. However, Prospero Ponce et al. (2009) believe that the OCT pachymetry maps are more accurate than the Scheimpflug maps. Shankar et al. (2008) have measured the peripheral corneal thickness in normal volunteers at 3mm from the central cornea using the Oculus Pentacam. I adopted a similar approach to measure both normal and post-LASIK patients at this position. In addition, Swartz et al. (2007) and

Shankar et al. (2008) have shown that the Pentacam is a reliable method to measure the front and back surface of the cornea. Jain et al. (2007) found the highest repeatability for measurements at the periphery of the anterior corneal curvature and the lowest for the horizontal meridian of the posterior corneal curvature. The current study also showed very good repeatability by using the Pentacam on imaging the front surface of cornea for post-LASIK patients. For the purposes of the current comparative study, therefore, we believe that the Pantacam provided reliable measurements of anterior/ posterior curvature and central/peripheral thickness.

The average values of CH in both normal and LASIK groups was in accordance with previous reports (Shah et al. 2006; Shab et al. 2007; Ortiz et al. 2007; Touboul et al. 2008; Kamiya et al. 2008; Shah et al. 2009; Franco et al. 2009). The changes in CH after LASIK surgery might be due to the weakening and structural changes of the cornea caused by flap creation and corneal ablation. However, Kirwan and O'Keefe (2008) demonstrated a similar corneal hysteresis reduction after LASIK and LASEK; this indicated that CH is not correlated with the depth within the stroma from where tissue is removed. Although Ortiz et al. (2007) showed a significant difference in CH between post-LASIK and keratoconus patients, Shah and Laiquzzaman (2009) demonstrated that CH is very similar between these two groups. The low value of CH could be considered a sign of keratoconus or keratectasia (Shah et al. 2007). As a result of the reduction of bending stiffness that occurs in the cornea after LASIK surgery (Hjortdal et al. 2005), the reduction of CH was expected as the weaker corneas tend to have a lower CH measurement. It is known that within the first year of LASIK, the patient should be followed-up during this period for fear that there will be any changes occurring

in the cornea after LASIK procedure.; therefore, the length of time since the operation has been done was taken into account in this study.

Because we confirmed (in the last chapter) that CRF is related to CH and they are similarly affected by most factors, we expect that LASIK affects the CRF values similarly to the CH values (result not shown).

Khoramnia et al. (2008) believe that there is no correlation between age and change in both the centre and peripheral corneal thickness measured using the Pentacam. Therefore, the disparity of the ages of patients in our study is unlikely to affect the corneal thickness measurements. Measurements of the central corneal thickness showed good repeatability and this was comparable at the pupil centre and at the corneal vertex, however, the peripheral corneal thickness at 3mm from the centre showed better repeatability when the corneal vertex was used as the central reference point. This is in agreement with Shankar et al. (2008) and so it was decided to use the corneal vertex in this study. Following LASIK surgery, the peripheral corneal thickness was also reduced, albeit less than the central thickness. The standard optical zone for LASIK surgery is about 6mm in diameter, and this would just encompass the peripheral corneal position examined here.

The purpose of LASIK surgery is to increase the focal distance of the cornea by increasing its anterior radius of curvature. Although the posterior curvature of the cornea plays a minor role in the refractive properties, it does contribute to the refractive power, thus it was interesting to see if LASIK had any effect on this surface or not. Our results indicated that the posterior radius of curvature is unaffected by LASIK. However, the

result is of interest from a mechanical point of view. It suggests that, even though, following LASIK, the posterior lamellae alone are required to withstand the stress created by the intraocular pressure, and are therefore under increased tension, no changes seem to occur in their curvature.

We and others found no significant correlation between CH and anterior corneal curvature (Liu et al. 2008; Franco and Lira 2009). However, in addition to that, we also found that there is no significant correlation between CH and posterior corneal curvature (Figure 4.1), which confirms the above finding that the posterior corneal curvature is unaffected by LASIK.

The results showed a significant correlation between CH and CCT (Figure 4.2). This is supported by Luce (2005), Shah et al. (2006), Montard et al. (2007), Liu et al. (2008), Kamiya et al. (2008), Fontes et al. (2008), Schroeder et al. (2008) and Mangouritsas et al. (2009) all of whom showed a significant correlation between central corneal thickness and CH. However, some of them believed that this correlation is significant but weak (Luce 2005) or moderate (Shah et al. 2006). However, we also demonstrated that there is the same correlation between CH and *peripheral* corneal thickness in normal and post-LASIK patients (Figure 3); this suggests that much of the decrease in CH after LASIK is therefore a direct consequence of the reduction in corneal thickness, this can be clearly observed in figure 4.3 as the two slopes of normal and post-LASIK patients appear to be consecutive. Because there is a relationship between corneal thickness and CH, and we know that the corneal thickness is a fundamental consideration in LASIK surgery, CH might become a basic qualification

parameter in refractive surgery. However, Touboul et al. (2008) suggested that the CCT should continue as a useful and major parameter in LASIK.

For the first time, this study has shown that there is no significant correlation between CH and posterior corneal curvature in either normal or post-LASIK subjects; however, it has shown that there is a significant correlation between CH and peripheral as well as central corneal thickness, in both normal and post-LASIK patients. This result gives us more comprehensive knowledge about the factors that are related to changes in CH but further studies are needed to clarify the influence of refractive surgery on these parameters.

Chapter 5: Infra-red spectrometer study of the cornea cross-linked with riboflavin and Ultraviolet A

5.1 Introduction

The interaction of electromagnetic waves with the bonds of molecules defines molecular spectroscopy. In this way spectroscopy uses radiation to determine and investigate the structure and properties of a sample. Organic and inorganic compounds can be investigated using infrared (IR) spectroscopy. IR radiation has wavelength in the range from 1 μ m to 300 μ m and when a sample absorbs radiation this induces vibration or rotation of molecular bonds (Figure 5.1). There are many factors that influence a molecule's vibration; these are the type, size and weight of atoms, and the type, elasticity, length and strength of the bonds between the atoms (Banwell and McCash 1994).

Spectroscopy is a term that describes the analysis of the emission or absorption of radiation in terms of chemical and physical properties; in other words, it can investigate the chemical composition of a sample (Banwell and McCash 1994).

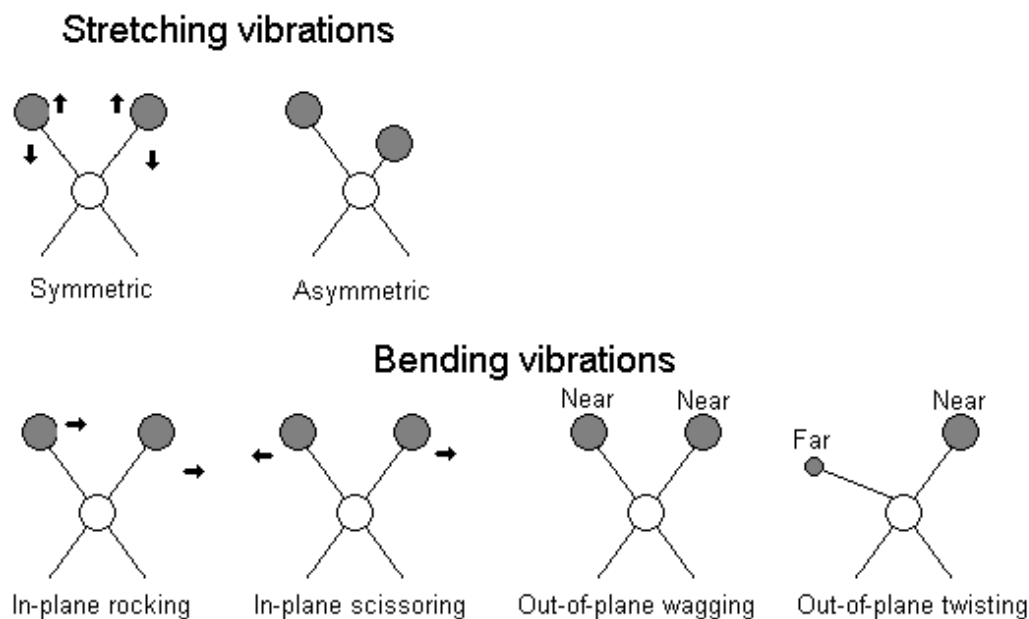


Fig 5.1 IR-sensitive Molecular Vibrations

From figure 5.1 it can be clearly seen that there are two types of vibrations: stretching vibrations (changes in bond length) and bending vibrations (changes in bond angle).

Unlike X-ray diffraction, the secondary structure of proteins can be detected using Fourier transform infrared (FTIR) spectroscopy. The FTIR spectroscopy mechanism works by passing infrared rays through a sample and monitoring the sample's absorption in the infrared region. A plot of the spectrum of absorption with wavelength allows determination of bond-types.

There are two Fourier Transformation Spectrometers that were suggested to be used in this study in order to decide which of them was appropriate for our experiment; these are: the 'Bowmen' and the 'Ade-FTS'. The main difference between them is the

wavelength region for each one, the Bowmen works in the mid-IR wavelength region while the Ade-FTS works in the far IR wavelength region. They are both interferometers, however, the Bowmen was used because we were looked to the fingerprint region which is in the range between (500 to 1500 cm^{-1}).

5.2 Material and methods

As mentioned in the introduction, this kind of research can use either of these two interferometers (Bowmen and Ade-FTS), depending on which region the researcher is looking at. So if the experiment has to work in the IR region between 400cm^{-1} and $14,000\text{cm}^{-1}$, the Bowmen should be used; however, if the experiment has to work in the far infrared region between 2cm^{-1} and 600cm^{-1} , then the Ade-FTS is preferred. In this experiment, therefore, the Bowmen were used. The general set-up of these two Fourier Transformation spectrometers is similar. An automated programme manages the major mirror and the instrument parameters (such as reading of the data (voltages)).

Before taking any measurements, the device must be calibrated, and then it will be ready to take measurements. After that, the background, with the chamber empty and just the mounting substrate without a sample (i.e. just polypropylene), must be recorded; then, we can take measurements of the sample. The sample is screened 30 times and this is repeated 3 times in order to give a good signal-to-noise ratio in the data. Then the data are Fourier transformed to give a spectrum.

5.2.1 Sample preparation

Substrate preparation

To carry out the interferometry, the sample needed to be mounted on a substrate. The choice of substrate depended on the region we were looking at. In this study, however, three different types of substrates were tested in order to decide which of them was suitable for our experiment; these were: polypropylene $3.3\mu\text{m}$, Mylar $0.8\mu\text{m}$ and common kitchen cling-film. After measuring the spectra of these substrates, it was confirmed that polypropylene had the lowest absorption in the region we were interested in. Polypropylene was also selected because it was supposed not to have many spectral lines in the region we were looking at. The samples used in this experiment were very thin so they had to be protected from anything that could affect them. Metal rings were used to secure the substrate. The range of diameters of these rings was between 2 and 6cm. In the preliminary experiments (data not shown), cyanoacrylate (super glue) was used to seal a ring onto the substrate, and then it was placed for about 30 minutes in an oven at 50°C . In later experiments, we found that vacuum grease was better than super glue or varnish in terms of leakage and protecting the samples. The substrate was very sensitive; therefore, care had to be taken to avoid any ripping. Finally, the sample was placed in the centre of the substrate and then two rings were fully glued to each other with the sample between them. Care was also taken to ensure that the sample was hydrated and in the correct position.

5.2.2 Preparation of the cornea

As mentioned in Chapter 2, ovine eyes were obtained from the abattoir at Cinderford, Gloucestershire, within hours of death, and were usually fresh. By using a scalpel we dissected the cornea from the eye (treated or non-treated) with its sclera rim. Because we needed very thin samples, we cut a small thin layer from the cornea (the thickness depended on the experiment type) using the Sledge Microtome (Microm HM 440E (Thermo Fisher, UK)). The cutting procedure was as follows:

- 1- The machine was turned on and the temperature reduced to -16°C .
- 2- It was ensured that the blade was in a tight secure position by locking it at the beginning of the procedure.
- 3- A small amount of water was applied to the platform to build up a dome of ice on which to place the cornea.
- 4- It was necessary for the dome of ice to be as close as possible to the cornea in shape, so that when the cornea was placed on it, the cornea maintained its original shape.
- 5- After adjusting the cornea on the dome carefully, the platform was covered with laboratory plastic glass (to protect the sample during temperature changes), then the temperature was decreased to -30°C .
- 6- When the temperature reached -30°C , and it was confirmed that the cornea was frozen, the knife was adjusted carefully, to the very anterior edge of the cornea;

an appropriate depth of cut was chosen in order to have a specific thickness of the sample.

- 7- The cutting was continued until a small hole appeared in the centre of the sample.
- 8- After this hole appeared, the temperature was increased to melt the ice and remove the remaining tissue for disposal.
- 9- Finally, the selected sample was placed between two layers of substrate (as mentioned previously) and sealed between them using super glue (cyanoacrylate).

In this experiment we had two groups, a control group and a treated group. The preparation of the riboflavin solution and the treatment procedure were carried out exactly as explained in Section 2.5.2. The same procedure was carried out on both treated and control samples in terms of sample cutting and placement in the substrate.

5.3 Results

5.3.1 Substrate Choice:

As mentioned previously, in Section (5.2.1), we used three different substrates to investigate which of them was more suitable for use in this experiment.

In Figure 5.2, it can be seen that we used IR to observe the absorption bounds for these three substrates. It is apparent that polypropylene and Mylar (3.3 μm and 0.9 μm respectively) have low absorption in the IR. In contrast, clingfilm has a higher absorption in the IR, therefore, clingfilm was excluded before making any comparison between them. By using two layers of the substrate to hold the sample between them, we were able to measure two layers of the substrate together and the final results are presented in Figure 5.3.

After excluding the clingfilm, we compared the absorption of Mylar and polypropylene (Figure 5.3) and found that, in the fingerprint region of the corneal collagen, Mylar has strong absorption features. On the other hand, polypropylene has absorption features around the region where CH_2 and CH_3 are located. In this experiment, therefore, polypropylene was used. Before moving to the next part of the results, it should be noted that the transmission of Mylar appears to go to 1 which is impossible. This may have been due to some problem with the spectrometer, but was more likely due to the thickness and/or the hydration of the Mylar.

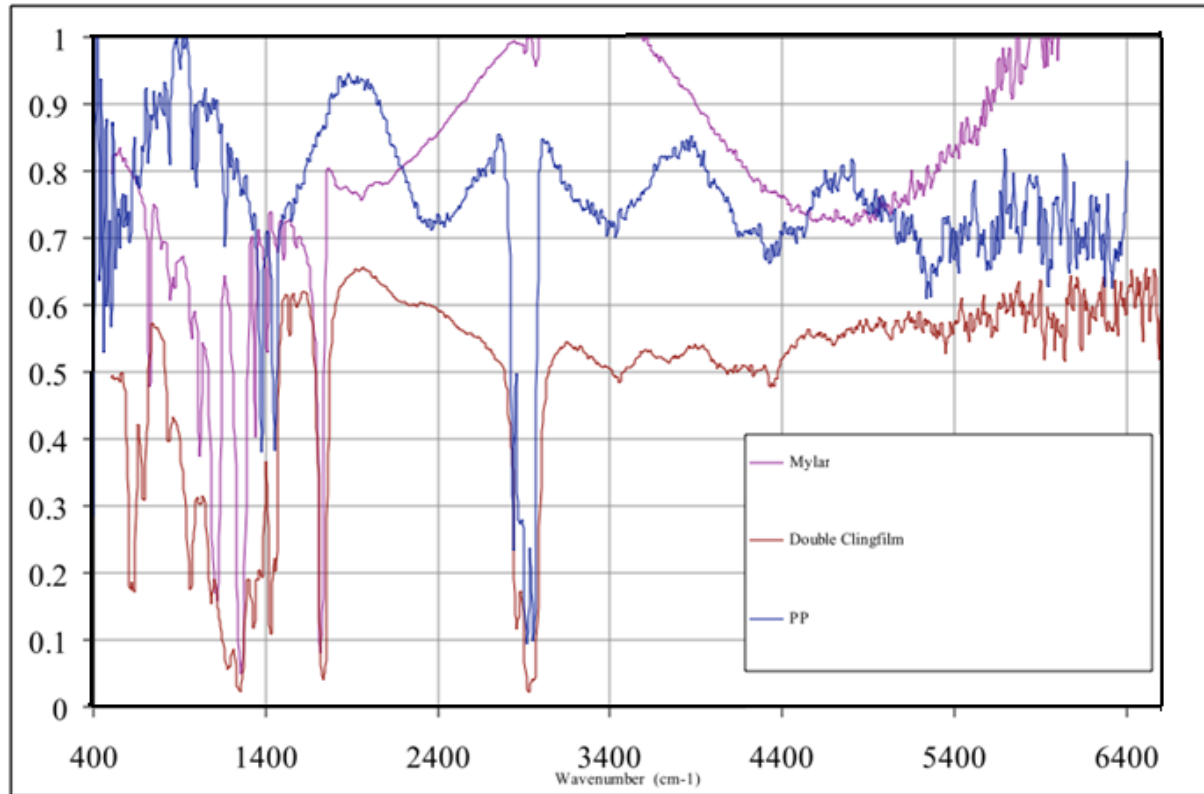


Figure 5.2: The absorption of Mylar, polypropylene and clingfilm (y-axis represent the transmission of substrates and x-axis represent the wavenumber).

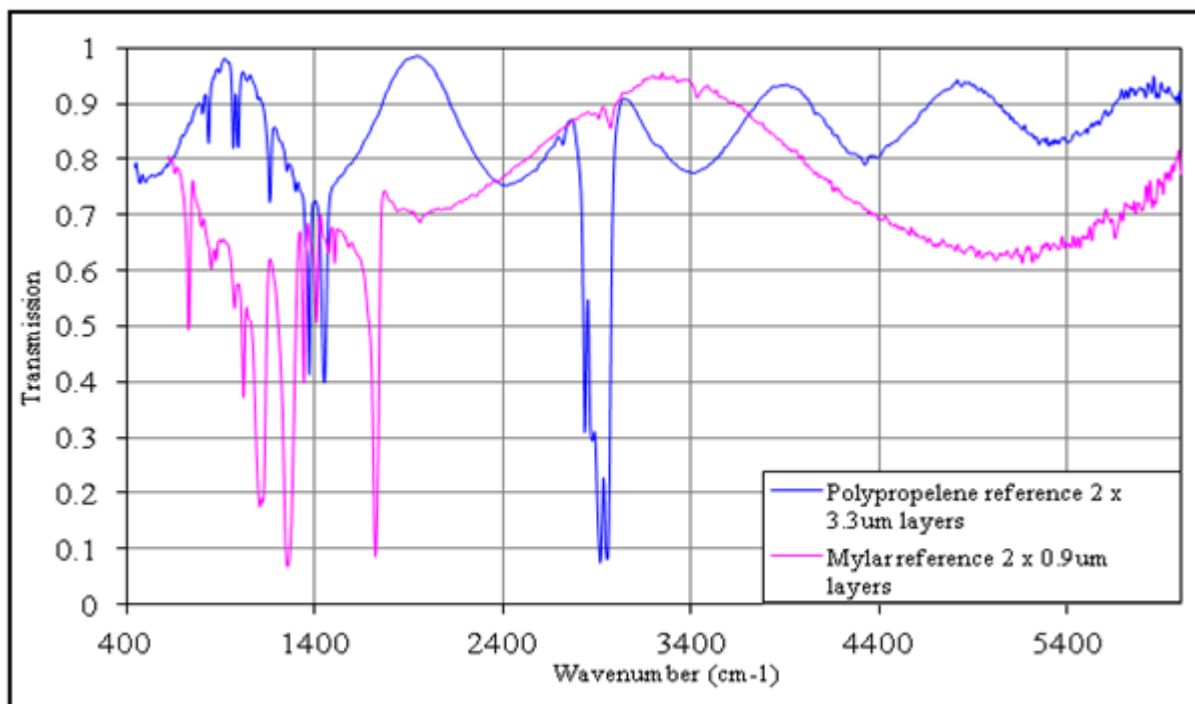


Figure 5.3: A reference of polypropylene and Mylar substrate to demonstrate the choices of substrate made. Mylar has a strong absorption feature between 1000 and 1800; therefore, Polypropylene is more suitable for investigations in this region. However, Polypropylene has a strong absorption feature between 2700 and 3100; therefore, Mylar is more suitable for investigations in this region.

5.3.2 Sample Thickness

After choosing a suitable substrate, we then looked at sample thickness to decide which thickness we should use in this experiment. Two different thicknesses were investigated, 100um and 200um. Figure 5.4 shows that samples with thickness 200um in the fingerprint region were almost drowned out due to saturation. Due to the 200um thickness results, we decided to use a sample with maximum thickness of 100um. Ideally we needed to use a sample of 50um thickness (in a previous study by Catherine Jackson in 2008, it was shown that at 50um the transmission is much greater).

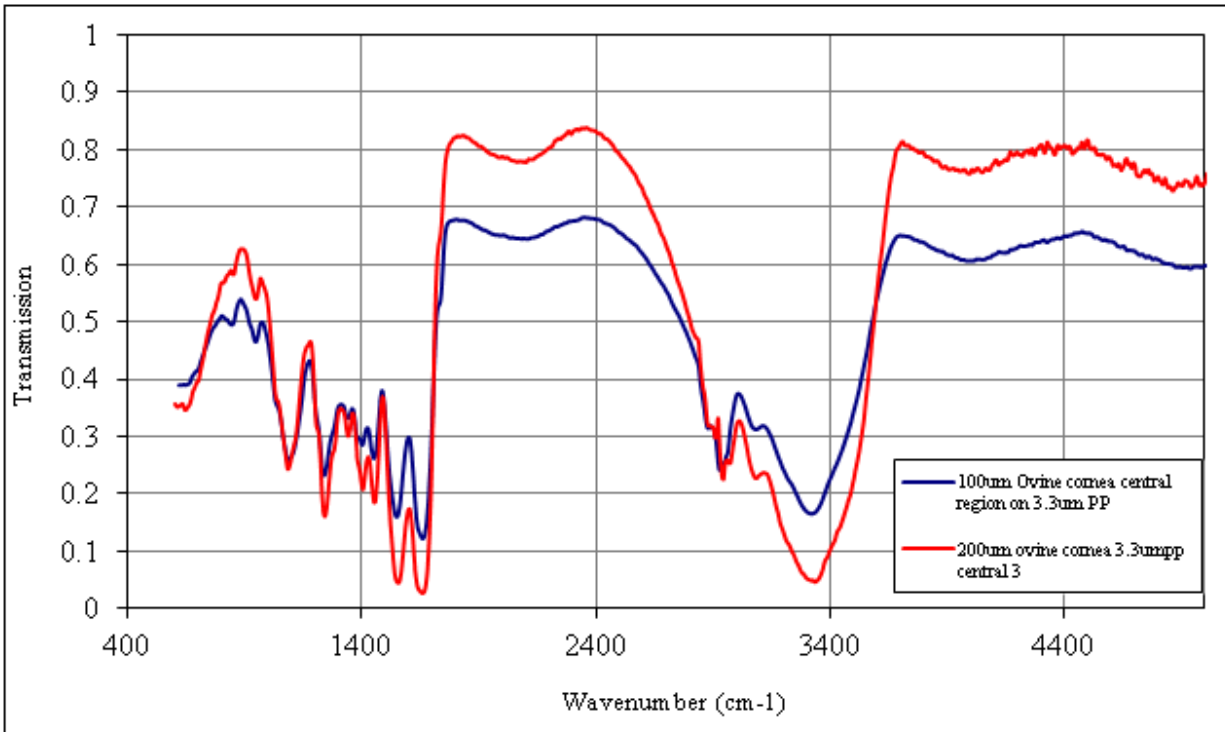


Figure 5.4: Comparison of 100 and 200um Ovine Cornea in the central region

5.3.3 Depth of section

Before moving to the treated and untreated sections, we had to know if the depth from where the section was removed from the sample affected the absorption features or not.

Figure 5.5 indicates that the absorption features become stronger in the deeper layers. It also shows that the spectra of the two samples, at depths 200um and 300um, appear almost flat and are saturated between 3,000 and 4,000 cm^{-1} . If this is a correct outcome (as the spectrometer would occasionally overheat and stop working which would have caused these anomalies), then there should be no lower absorption than this; however, the samples with depth 400um and 500um appear lower than this.

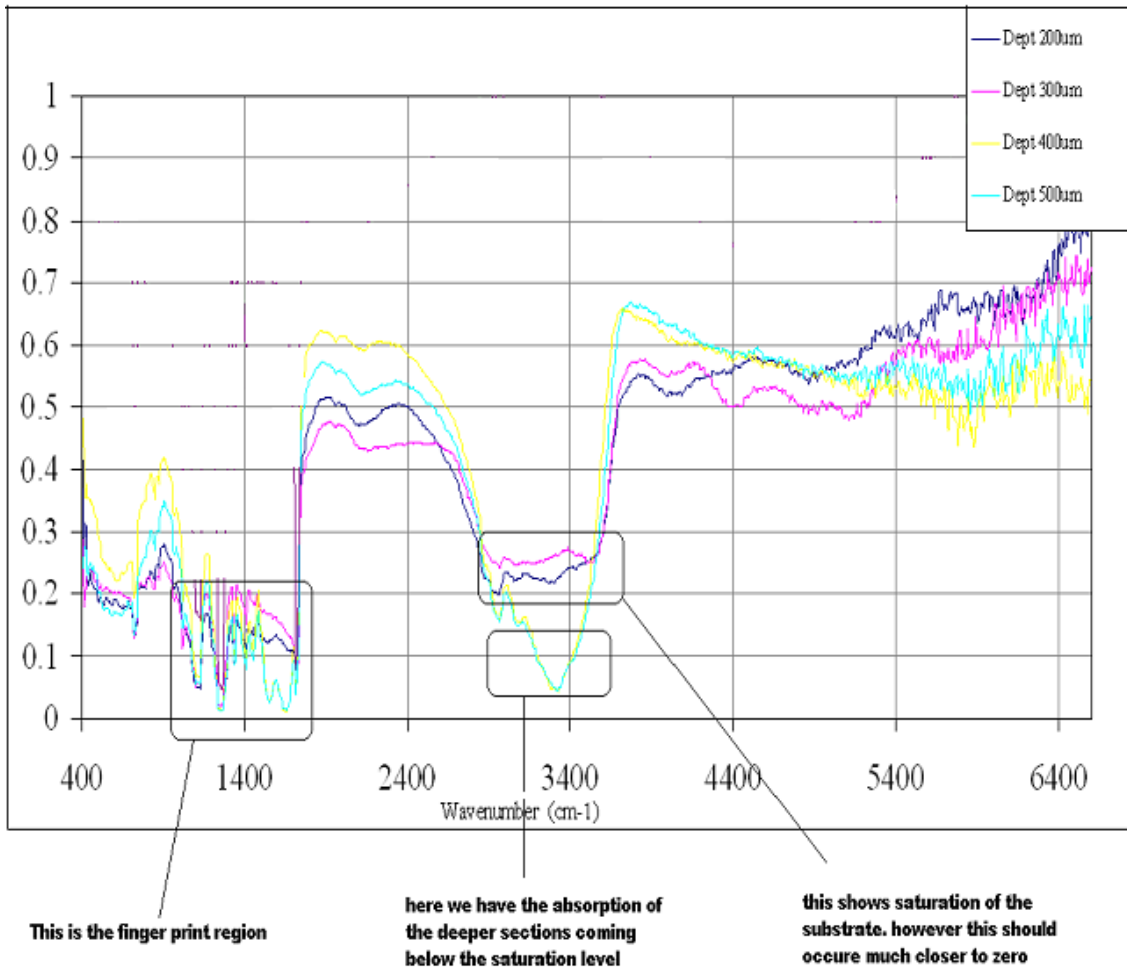


Figure 5.5: Comparison of the absorption features depending on the depth of the section.

5.3.4 Comparing treated and untreated samples

This experiment included 20 samples (10 treated and 10 untreated) and similar results were achieved from all treated samples and untreated samples. Therefore, four samples (two treated and two untreated) are presented in Figure 5.6 to compare the treated and untreated results.

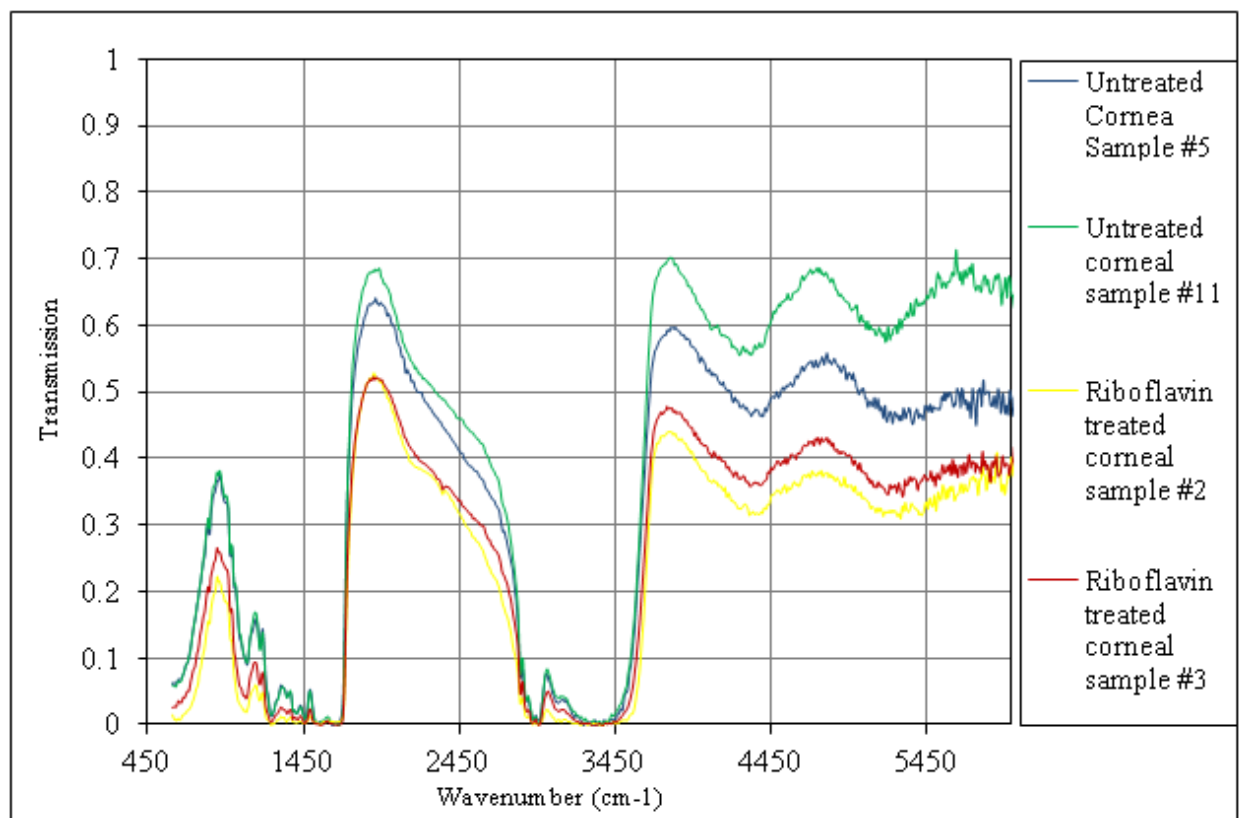


Figure 5.6: Comparison between treated and untreated samples.

Figure 5.6 shows clearly that the treated sample is saturated in both regions; this means that there are some structural changes that have occurred in the treated sample.

However, we cannot clearly distinguish between structural differences and hydration effects. Therefore, we decided to dehydrate the samples to reduced the water content and repeat our measurements.

5.3.5 Effect of tissue hydration and substrate

Another important factor needs to be taken into account; the hydration of samples was a fundamental problem that could affect our measurements. This is because water is highly absorbing of IR radiation. Having excised the whole eye from in vivo, the cornea became swollen, which resulted in a reduction in its corneal transparency.

On the other hand, the sample would become dehydrated after we sectioned the cornea. However, directly after we cut it, we placed the sample between two rings of substrate and sealed them together using vacuum grease.

Therefore we decided to dehydrate the specimens. When doing a dehydrated sample, we have the added advantage of no longer requiring a supporting medium/substrate (polypropylene) as the sample was strong enough to stand alone. Therefore there was no need to ratio out background effects. Data in figure 5.7 shows results for free-standing untreated and treated corneas.

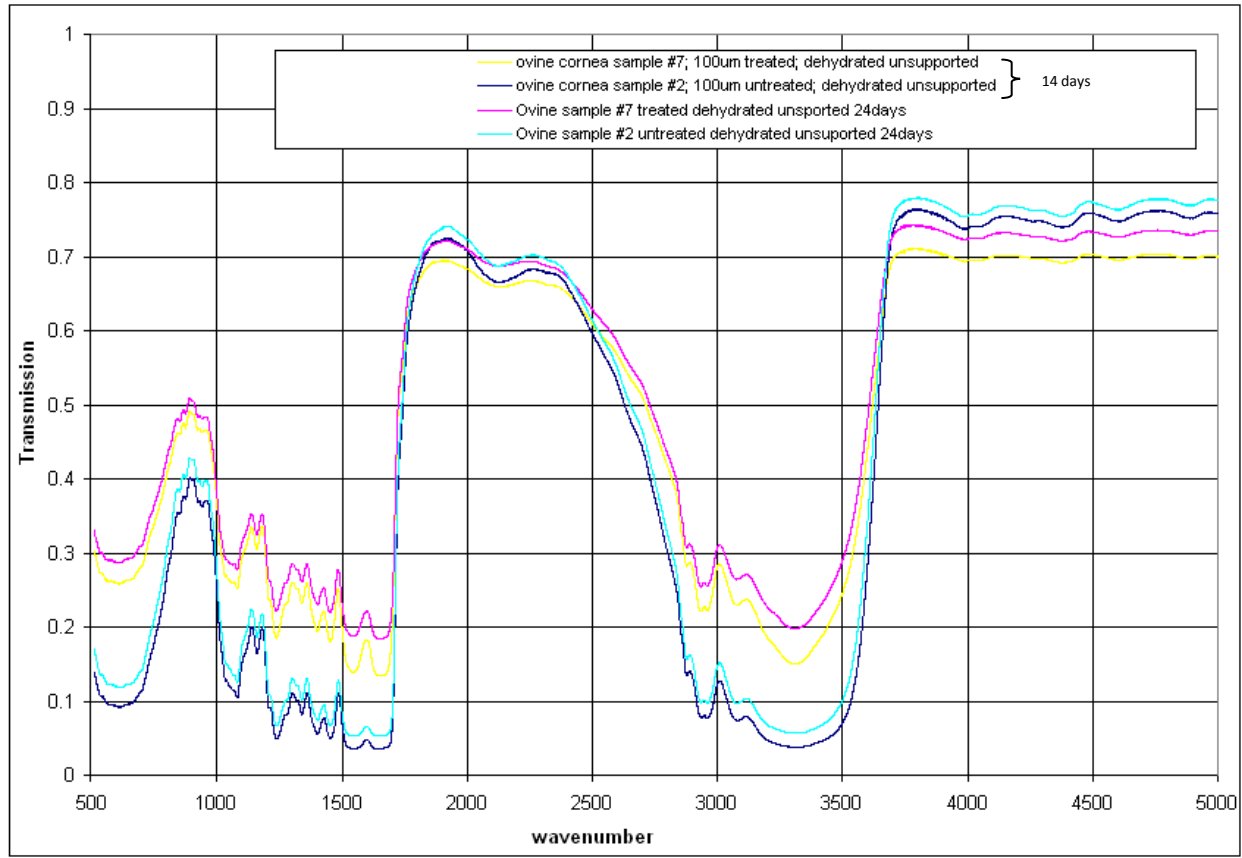


Figure 5.7: The effect of hydration and substrate in the sample absorption.

We left the samples a long time (two weeks) for complete dehydration but, as with the hydrated tissue, the peaks were less strong for the treated sample. This suggests that it was the substrate that was drowning out the features in the hydrated samples. However, it is interesting that, for the same thickness of sections, all untreated specimens saturated whereas all treated specimens did not. This suggests that there may be some structural changes that occur to the cornea after cross-linking treatment, but the fact that the untreated samples were still saturated even after dehydration does not allow us to be certain about this.

5.4 Discussion

Corneal collagen cross-linking with riboflavin and UV is a quantum leap in vision research. It shows a significant increase in the stability of the cornea after treatment; however, to date it is not yet approved by the NHS. Previous study have shown good results with treated keratoconus patients and demonstrated an increase in the biomechanical properties of the cornea (Wollensak et al. 2003a and 2003b). Therefore, research in this field should continue to be carried out to provide better understanding of cross-linking treatments.

In this study we investigated the structural changes in the cornea, after treating it with riboflavin and UV, by comparing the IR spectra between normal corneas and cross-linked corneas. The molecular structure of the treated corneas was changed after treatment; however, it was not a significant change built upon significant structural changes.

Unfortunately, the experiment was confounded by a number of experimental problems, such as hydration of the sample, the instrumentation and the thickness of the sample. Due to these factors there were not enough reliable data to allow any statistical analysis to be carried out. Therefore, if repeating this experiment or doing any similar investigation, samples would have to be prepared that are much thinner - maybe by a factor of 10, so up to 20um thickness. In this way absorption features will be well defined and mathematical fitting techniques (Gaussian line-shape fitting) could be applied. Then, for statistical analysis, many samples of treated and untreated corneas of similar thickness would need to be measured. It would then be possible to correct the

data for thickness variation; then a basic analysis of depth, width, frequency location of absorption peaks could be done. If the above is difficult to achieve and all corrected spectra look similar, then more detailed mathematical fitting tools could be applied, such as Principle Component Analysis.

Despite the problems encountered, our results did indicate that if changes in the absorption features do occur after crosslinking, they are only small. This suggests that the large changes that are known to occur in the structural stability of the cornea arise from only small changes in the number of cross-linkages. Thus, further studies to compare keratoconus corneas before and after cross-linking are required to determine structural changes in the cornea before and after cross-linking, and also to observe the amount of change in absorption features.

Chapter 6: An investigation of trans-epithelial stromal Riboflavin absorption with Ricrolin TE[®] (Riboflavin 0.1% with trometamol and sodium EDTA) using spectrophotometry.

6.1 Introduction

Keratoconus is a non-inflammatory, degenerative disorder characterized by corneal stromal thinning with resultant conical ectasia, irregular astigmatism and visual loss (Krachmer et al. 1984). Its patho-physiology is not understood and is thought to include biochemical, physical and genetic factors. It is likely that keratoconus is the final common pathway for several different disorders. It is the commonest corneal dystrophy and affects around 1 in 2000 individuals (Ucakhan et al. 2006). Mild and sub-clinical cases may be effectively corrected with spectacles and soft toric contact lenses. In moderate and advanced cases, rigid contact lenses are necessary to provide adequate visual rehabilitation. Progressive disease can result in severe ectasia with corneal scarring and associated contact lens intolerance and/or inefficacy. Surgical intervention in the form of corneal transplantation is required in 10-25% of cases (Javadi et al. 2005; Reeves et al. 2005; Mamalis et al. 1992; Al-Yousef et al. 2004).

Over the past decade a new therapeutic modality Riboflavin (vitamin B₂)/ultraviolet A (UVA) (370nm) corneal collagen cross-linkage (CXL) has been developed, which is thought to induce cross-linking of stromal collagen photo-chemically (Andley U et al. 1992). It appears to be the first treatment which can stabilize the keratoconic process (Wollensak 2006). Laboratory studies have shown CXL to increase the stress-strain measurements of corneal tissue and augment its resistance to

enzymatic digestion, thermal damage and hydration (Wollensak et al. 2003b, Spoerl et al. 2000, Spoerl et al. 2004a and 2004b; Wollensak G et al. 2007).

Riboflavin is a key component of this photochemical cross-linking process. It acts both as a photosensitiser for the production of oxygen free radicals to activate the cross-linking process (Andley et al. 1992) and concentrates and absorbs the UVA irradiation within the stroma to prevent damage to deeper ocular structures such as the endothelium, lens and retina (Wollensak et al. 2003a and 2003b; Spoerl et al. 2007; Spoerl et al. 1998). Riboflavin is a hydrophilic compound and cannot easily cross the intact epithelial barrier. Spoerl et al demonstrated the need for complete central epithelial debridement to allow adequate stromal absorption of Riboflavin, identifying no alteration in the biomechanical properties of corneal tissue where the technique had been performed with the epithelium intact (Spoerl et al. 1998). On the basis of this preliminary study, the epithelium was removed prior to corneal Riboflavin administration in the first published clinical studies (Wollensak 2006 and Wollensak et al. 2003a).

Despite this recommendation a number of clinicians have elected to perform the technique with the epithelium intact or partially disrupted in order to reduce post-operative discomfort and speed visual recovery (Chan et al. 2007; Samaras and Lake 2010). The use of multiple applications of tetracaine 1% to attempt to loosen epithelial tight junctions has been advocated by some (Chan et al. 2007). Others have employed limited full-thickness epithelial debridement in a grid-like pattern, with islands of intact epithelium to facilitate more rapid post-operative epithelial healing (Samaras and Lake 2010).

In two previous publications (Hayes et al. 2008; Samaras et al. 2009), authors indirectly measured stromal Riboflavin absorption using spectrophotometry following complete epithelial debridement, superficial epithelial trauma or no epithelial trauma with concomitant administration of topical tetracaine 1% and with and without UVA exposure (Hayes et al. 2008) and investigated techniques to assist Riboflavin uptake by either loosening the epithelial tight junctions with application of 20% Alcohol solution or by removing the epithelium in a grid pattern rather than complete debridement (Samaras et al. 2009). These results indicated that complete removal of the epithelium was essential to permit adequate absorption of Riboflavin with all other tested methodologies being insufficient.

In this present study we investigated the use of a specially formulated Riboflavin solution, RICROLIN TE[®] (RTE) (Sooft Italia S.p.A.), in which, two enhancers, trometamol (Tris-(hydroxymethyl)aminometane) and Sodium Ethylenediaminetetraacetic acid (EDTA) have been added to help Riboflavin penetrate into the corneal stroma through an intact epithelium. We employed spectrophotometry to indirectly measure Riboflavin stromal absorption in ex-vivo rabbit corneas and porcine corneas.

6.2 Materials and Methods

Seventy animal eyes were transported from a local abattoir within 24 hours of death (thirty-five porcine eyes and later on thirty-five rabbit eyes). A visual examination of each specimen for the presence of corneal scarring or opacity was undertaken.

In the first set of experiments we used porcine corneas to investigate transepithelial stromal riboflavin absorption with Ricrolin TE by analyzing light transmission properties of these ex-vivo porcine corneas. However, we decided to repeat this experiment and use ex-vivo rabbit corneas instead of porcine corneas; the reasons for repetition and using different tissue were:

- 1) The two control groups (no treatment and intact epithelium and riboflavin TE) showed statistically significant higher transmission at all measured wavelengths and we thought that because we measured these control eyes first it may represent a hydration problem with the porcine eyes from the abattoir.
- 2) The preliminary experiments convinced us that it was necessary to set up all the eyes on the bench at the same time and expose all eyes for the same time.
- 3) In the repeat experiment we decided to randomly measure the eyes with the spectrophotometer, rather than measuring them in groups (because randomly measuring the eyes should negate errors such as may be caused by the spectrophotometer as it warms up etc).
- 4) We decided to use rabbit eyes rather than porcine eyes. The epithelium is very thick in porcine eyes; rabbit epithelium is much thinner. We were supplied with fresh rabbit eyes (within 12 hours of death), thus eliminating the hydration

problems encountered by using porcine eyes from the abattoir.

Twenty-eight eyes were selected from each group for inclusion in the study (initially from the porcine group then later from the rabbit group) and divided into the following treatment regimens:

1. Controls: Balanced saline 0.9% drops (BSS) were administered to 4 eyes every three minutes for 1 hour. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes.

2. RTE, intact epithelium: In order to improve riboflavin penetration and retention, a specially designed ring-shaped silicone container (Fig. 6.1), 12 mm in diameter, 3 mm high, with a flange 2 mm wide and 0.3 mm thick at its base was placed over the cornea. In vivo this device also obviates the need for a blepharostat. RTE drops were then administered every three minutes for 1 hour to 4 eyes. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes.

3. RTE, superficial grid-pattern epithelial trauma: Prior to administration of RTE drops a 27 gauge canella was used to create superficial scratches over the corneal surface (10 horizontally and 10 vertically) in a grid pattern in 4 eyes. Following placement of the silicone ring container, RTE drops were administered every three minutes for 1 hour. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes.

4. RTE, central epithelium completely removed: Prior to administration of RTE drops a scalpel blade was used remove the epithelium over the central cornea with a diameter of approximately 8.00 millimeters (mm) in 4 eyes. Following placement of the silicone

ring container, RTE drops were administered every three minutes for 1 hour. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes.

5. Ricolin[®] (Riboflavin 0.1%, dextran T500) (Rnorm), intact epithelium: Following placement of the silicone ring container, Rnorm drops were administered every three minutes for 1 hour in 4 eyes. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes.

6. Rnorm, superficial grid-pattern epithelial trauma: Prior to administration of Rnorm drops a 27 gauge canella was used to create superficial scratches over the corneal surface (10 horizontally and 10 vertically) in a grid pattern in 4 eyes. Following placement of the silicone ring container, Rnorm drops were administered every three minutes for 1 hour. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes (this treatment for rabbit corneas only).

7. Rnorm, central epithelium completely removed: Prior to administration of Rnorm drops a scalpel blade was used remove the epithelium over the central cornea with a diameter of approximately 8.00 millimeters (mm) in 4 eyes. Following placement of the silicone ring container, Rnorm drops were administered every three minutes for 1 hour. Tetracaine 1% drops were also administered every 5 minutes for the first 30 minutes.

Prior to treatment to minimize any possible effects in light transmission caused by differences in stromal hydration and thickness, central corneal pachymetry (Pachmate DGH55, DGH Technology Inc., PA, USA) was performed in all rabbit eyes and only those within 50 micrometres (um) of 400um were included.



Figure 6.1: Silicone container

Immediately following treatment each cornea was washed with BSS for 10-15 seconds. A 3mm scleral rim was dissected from the globe and placed into a specially designed sample holder (as described in chapter 2). The natural curvature of the cornea was maintained by clamping the scleral rim within the sample holder and injecting silicone oil (Dow Corning 200/5cS, BDH Laboratory Supplies, Poole, UK) into the chamber behind it. Silicone oil was also injected into the front chamber of the holder so as to maintain a uniform refractive index and reduce light scatter (Kostyuk et al. 2002). The sample holder was then positioned into the spectrophotometer (PYE Unicam, SP8-100 UV/VIS) in such a way that light passed through the centre of the cornea in the anterior-posterior direction. The optics of the unit and the aperture were set to give a slit of (no larger than) 1x1mm on the surface of the cornea i.e. at the point where the centre of the cell lies, and it was ensured that the cell lay such in the path length that the beam was always incident on the centre. A transmission spectrum was measured for each cornea at 10nm intervals within the range of 400 to 700nm. Although this spectrum is within the visible spectrum, it is outside the treatment wavelength of 350 to 380nm. However, it does include one of the peak absorption spectra of Riboflavin at 400 to 490nm and is therefore entirely relevant in its aspiration to detect

changes in light transmission due to stromal absorption of Riboflavin. Using the method detailed by Kostyuk et al. (2002), the transmission spectrum for each sample was normalised against a baseline transmission spectrum of the chamber filled with silicone oil. A further transmission spectrum over the same wavelength range (400-700nm) was obtained for the Rnorm solution alone.

Student t-tests were used to compare transmission values. Results with $p < 0.05$ were considered statistically significant.

6.3 Results

6.3.1 Porcine corneas

As mentioned, the initial experiments were carried out using porcine eyes. Figure 6.2 shows the difference in light transmission in porcine corneas between these 6 groups (controls, intact epithelium and RTE, superficial grid trauma and RTE, central epithelial debridement and RTE, intact epithelium and Rnorm, central epithelial debridement and Rnorm). The results showed the effects of penetration of Rnorm and RTE (minimum near 450nm) but seemed to suggest that transmission levels vary at higher wavelengths between the groups. This was likely due to experimental artifact and so, for the reasons previously mentioned, we switched to rabbit corneas, modifying the protocols as described above.

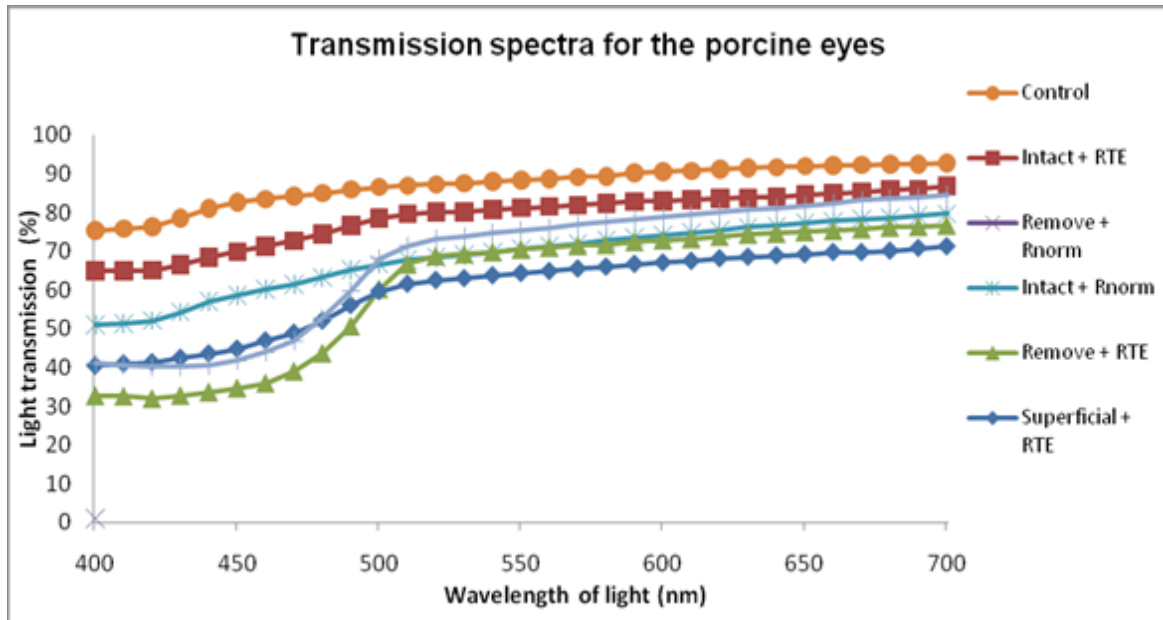


Figure 6.2: Transmission spectra for the six porcine groups.

6.3.2 Rabbit corneas

There were no differences in the central pachymetric measurements between the treatment groups. Central corneal thickness was 401.2um +/- 25.8 for group 1 (controls), 400.4um +/- 22.7 for group 2 (Intact epithelium and RTE), 413.2um +/- 26.2 for group 3 (superficial grid trauma and RTE), 404.3um +/- 25.9 for group 4 (central epithelial debridement and RTE), 406.8um +/- 23.3 for group 5 (superficial grid trauma and Rnorm), 409.7 +/- 32.1 for group 6 (Intact epithelium and Rnorm) and 422.2 +/- 19.5 for group 7 (central epithelial debridement and Rnorm).

Control corneas showed a gradual increase in light transmission between 400 and 700nm in a similar manner to previous studies (Hayes et al. 2008; Samaras et al. 2009) (figure 6.3). Similar light transmission spectra to control eyes were seen in group

2 (RTE, intact epithelium) (Figure 6.3), group 5 (Rnorm, intact epithelium) and group 6 (Rnorm, superficial grid-pattern epithelial trauma) although there was a slight dip in transmission between 400 and 490nm (Figure 6.3), which was greater in group 6 compared to groups 1, 2 and 5 ($p < 0.001$). In group 3 (RTE and superficial epithelial trauma) there was a more pronounced dip in light transmission between 400 and 490 nm compared to groups 1, 2 and 5 and eyes in group 6 ($p < 0.00001$) (Figure 6.3).

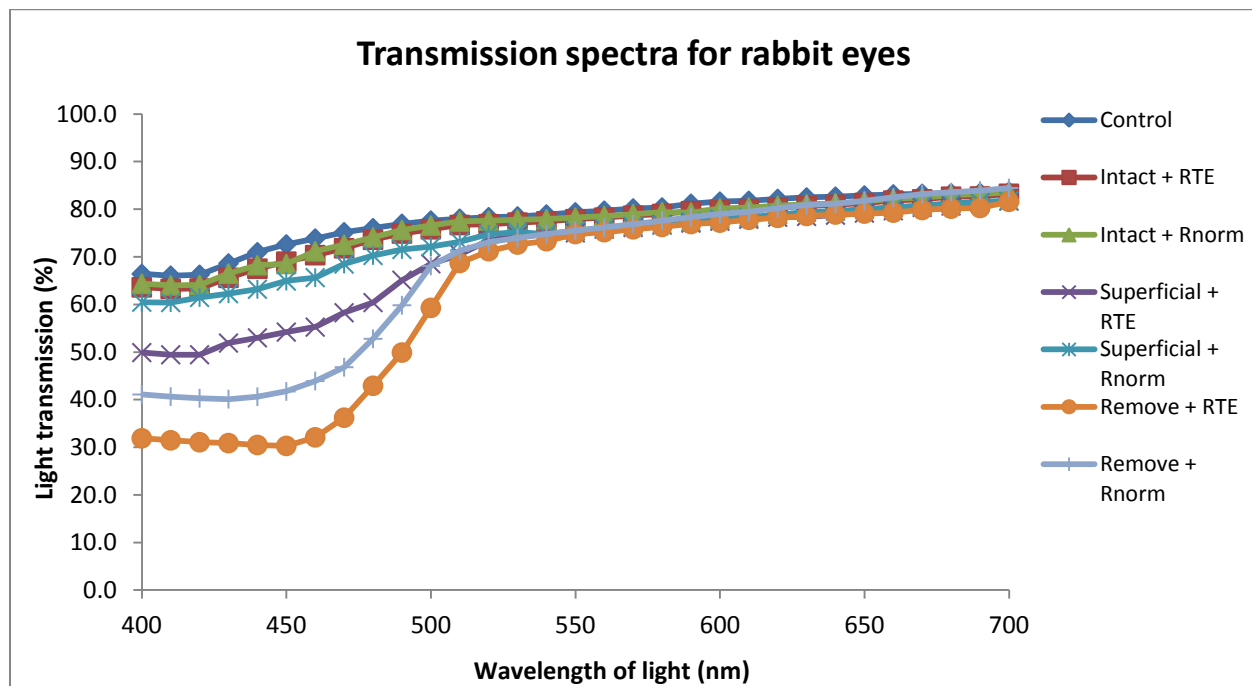


Figure 6.3: Transmission spectra for the seven rabbit treatment groups.

Direct observation of the eyes in group 3 showed an obvious central homogeneous yellow discoloration of the central corneal stroma consistent with Riboflavin absorption (Figure 6.4a) and also present in the eyes of group 4 (Figure 6.4b). However, it was not present in the eyes of group 2 (Figure 6.4c), or group 6 (Figure 6.4d). The dip in light transmission between 400 and 490nm in group 3 was less

than that in eyes for which epithelial removal was complete in groups 4 and 7 ($p < 0.0001$), where the spectrum showed a similar pattern to that of the RTE solution (figure 6.5). Interestingly, there also appeared to be a greater dip in light transmission between 400nm and 490nm for eyes in group 4 (RTE, complete epithelial removal) compared to group 7 (Rnorm, complete epithelial removal) ($p < 0.0001$).



Figure 6.4a: Colour photograph of rabbit cornea with superficial epithelial scratches and RTE for 60 minutes. There is an obvious homogeneous yellow discoloration of the central cornea consistent with Riboflavin stromal absorption.



Figure 6.4b: Colour photograph of rabbit cornea with removed epithelium layer and RTE for 60 minutes. There is an obvious homogeneous yellow discoloration of the central cornea consistent with Riboflavin stromal absorption.

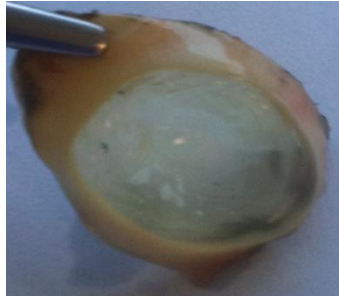


Figure 6.4c: Colour photograph of rabbit cornea with an intact epithelium receiving RTE for 60 minutes. Most of the central cornea is clear.



Figure 6.4d: Colour photograph of rabbit cornea with a superficial scratches and Rnorm for 60 minutes. Most of the central cornea is clear with no homogeneous yellow discoloration.

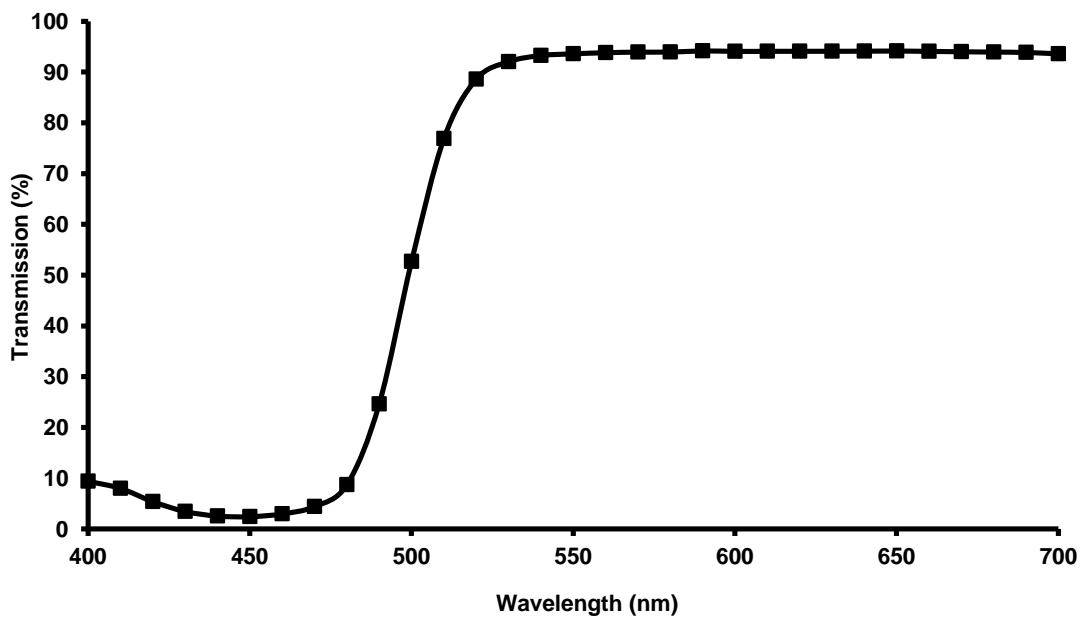


Figure 6.5: Light transmission spectrum of riboflavin solution.

6.4 Discussion

Riboflavin/UVA corneal collagen cross-linkage is the first treatment modality that appears to halt the progression of keratoconus and post-keratorefractive surgery ectasia (Wollensak 2006; Wollensak et al. 2003a). Riboflavin increases UVA absorption in the cornea up to 95% (Spoerl et al. 2000), thereby optimizing the cross-linking process and protecting internal ocular structures such as the endothelium (Wollensak G et al. 2003c; Spoerl et al. 2007). Published clinical and laboratory studies have advocated the complete removal of the central epithelium to allow adequate penetration of the riboflavin into the corneal stroma (Wollensak 2006; Wollensak et al. 2003a; Spoerl et al. 2000; Spoerl et al. 2004a; Spoerl et al. 2004b; Wollensak et al. 2007; Wollensak et al. 2003b; Spoerl et al. 2007; Spoerl et al. 1998).

Removal of the epithelium, however, whilst simple to perform is associated with considerable early post-operative discomfort and reduced visual performance. Therefore a number of clinicians have advocated performing cross-linking with the epithelium intact or partially disrupted (Chan et al. 2007; Samaras et al. 2010). Techniques described include multiple applications of topical tetracaine 1% (Chan et al. 2007) or benzalkonium chloride (Kissner et al. 2010) in an attempt to loosen epithelial tight junctions and limited grid-pattern full-thickness epithelial debridement (Samaras et al. 2010).

Previous publications, indirectly measuring stromal Riboflavin absorption using spectrophotometry have demonstrated that complete removal of the epithelium appears to be necessary to permit adequate absorption of Riboflavin with all other tested

methodologies such as administration of topical Tetracaine 1%, 20% Alcohol application or grid pattern epithelial removal being insufficient (Hayes et al. 2008; Samaras et al. 2009).

In this study spectrophotometry has been used to indirectly measure Riboflavin stromal absorption. We investigated the use of a new formulation of Riboflavin, in which trometamol (Tris-(hydroxymethyl)aminometane) and Sodium Ethylenediaminetetraacetic acid (EDTA) had been added to help Riboflavin penetrate through an intact epithelium into the corneal stroma. Our results showed that whilst the light transmission spectrum was fairly similar between control eyes and those with a completely intact epithelium and receiving RTE and Rnorm (figure 6.3), those eyes with superficial epithelial scratches and treated with RTE showed a very significant and obvious dip in light transmission between 400 and 490nm which corresponds to the light transmission spectrum of the Riboflavin solution itself (figures 6.3 and 6.4a). These results indicate that the administration of RTE, for 60 minutes, in the presence of superficial epithelial scratches allows enough Riboflavin uptake into the corneal stroma to very significantly alter its light transmission spectrum. Indeed visual inspection of the corneas in this group showed an obvious homogeneous yellow discoloration of the central cornea (figure 6.4a). This was in contrast to eyes with superficial corneal epithelial scratches receiving Rnorm where the light transmission spectrum although slightly reduced between 400 and 490nm was not dissimilar to controls (figure 6.3) and visual inspection of the corneas showed only slight heterogenous yellow discoloration (figure 6.4d). These changes are also in contrast to a previous published studies where the use of full thickness epithelial scratches whilst achieving stromal Riboflavin

absorption in the areas of complete thickness epithelial debridement did so in a non-homogeneous manner with no obvious uptake in non-debrided areas (Samaras and Lake 2010; Samaras et al. 2009).

We measured a greater dip in light transmission between 400nm and 490nm for eyes with complete epithelial debridement and receiving RTE and Rnorm compared to our group with superficial scratches and RTE. This does suggest greater stromal Riboflavin uptake with complete epithelial debridement. Interestingly, we also found a greater dip in light transmission with RTE compared to Rnorm in the presence of epithelial debridement, suggesting that RTE facilitates Riboflavin absorption even in the presence of epithelial removal.

At present, the optimum stromal concentration of Riboflavin for Riboflavin/UVA corneal collagen cross-linking is undetermined. In addition, our study has a number of limitations as our measurements of stromal Riboflavin were indirect, the study eyes were ex-vivo and rabbit eyes/corneas differ from human eyes with a thinner epithelium, etc. However, our results indicate significant homogeneous stromal riboflavin absorption with superficial epithelial grid-pattern trauma and RTE administration and that avoidance of full-thickness large area epithelial debridement is likely to offer patients significant advantages in terms of reduced pain, faster visual recovery and possibly reduced incidence of post-operative infection. Administration of RTE and superficial epithelial scratches allows sufficient Riboflavin stromal absorption to alter the transmission spectra of ex-vivo rabbit corneas. This did not occur with RTE or Rnorm with an intact epithelium or Rnorm with superficial scratches. Further laboratory and clinical studies are clearly indicated to evaluate the efficacy of this technique and to

compare it to the standard procedure with complete epithelial removal both with Rnorm and RTE.

Chapter 7: Investigation of the influence of corneal cross-linking with riboflavin and RTE on the biomechanical properties of the cornea to determine the impact on the whole eye in measurements of corneal biomechanics.

7.1 Introduction

In this chapter, a number of experiments were carried out to confirm or disprove some of the results and inferences from previous chapters. In Chapter 6, we compared R_{norm} and RTE in terms of penetration and light transmission to detect whether there is any difference between them, and to confirm whether or not we need to remove the epithelium layer before cross-linking. In this chapter, we compare these crosslinking promoters in terms of their effects on corneal biomechanics to see how these results can support our results in that chapter.

Over recent years, some groups (Spoerl et al. 1998; Wollensak et al. 2003b; McCall et al. 2010; Kling et al. 2010) have investigated the corneal properties ex-vivo after CXL, and have shown how the cornea becomes more stable after treatment. On the other hand, measurement of corneal stability after CXL in vivo was usually done by observing the corneal topography reading (which is a mean indirect measurement of the biomechanical properties of the cornea). However, 2005 saw the arrival of the Ocular Response Analyzer (ORA) which provides two biomechanical parameters of the cornea, CH and CRF; as seen in earlier chapters, this has allowed measurements of corneal biomechanical properties in vivo.

Goldich et al. (2009) and Spoerl et al. (2011) demonstrated that there is no significant change in the biomechanical properties of the cornea after corneal cross-linking with Riboflavin (Rnorm) and UVA measured by the ORA. However, Spoerl et al. found a significant change in the peak 2 area which increased by 35% one year after cross-linking. In this chapter we look at the differences in parameters and signals by using ORA and determine whether the new riboflavin (RTE) affects the measurement of corneal biomechanics or not. Specifically we examine the superficial grade group, as there was a significant difference in light transmission between them, so there may also be some difference in their biomechanical properties. In addition, we took the opportunity to investigate if crosslinking in dark conditions makes any difference to the usual method of crosslinking in natural daylight.

In a different context, we found a significant reduction in CH and CRF in post-LASIK patients compared to normals (see Chapter 4); however, we believe that this reduction after surgery is not due to a decrease in the biomechanical properties of the cornea but is because of a reduction in central corneal thickness (CCT); therefore, the CH and CRF decreased as they have a strong positive relationship with CCT. In this chapter, we have measured CH and CRF before and after a laser in situ keratomileusis-like flap procedure to confirm our assertion that the flap has no significant effect on the biomechanical properties.

7.2 Materials and methods

Thirty-eight fresh ovine eyes were included in this study and were divided into 9 groups:

Group 1 (Control; n= 6).

Group 2 (Rnorm +UVA; n = 4).

Group 3 (RTE + UVA; n = 4).

Group 4 (Rnorm in dark (No UVA) n = 4).

Group 5 (RTE in dark (No UVA) n = 4).

Group 6 (Rnorm + superficial; n = 4).

Group 7 (RTE + superficial; n = 4).

Group 8 (Before LASIK-flap; n = 4).

Group 9 (After LASIK-flap; n = 4).

The new ORA software (2.04) was used to measure CH and CRF before/after treatment, before/after creating the flap and before/after excising the cornea from the whole eye.

Laser in situ keratomileusis-like flaps were created by using mickrokeratome (Bausch and Lomb Hansatome Microkeratome).

In the first part of this study and before treating any eye, all eyes were checked and it was confirmed that they did not have any ocular disorder. Immediately afterwards,

cross-linking with Rnorm and RTE was performed according to the standard procedure described in Chapter 2 (General materials and methods).

After completion of the treatment, four measurements were taken by using ORA, and the best signal which had the highest WS (wavescore) was chosen.

Student t-tests were used to compare values before and after cross-linking; the results with $p < 0.05$ were considered statistically significant. To study the relationship and the differences between values, a Pearson correlation coefficient was calculated.

7.3 Results

Figure 7.1 shows that neither CH nor CRF are significantly altered after crosslinking using either Rnorm (7.1a) or RTE (7.1b). Furthermore, there were no differences using either Rnorm or RTE; the mean CH and CRF for Rnorm were 12.13 ± 2.9 and 11.82 ± 1.9 , respectively and for RTE were 12.36 ± 1.7 and 11.74 ± 1.4 respectively.

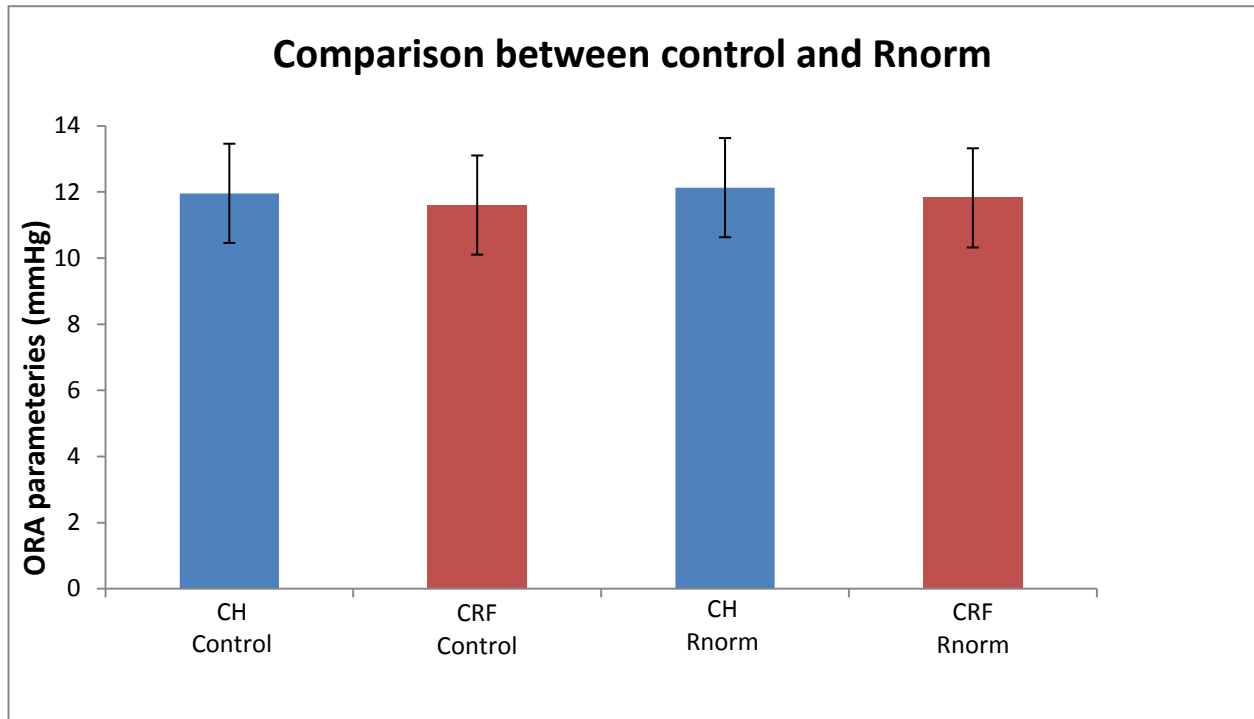


Figure 7.1a: The biomechanical properties of the cornea before and after CXL with Rnorm

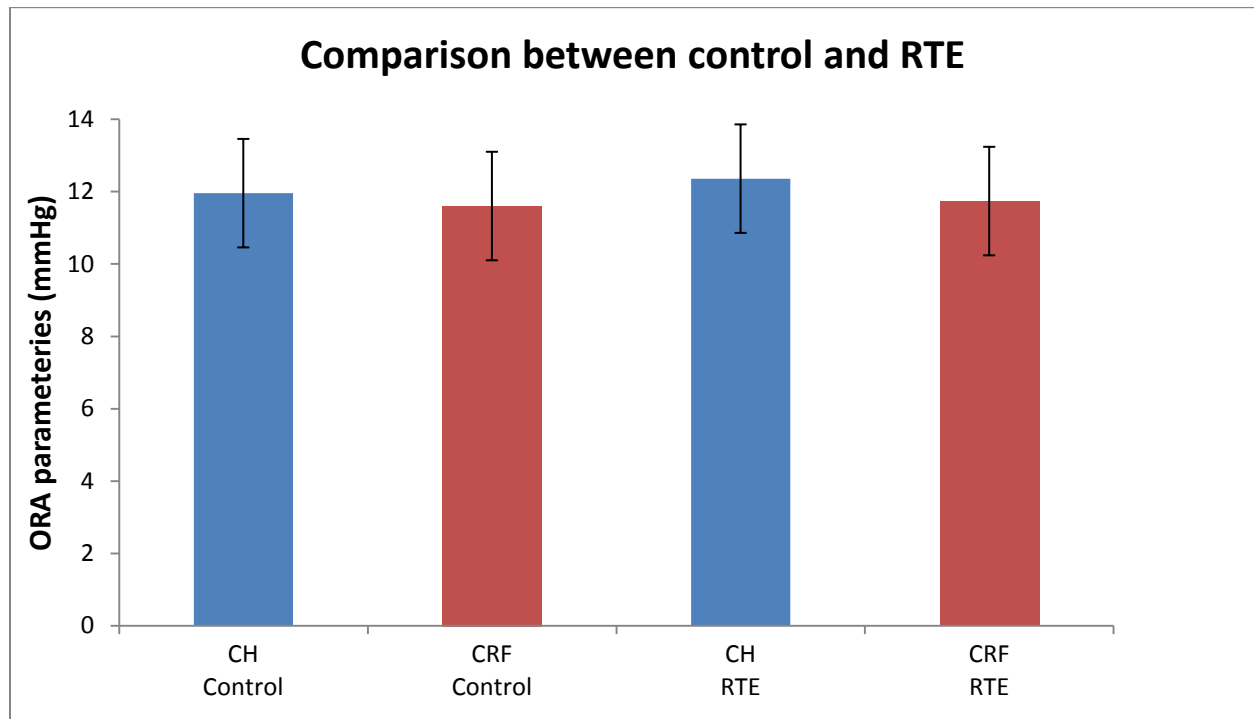


Figure 7.1b: The biomechanical properties of the cornea before and after CXL with RTE

However, the shape of the ORA signals was affected, where more noise and a flatter signal appeared after the treatment (Figure 7.2a and 7.2b).

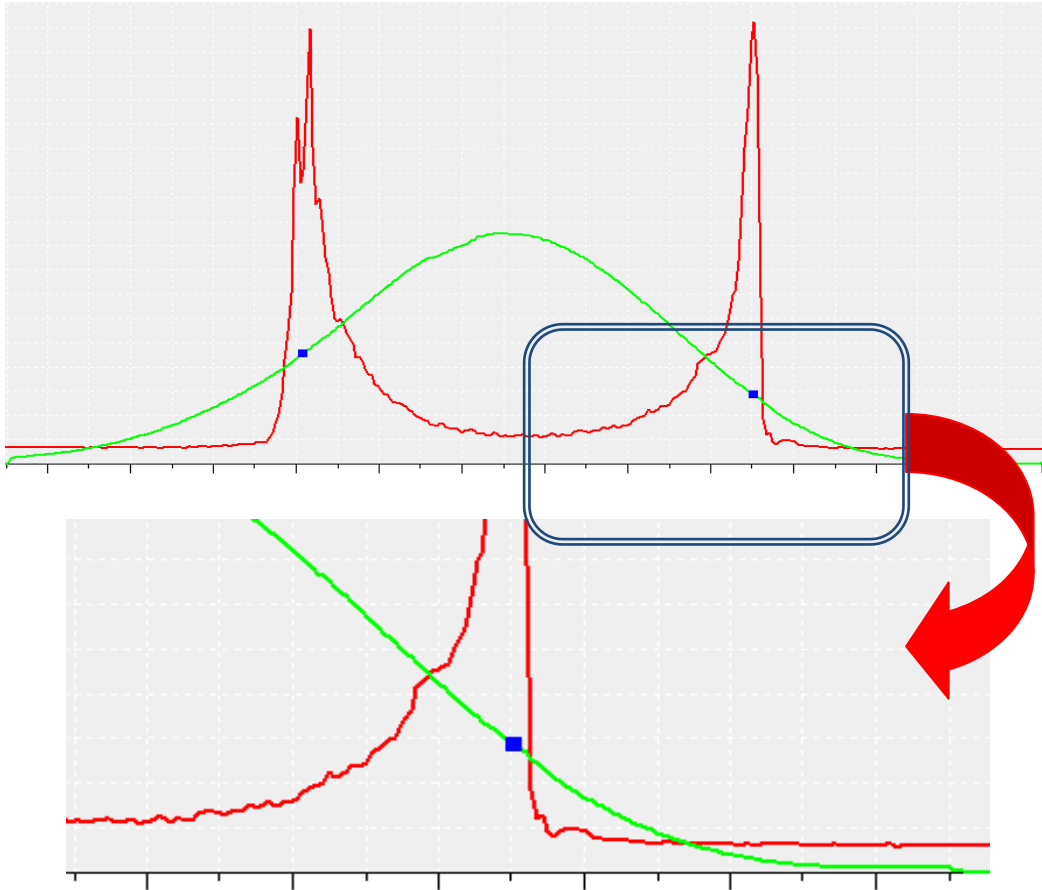


Figure 7.2a: The signal before CXL

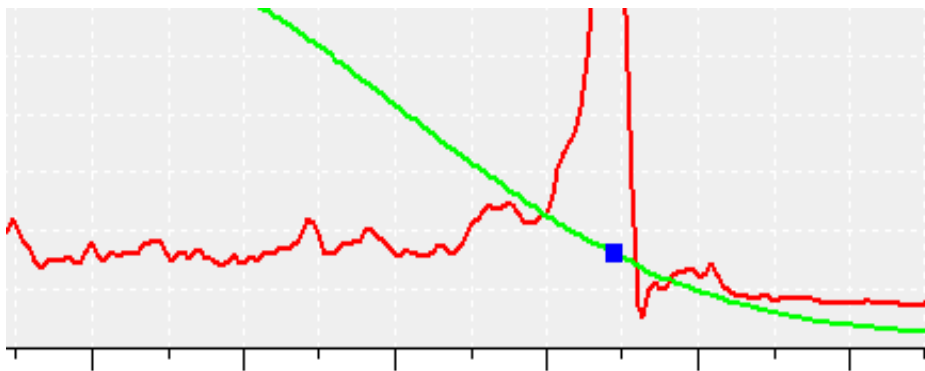


Figure 7.2b: The signal after CXL

The biomechanical properties were also measured after crosslinking in the dark (i.e. without any light, neither UVA or visible) using Rnorm and RTE, to investigate whether UVA or normal blue light affected the measurements, but our results showed no significant change between them ($p > 0.1$) (Figs. 7.3a, 7.3b). The mean CH for Rnorm in the dark was 11.87 ± 2.4 and 11.64 ± 2 for RTE. The CRFs were very close at 11.53 ± 2.1 and 11.39 ± 1.9 , respectively.

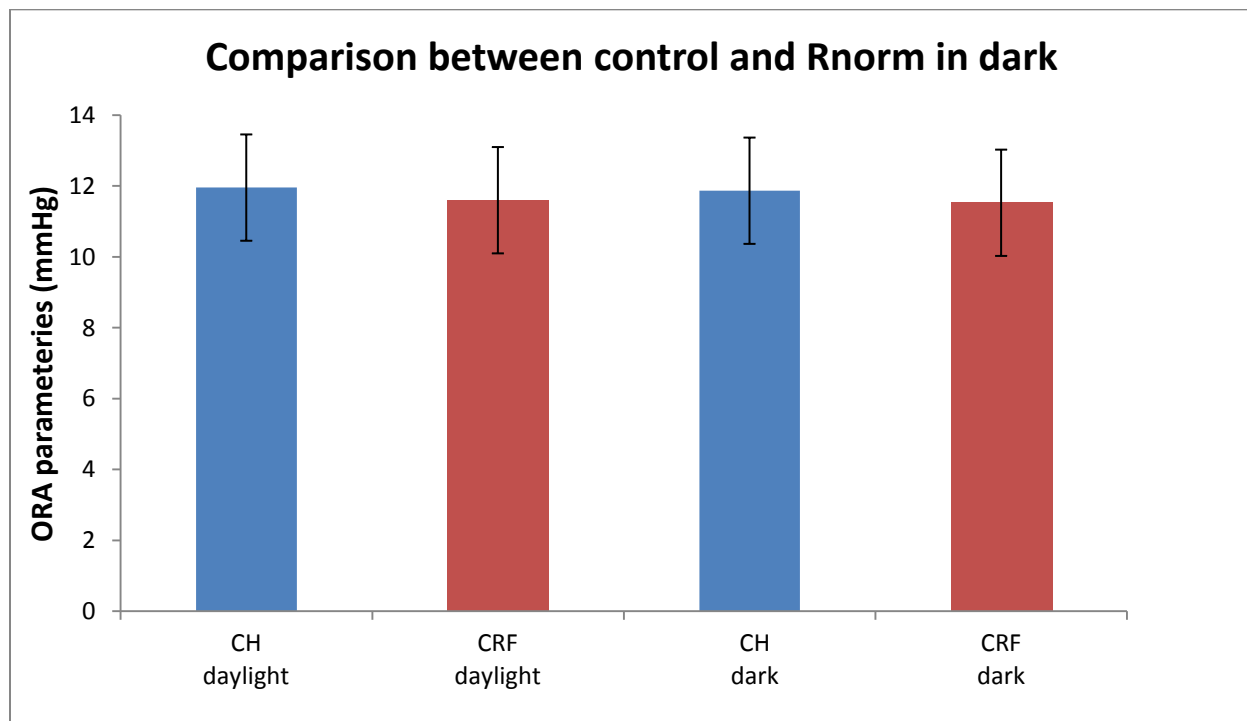


Figure 7.3a: The biomechanical properties of the cornea in dark (Rnorm)

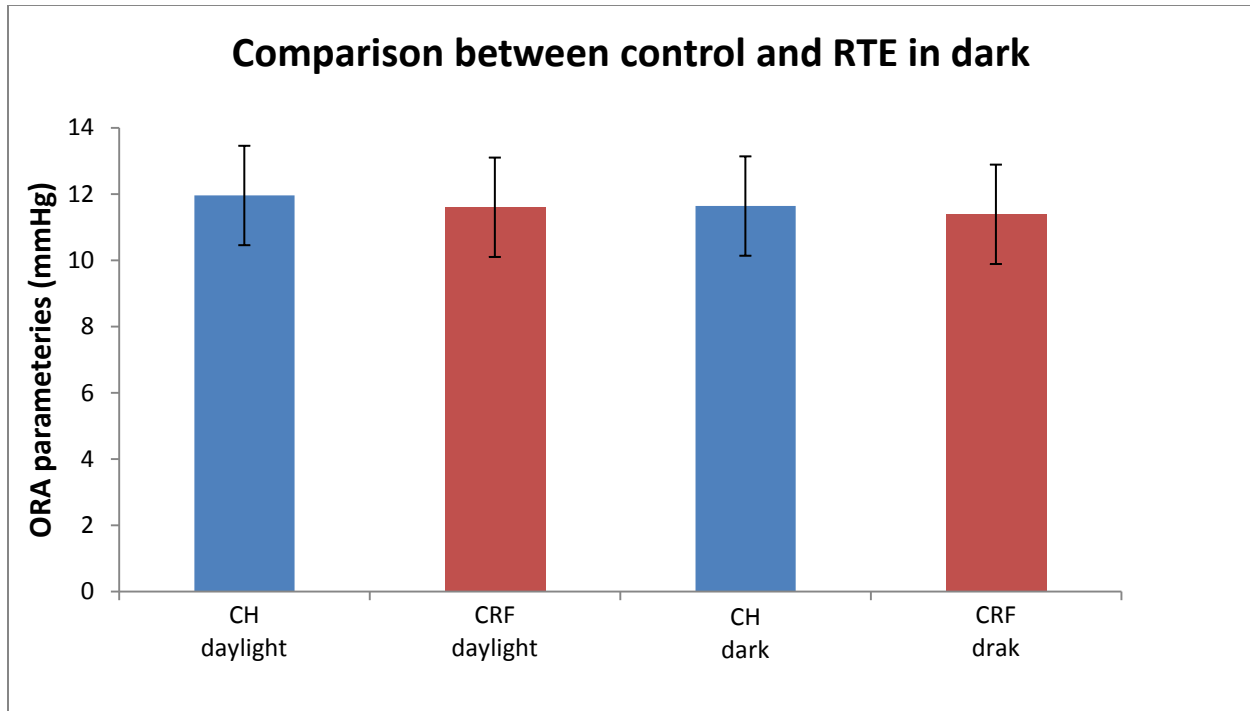


Figure 7.3b: The biomechanical properties of the cornea in dark (Rnorm)

As we found a statistically significant difference between Rnorm and RTE in the superficial grid group in terms of light transmission, we decided to compare the differences in their biomechanical properties. Firstly, these results confirm that there was not a significant difference between the removed epithelium group and the superficial grid group in CH and CRF values, whether using Rnorm or RTE. Secondly, our results show that both biomechanical properties for the superficial grid group with RTE were higher than those for the Rnorm group; however, it was not a significant difference ($p > 0.01$)(Figs. 7.4a, 7.4b); the mean CH was 10.54 ± 2.3 for Rnorm and 10.85 ± 1.6 for RTE, CRF was 9.88 ± 2.4 for Rnorm and 10.17 ± 1.8 for RTE.

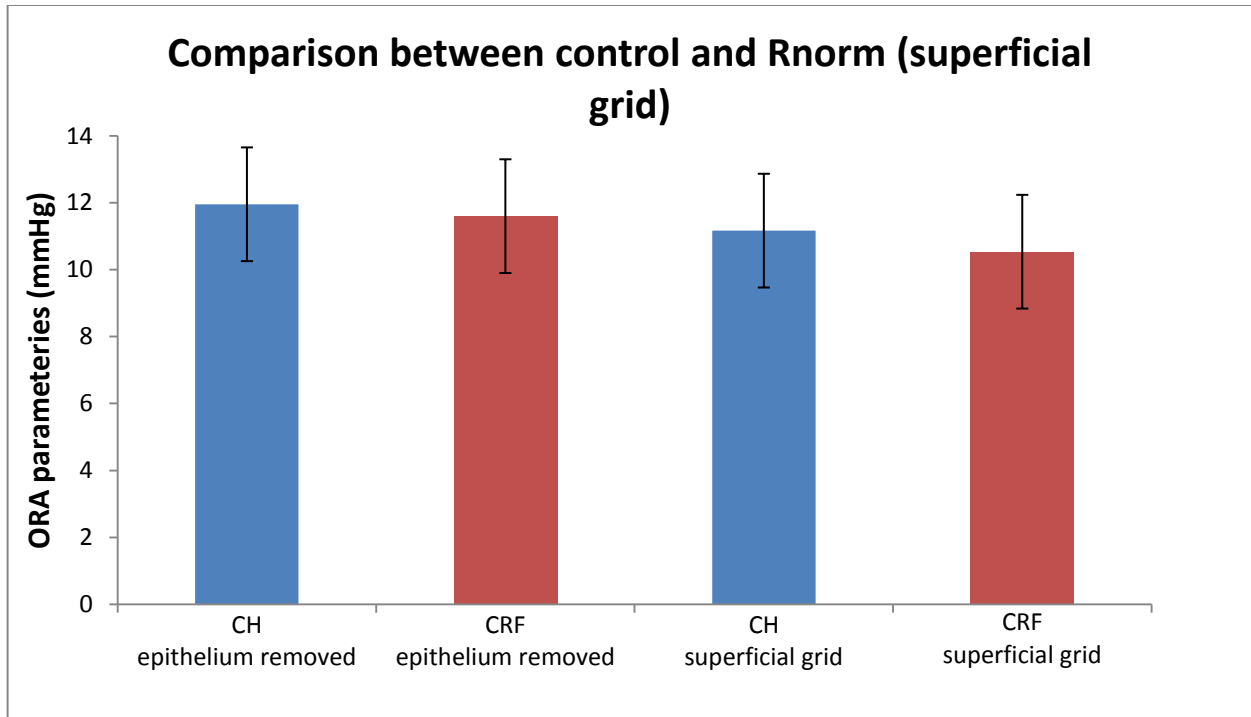


Figure 7.4a: The biomechanical properties of the cornea using Rnorm (superficial grid)

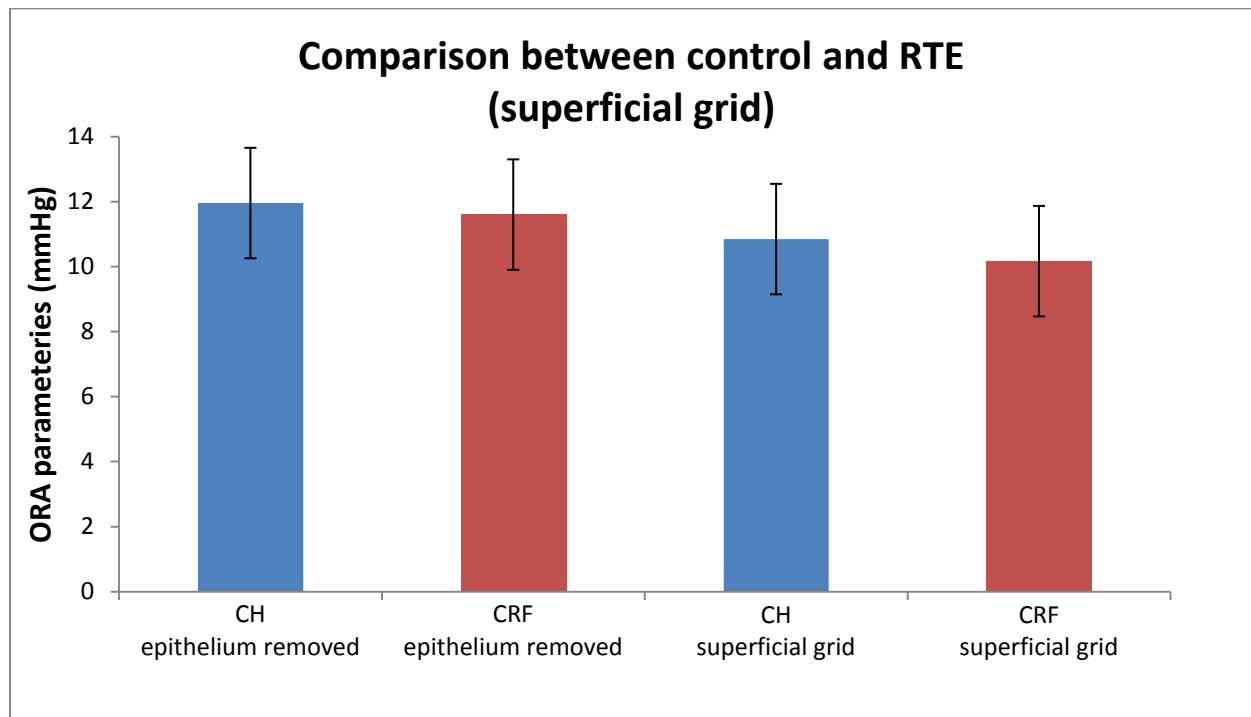


Figure 7.4b: The biomechanical properties of the cornea using RTE (superficial grid)

We showed in Chapter Four in this thesis that the reduction in the measured biomechanical properties of the cornea is caused by the decrease in central corneal thickness, which means that the flap itself does not affect measurements of CH and CRF. To confirm this, we measured the CH and CRF before and after creating a laser in situ keratomileusis-like flap. We found that there was no statistically significant change after making the flap (before: CH = 11.51 ± 1.4 , CRF = 11.12 ± 1.7 ; after CH = 11.32 ± 1.8 , CRF = 11.17 ± 1.5) (Fig. 7.5). However, there was clear noise and no clear/smooth signal which could be detected after creating the flap (Fig. 7.6a and 7.6b).

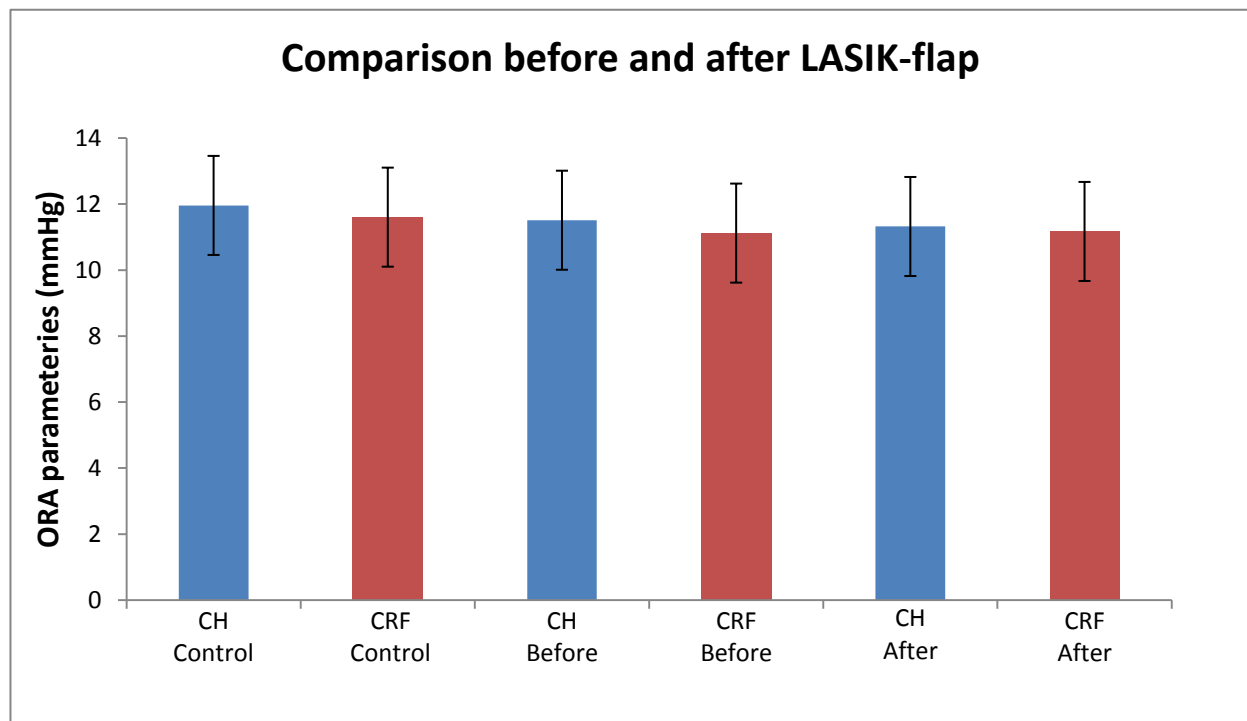


Figure 7.5: The biomechanical properties of the cornea before and after LASIK-flap

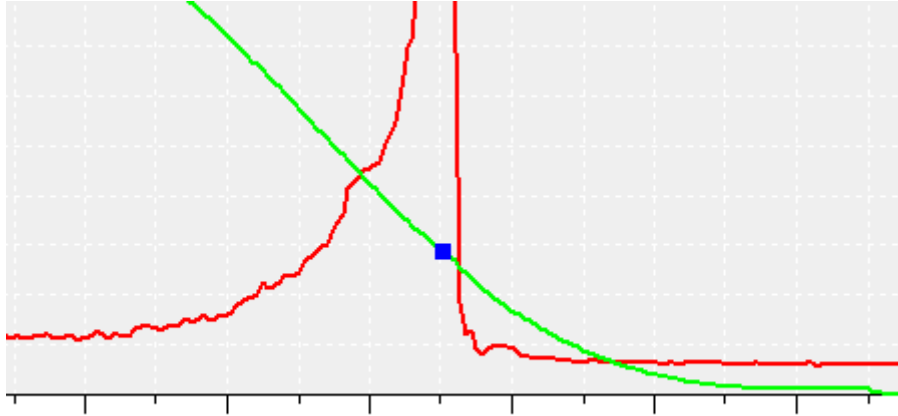


Figure 7.6a: Signal before LASIK-flap

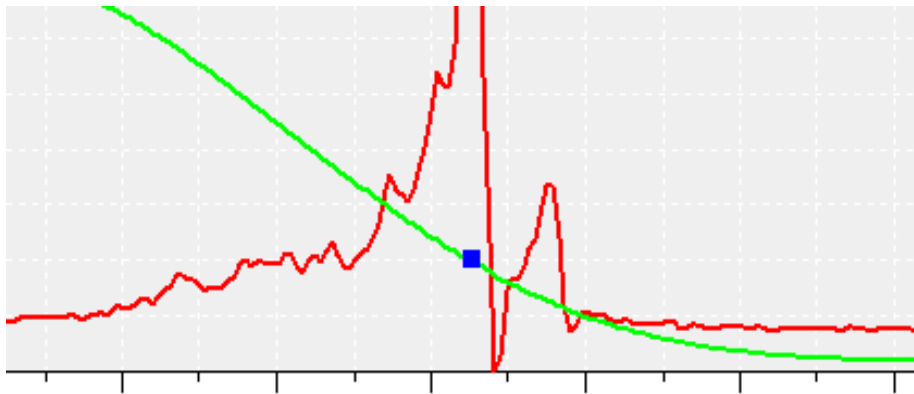


Figure 7.6b: Signal after LASIK-flap

Figure 7.7a and b shows a general comparison between all these groups and demonstrates that there were no statistically significant differences between them.

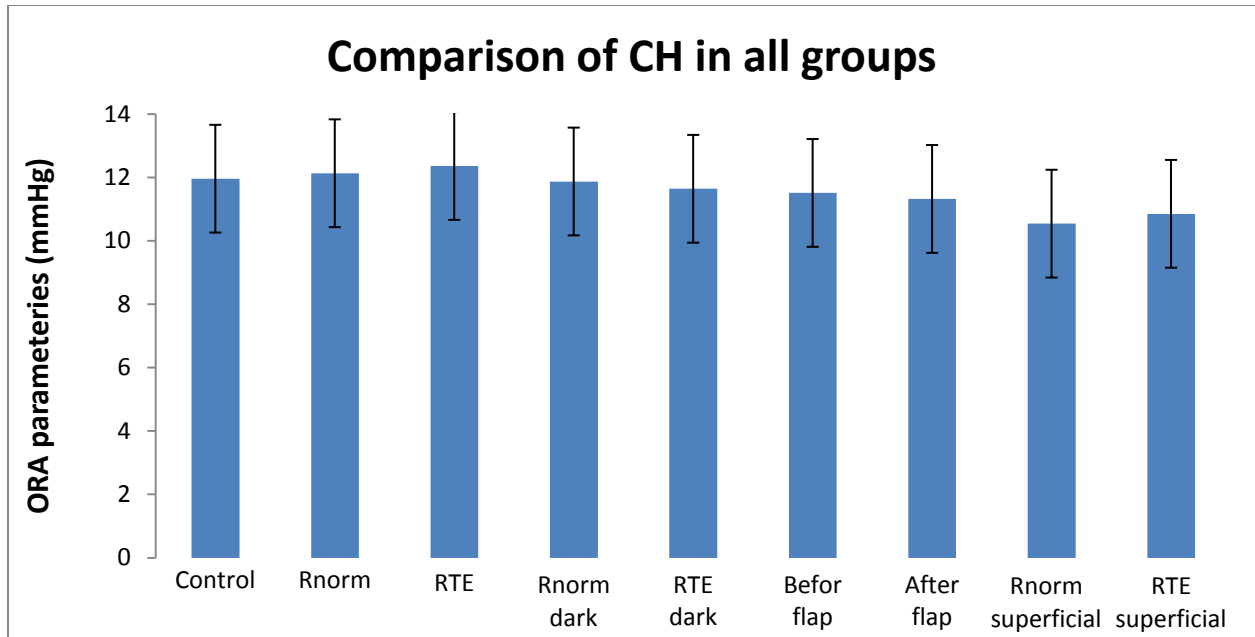


Figure 7.7a: CH of all groups

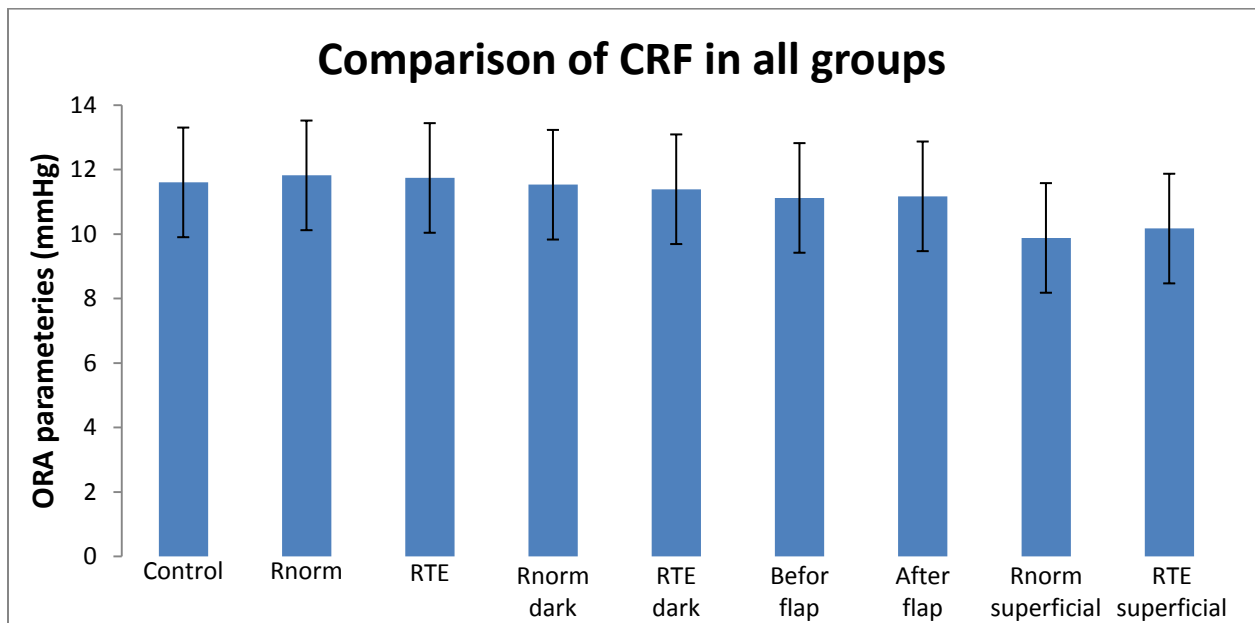


Figure 7.7b: CRF of all groups

7.4 Discussion

From their in vitro studies, Wollensak et al. (2003b) stated that the biomechanical properties of the cornea are affected after the crosslinking procedure and that the biomechanical rigidity of the cornea increases by 300% after irradiation. In another experiment (Wollensak et al. 2004), the researchers believed that the collagen fibrils' diameters increase after the procedure. However, these rigidity measurements were not made using the ORA but by using a tensometer (i.e. they were biomechanical stress-strain measurements); this is because Wollensak believed that ORA is not the appropriate machine for measuring corneal biomechanics because it measures corneal biomechanics in the sagittal direction only, unlike biomechanical stress-strain measurements which are taken in the tangential direction as well. In this respect maybe it is not surprising that neither CH nor CRF values showed any significant changes after cross-linking with Rnorm which is consistent with some previous reports by Goldich et al. (2009) and Spoerl et al. (2011). However, we went further in this investigation by cross-linking with RTE (besides Rnorm) and we showed, for the first time, that although there was a significant difference between Rnorm and RTE in the spectrum of light transmission, there was no significant difference in the biomechanics of the cornea as measured by the ORA (either after superficial grid or epithelial removal). However, clear noise appeared in the applanation signal after CXL, as can be seen in figure 7.2b and it may be this noise that reveals more about the effects of crosslinking than the actual values of CH and CRF.

The same procedure was performed on Groups 4 and 5, but in the dark to investigate if diurnal light affects the CXL procedure or not, as we know that riboflavin is

sensitive to light. The result showed a drop in the CH and CRF of the cornea in Groups 4 and 5 (in the dark); however, this was not a statistically significant reduction.

In Chapter 4 of this thesis we did a clinical study and one of our findings was that the reduction in CH and CRF values was due to the lower than normal thicknesses of the corneas after LASIK; therefore, we measured the biomechanical properties of the cornea before and after creating a laser in situ keratomileusis-like flap. Our results in this study are in agreement with our previous clinical study (in Chapter 4), which indicated no significant changes after creation a LASIK-flap, confirming that the reduction in the parameters after LASIK surgery is because of the reduction in corneal thickness. However, we found a clear difference in the ORA signal before and after the flap. This also indicates that the ORA signal seems to be more accurate than the CH and CRF values in some cases and should be taken into account when measuring the biomechanical properties of the cornea, especially in cases where the CH and CRF are not affected or do not change significantly.

Chapter 8: General Discussion and Concluding

Understanding the biomechanical properties of the cornea is a fundamental requirement for developing a compatible understanding of overall ocular behaviour, and response to some other factors caused by disease, before/after surgery. It is also essential for establishing accurate medical devices for ocular behaviour, which have several clinical and laboratory applications, including developing the accuracy of any anterior segment machine, the treatment of refractive errors including contact lens and refractive surgery, and follow-up after any ocular surgery.

The Ocular Response Analyzer (ORA) was produced by Reichert in 2005. This is a non-contact tonometer that produces new parameters (CH and CRF) and it is believed that these new parameters provide a measure of corneal biomechanics. However, the true meaning of these new parameters and their relationship with some more conventional biomechanical measures such as strength and rigidity is not yet fully understood.

This thesis, however, has examined the clinical and laboratory areas where corneal biomechanics, post-LASIK and corneal collagen cross-linking research are most needed. However, it must be clarified that while work to investigate these areas is needed, the biomechanical properties of the cornea cover a very large area which cannot be covered in one thesis. Nevertheless, in this thesis, I have tried to cover it as one coherent story which begins with general experimentation and moves on to more specific and specialized experiments at the end.

In this thesis I have shown for the first time that it is possible to obtain meaningful results using the ORA from whole enucleated eyes, and this opens up the possibility of using the ORA in controlled ex vivo studies, which will allow us to understand better the relationship between CH measurements and conventional biomechanical properties, such as stiffness and elasticity. Furthermore, I have shown that the ORA is designed to measure one biomechanical property of the cornea, but the differences between CH/CRF measurements from whole eyes in vivo and excised corneas in vitro suggest that the measurements depend on the presence of other parts of the eye. This was confirmed by making measurements on the same corneas before and after removal from the eye. Combining in vivo measurements with in vitro measurements has allowed me to compare the factors that influence the measurement of corneal hysteresis, the in vitro data allowing a much wider range of experimental conditions to be examined, and also allowing parameters to be adjusted within individual corneas. This showed clearly which factors influence CH measurements and which do not.

After finishing this comprehensive study I conducted my second experiment, in Chapter 4, which determined the relationship between corneal biomechanical properties, anterior/posterior corneal curvature and peripheral/central corneal thickness accompanying LASIK surgery. In this study I found, as expected, that the anterior radius of corneal curvature increased after LASIK; however, there was no statistically significant change in posterior corneal curvature. CH was significantly lower after LASIK, as was peripheral corneal thickness. For the first time, this study has shown that there is no significant correlation between CH and posterior corneal curvature in either normal or post-LASIK subjects; however, it has shown that there is significant

correlation between CH and peripheral corneal thickness in both normal and post-LASIK patients. This experiment suggests three important points:

- 1) LASIK flaps do not affect the posterior layers of the cornea and visco-elastic changes may be confined to the anterior layers.
- 2) The biomechanical properties of the tissue determined by the ORA do not change significantly after LASIK.
- 3) The responses in pre- and post-LASIK are similar.

This experiment provided more comprehensive knowledge about the factors that are related to changes in CH and shows some other factors that may influence the corneal structure and biomechanical properties of the cornea, before and after LASIK.

Before doing the last two series of experiments (which concentrated on corneal cross-linking), I did my third experiment, Chapter 5, an infra-red spectrometry study of corneas cross-linked with riboflavin/Ultraviolet A. In this study I tried for the first time to investigate the effect of corneal cross-linking at the molecular level. The final result of this chapter was good, but not significant. This was partly due to a number of experimental difficulties that were identified during each repeated attempt at this work. It was concluded that the main confounding factor is the reduction of thickness of the specimen needed to avoid saturation. For future work it was concluded that partial drying of the sections or using a species with a much thinner cornea will produce good results. Nevertheless, even from this preliminary work, it was clear that molecular crosslinking should be detectable by this technique.

After using normal riboflavin in Chapter 5, I investigated the use of a specially formulated Riboflavin solution, RICROLIN TE[®] (RTE); for Chapter 6. I employed spectrophotometry to measure Riboflavin stromal absorption indirectly in ex-vivo rabbit corneas and porcine corneas. I showed that RTE can penetrate the epithelium with only partial removal of epithelial cells and so may represent an improved and less painful method of corneal crosslinking.

In the final results chapter in this thesis, Chapter 7, I aimed to cover any questions that arose during our investigations and experiments in the previous chapters; however, a new aim was added. This was a study investigating the influence of corneal crosslinking with riboflavin and RTE on the biomechanical properties of the cornea. The results showed that crosslinking did not affect the measured values of CH and CRF whereas it is known to increase corneal rigidity. We concluded that the values of the two biomechanical parameters output from the ORA may not fully describe the biomechanical changes in the tissue, and other parameters, such as the shape and noise level in the ORA curves, may need to be considered.

Future work

The ORA is currently the only device that claims to measure corneal biomechanical parameters in vivo. However, our results have indicated that additional studies are needed to find more relations and associations between the corneal biomechanical parameters measured by different devices. This should be easier now that we have established the ability of the ORA to be used on excised eyes.

Some other factors need to be examined, these include the effect of swelling (following sleep or wearing contact lenses) on corneal biomechanical behaviour, changes in microstructure associated with the progression of diseases such as keratoconus, the effects of long-term exposure to high levels of UVA in cross-linking, the wound-healing process following surgery and whether stromal tissue is able to regain its mechanical integrity after wound healing and determining if this healing is affected by cross-linking procedures or not in different ways and devices. All these effects must be quantified experimentally and/or clinically before they can be accurately considered as fundamental factors.

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Publications and presentations

- Study of the relationship between Corneal Hysteresis and the Intra-ocular Pressure in the human eye. Speaking of Science, Cardiff University, UK, 2009. Talk.
- Study of the relationship between Corneal Hysteresis and the Intra-ocular Pressure in the human eye. Poster day at Cardiff University. UK, 2009. Poster.
- The relationship between the biomechanical properties of the cornea and IOP in vivo and vitro. Corneal conference, Cardiff University, UK, 2009. Poster.
- Factors that influence the measurement of corneal hysteresis and corneal resistant factor after LASIK surgery. The 4th Saudi International Conference. University of Manchester, UK, 2010. Talk.
- Comparison of factors that influence the measurement of corneal hysteresis and corneal resistant factor in vivo and in vitro. Saudi Ophthalmology Conference. King Khaled Eye Specialist Hospital, KSA, 2010. Talk.
- Comparison of factors that influence the measurement of corneal hysteresis in vitro and in normal/post-LASIK patients. The Association for Research in Vision and Ophthalmology (ARVO). Fort Lauderdale, USA, 2011. Poster.
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