Central and Peripheral Cornea and Corneal Epithelium Characterized Using Optical Coherence Tomography and Confocal Microscopy

By

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Both in the closed and open eye state the superior limbus is covered by the upper lid. This region is of physiological interest and clinical importance because in chronic hypoxia, neovascularization of the cornea commonly occurs here. The limbal region in general is additionally of importance as the stem cells which are the source of the new corneal cells are located in the epithelium of the limbus and these are vital for normal functioning and are affected under certain adverse conditions. **Purpose:** In this experiment I examined corneal morphology in the limbal area and in particular under the upper lid in order to primarily examine the variation in the corneal limbal epithelial and total thickness as well as epithelial and endothelial cell density. **Methods:** I measured 30 eyes OD/OS (chosen randomly) of thirty healthy subjects aged from 18 to 55 years in the first study and twelve participants in the second study, with refractive error $\leq \pm 4$ D and astigmatism ≤ 2 D. The thickness and cell density of five positions: superior, inferior, temporal, nasal limbal and central cornea was determined with optical coherence tomography (OCT) and confocal microscopy. At least three scans of each position were taken in both studies with OCT. At least 40 of 100 adjacent sagittal scans of each image were measured using OCT software program. In the confocal study, image J software was used to determine cell densities. **Results:** The epithelial and corneal limbal thickness were significantly thicker than the epithelial and central corneal thickness ($p<0.05$). The limbal, inferior cornea is thinner than the three other positions and the temporal region of the cornea is the thickest both in epithelial and total cornea. Epithelial cell density was significantly lower in the superior cornea than the four other positions. There was no significant difference in the endothelial cell density. **Conclusions:** Using OCT with high resolution and cross-sectional imaging capability and confocal microscope with high magnification, I found that the limbal

cornea is significantly thicker than the central cornea both in total and in epithelial thickness. In the limbus, one might expect the superior cornea (under the lid) to be thickest (because of the expected hypoxia) whereas I found the temporal cornea was thickest. The epithelial cell density was lower in the superior cornea but there was no significant difference in cell densities in the endothelium. Further morphological investigation is of interest.

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I would like to begin by acknowledging the contribution of those individuals without whom this accomplishment would not have been possible.

I sincerely thank Dr Trefford Simpson for having me as a student, supporting me and guiding me through out my graduate study. I would also like to acknowledge the contribution of Dr Hovis who has been very helpful and generous and Dr Sivak who goes out of his way to help the students and facilitate their learning experience.

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I profoundly thank my family, friends, and well wishers without whom this would not have been entirely possible.

Dedication

I dedicate this work to my family, for their love and support.

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Chapter One

1.0 Introduction

1.1 Background

The cornea is the anterior optical system of the eye and consists of five layers: epithelium, Bowman's layer, stroma, Desment's membrane and endothelium. The transparency of the cornea results from its unique structure and constant control of its hydration. The thickness of the cornea, cell size and density and kind of the protein of the cornea are some of the parameters that vary in different regions of the cornea (Azen, Burg, Smith, & Maguen, 1979; Giasson & Forthomme, 1992). These differences are significant between central and limbal cornea. A variety of factors influence corneal metabolism. These in turn, may induce corneal thickness changes and affect corneal transparency. Corneal metabolism is dependent on oxygen derived from the atmosphere, with minor amounts supplied by the aqueous humor and limbal vasculature (Benjamin & Ruben, 1995). During sleep or under closed-eye conditions, oxygen is delivered to the cornea via the highly vascularized superior palpebral conjunctiva (Benjamin & Ruben, 1995). In open eye primary position, the upper eyelid covers about two millimetres of the superior cornea and is a partial oxygen uptake barrier in this part of the cornea. Studies show that oxygen uptake in the superior cornea is significantly higher, compared to other parts of the cornea immediately after retraction of the upper eyelid (Fitzgerald & Efron, 1986). When the entire cornea is exposed to a uniform oxygen environment, by retracting the upper lid for five minutes, the difference in oxygen uptake is reversed or eliminated (Benjamin & Ruben, 1995). Because about two millimetres of the superior cornea is covered by

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the upper eyelid during waking hours, we expect more difference between this region and other parts of the cornea. Tear evaporation and oxygen uptake (Benjamin & Hill, 1988; Benjamin & Rasmussen, 1988; Benjamin & Ruben, 1995; Erickson, Comstock, & Zantos, 2002) of the superior cornea may be affected due to the covering of the eyelid. This in turn affect corneal metabolism and we may expect a compensation mechanism. Studies have shown other differences between superior limbal cornea and other limbal positions as well as central cornea, for example: the distribution of specific keratins within the superior and inferior compare to lateral and central corneal regions (Wiley, SundarRaj, Sun, & Thoft, 1991), the size of the stem cells (Romano et al., 2003), corneal thickness (Remon, Cristobal, Castillo, Palomar, Palomar, & Perez, 1992a; Liu, Huang, & Pflugfelder, 1999), the stromal keratocyte density (Moller-Pedersen & Ehlers, 1995), the collagen fibril spacing and diameter (Boote, Dennis, Newton, Puri, & Meek, 2003), endothelial cell density (Amann, Holley, Lee, & Edelhauser, 2003a). However our knowledge about corneal structure variation, particularly under the lid, is very limited.

1.2 Study instruments

1.2.1 Optical coherence tomography (OCT)

There are a variety of morphometric methods that have been used for in vivo examination of human cornea, including optical pachometry, high-frequency ultrasound and optical coherence tomography (OCT).

Optical pachymetry has the disadvantage of being limited to situations where the media are optically transparent (Reinstein, Silverman, Trokel, & Coleman, 1994). Lower reproducibility is another reported disadvantage of the method (Azen et al.,

1979; Giasson & Forthomme, 1992). Most reports show a variation of reproducibility between 5.6 and 19μm (Azen et al., 1979; Giasson & Forthomme, 1992).

High-frequency ultrasound is not limited to optically clear media which is an advantage but a drawback is the contact mode of the method and need for topical anaesthesia (Reinstein, Silverman, Rondeau, & Coleman, 1994).

Optical coherence tomography (OCT) is a recent technique shown to be useful in studying human corneal structure (Feng & Simpson, 2005). It is a non-invasive, non-contact imaging modality with spatial resolution superior to that of conventional clinical imaging techniques (Hee et al., 1995; Puliafito et al., 1995). OCT is an emerging imaging technique that has the potential to be used in a wide range of applications. This technology uses infrared light to generate cross-sectional images of tissue on a micrometer scale (Huang et al., 1991). The nominal resolution is from 4 to 10 μm and the reproducibility of measuring corneal thickness is about 4.3 μm (Maldonado et al., 2000; Sin & Simpson, 2006).

The different layers of the cornea have different refractive indices. When directing coherent light to the cornea, the light will be reflected from various interfaces. By measuring the magnitude of backscattered light, the thickness of the structure can be obtained. Measuring the distance between peaks defines the thickness of layers.

In my study a Humphrey-Zeiss Optical Coherence Tomographer was used to capture corneal images with nominal resolution of from 10 to 20 μm (Figure 1-1).

Figure 1-1 OCT, Optical coherence tomography

1.2.2 Confocal microscopy

Confocal microscopy, invented in 1957 by Marvin Minisky (Bohnke & Masters, 1999; Masters & Bohnke, 2002), is a major development in instruments used to evaluate the structure of the eye. The slit lamp (biomicroscope) which currently is used in clinical examination provides us with reasonable magnification of the anterior segment of the eye, enabling us to diagnosis disease. But it is not possible to evaluate corneal structure at the cellular level using a slit lamp. The confocal microscope with higher magnification and lower scattering of light from outside the focal plane provides the ability to image thin layers of the cornea. However, since magnification is the inverse of the field of view, optical resolution is increased at the expense of the

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field of view reduction. The confocal microscope can produce gray-scale images of the different layers of the cornea at the cellular level *in vivo* or *in vitro*. It is a technique in which the tissue is illuminated with a focused spot of light. Putting a pinhole in front of the detector, allows only the light from an in –focus plane to pass and the scattered light from an out of focus plane is removed (Figure 1-2).
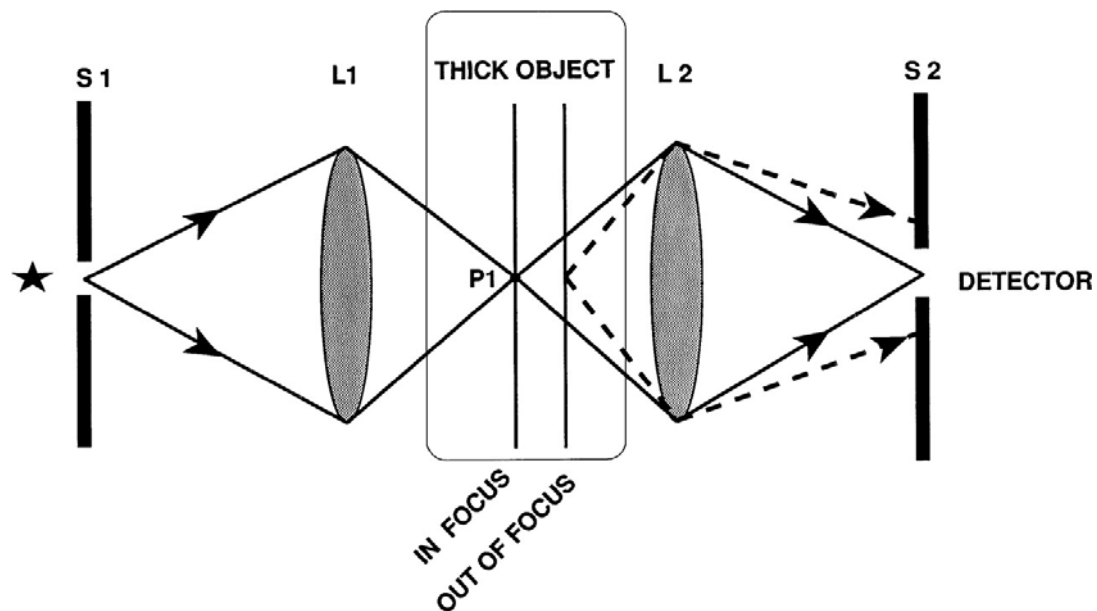
Figure 1-2 Schematic diagram illustrating the principle of a confocal microscope (reproduce with permission from Elsevier Limited)

S1 and S2 are confocal apertures. L1 and L2 are focusing lens for illumination and detection, respectively (see text). P1 is the focal volume that is illuminated with the point source of light from S1 and focused at P1 with lens L1. P1 is imaged by lens L2 to form an image at the aperture S2. (Bohnke & Masters, 1999; Masters & Bohnke, 2002).

The pinhole is put in front of the detector in such a way that it is conjugate with the focal plane and coincident with the pinhole enabling imaging in high resolution.

ConfoScan3, a fully digital confocal microscope, was used in this study (Figure 1-3). It is a type of slit confocal microscope and consists of one illuminated slit that projects light in to the cornea and a second slit that cuts off unwanted light from unfocused layers. The first slit is illuminated by a 100W / 12 V halogen lamp. The objective lens, a 40x / 0.75, is a water immersion lens which should be used with a gel while scanning. It provides a magnification of 500x and a field of view of [450-460] μm x [335-345] μm with a lateral resolution of 1 μm. Image scanning is very fast and a single scan consisting of 350 images can be captured in less than one minute (ConfoScan3: Operator's Manual rev5 020320. www.nidektechnologies.it).

Figure 1-3 ConfoScan3

1.3 Purpose of the study

➢ To determine limbal epithelial and corneal thickness in the superior region of the cornea (under the upper lid) and compare these to the inferior, temporal, nasal and central cornea.

➢ To investigate epithelial and endothelial cell density of the superior cornea and compare these to the inferior, temporal, nasal and central cornea.

Chapter two

2.0 Literature overview

2.1 Anatomy of the cornea

The human cornea is an avascular, clear transparent tissue that provides the eye with a clear refractive interface, tensile strength and protection from external factors.

2.1.1 Physical properties of the cornea

The horizontal diameter of cornea is slightly longer than the vertical dimension. The horizontal diameter is about 12.6 mm and the vertical diameter is 11.7 mm. The anterior surface of the cornea provides approximately 48 dioptres of the total refractive power of the eye. The central third of the cornea, called the optical zone, is generally spherical and the radius of curvature is about 7.8 mm. The peripheral portion of the cornea becomes more flat and thicker (0.65 mm) than the central portion (0.52 mm) (Pepose & Ubels, 1992).

2.1.2 Corneal transparency

The transparency of the cornea depends on the arrangement of the epithelial cells, physical arrangement of collagen fibrils which regularly and tightly packed together, not having blood vessels, and the mechanism that protects it from swelling

The tear film provides a clear refractive interface for the cornea. The cornea transmits radiation from approximately 310 nm in the ultraviolet to 2500 nm in the infrared and is most sensitive to ultraviolet radiation damage at 270 nm （Edelhauser and Ubels, 2002).

2.1.3 Corneal layers

The cornea consists of five layers: the epithelium, Bowman's membrane, stroma, Desment's membrane and endothelium.

2.1.4 Epithelium

The epithelium is the outermost layer of the cornea, five to seven cells thick and about 50-70 μm thick. It consists of three types of cells: Superficial cells that are outermost cells, wing cells which lie beneath the superficial cells, and basal cells that are innermost cells in which mitosis occurs. The basal cells are arranged in a single layer on the basal membrane. The next three layers are composed of wing cells which become flatter in the layers towards the surface. The upper-most layer, the superficial cells, consists of large, flattened surface cells. The different cell layers represent different stages of cell development. The basal cells are the only source of new cells within the corneal epithelium so they have more mitochondria and glycogen. The basal cells are pushed towards the surface and are replaced by new cells. As they move upward, they gradually flatten and become desquamated. Finally, they degenerate and are sloughed from the corneal surface. This results in turnover approximately every seven days. Although in this process a portion of these cells are dying and will be sloughed, these superficial cells form an essential barrier. The wing cells form two to three layers of cells beneath the superficial cells. The basal cells are single layered and the innermost layer of the epithelium rests on a basement membrane. The epithelium cells are joined together by numerous desmosomes. The entire epithelium adheres to the basement membrane and stroma by the hemidesmosomes found in the basal layer. Hemidesmosomes are linked to anchoring

fibrils that pass through Bowmane's layer into the stroma. The arrangement of cells in the epithelium is extremely uniform (Edelhauser and Ubels, 2002).

2.1.5 Bowman's layer

Bowman's layer is a modified superficial layer of the stroma, which lies beneath the basement membrane. The basal cells of the epithelium rest on a basement membrane about 10 to 15 μm thick (Edelhauser and Ubels, 2002).

2.1.6 Stroma

The stroma comprises 90% of the total corneal thickness. The stroma is an extra cellular matrix consisting of proteoglycans and a lamellar arrangement of collagen fibrils running parallel to the cornea surface. The collagen fibrils and extra cellular matrix are maintained by flattened cells called keratocytes lying between the collagen lamellae.

The keratocytes maintain, renew and repair the stromal fibrils. Each lamella is laid on top of another one and each runs in a different direction to its neighbour. Such lamella arrangement forms tight junctions which gives the cornea great strength and resilience. The collagen fibrils have a mean diameter of between 22.5 and 32nm, thickening toward the periphery. The 300 or more collagen lamellae extend from limbus to limbus and may wrap around the circumference of the cornea or fuse together to form larger fibrils. The fibrils of the lamellea have high tensile strength and are embedded in a ground substance, which keeps them apart and prevents their compression. This arrangement allows the transfer of substances through tissue for adequate nutrition (Edelhauser and Ubels, 2002).

2.1.7 Descemet's membrane

The corneal endothelium rests on a basement membrane, known as Descemet's membrane, which in the adult eye is 10 to 15 um thick. This membrane is secreted by the endothelial cells and increases in thickness throughout life (Edelhauser and Ubels, 2002).

2.1.8 Endothelium

The posterior surface of the cornea is covered by a single layer of cells known as the endothelium. The cells are about 20nm in diameter and 4 to 6 nm thick. In this monolayer cells are hexagonal in shape and extremely regular in size. At birth, endothelial cell density is about 4000 cells/mm^2, but because these cells are amitotic the density decreases throughout life to about 2000 cells/mm^2 in the eighth to ninth decades of life. It is still well above the minimum level of 400 to 700 cells/mm^2 required for maintenance of normal corneal function, indicating that adequate cells are available for a lifetime of well over 100 years. In contrast to the epithelium, endothelial cells are not replaced as a normal process during adult life. They stop dividing in the late teens and the population of cells present at this time must last the lifetime. When a cell is lost from the matrix, others around the space grow in size to fill the hole resulting in a structural change. As this process continues, the cells become less regular in shape and more variable in size. This is called polymegethism (Edelhauser and Ubels, 2002).

2.2 Stromal and endothelial physiology

Water evaporates from the corneal surface. Evaporation accounts for a 5% thinning of the cornea during the day. The stroma tends to absorb water because of the water

binding capacity of the proteoglycans in the extra cellular matrix. Two factors contribute to the prevention of stromal swelling and the maintenance of its water content at 78%. These are the barrier and pump functions of the endothelium. The barrier is incomplete compared to the epithelial barrier. Fluid moves into the stroma from the aqueous humer and this is a vital source of nutrients including glucose and amino acids for the cornea. Pump functions of the endothelium remove fluid from the stroma. The activity of Na/K ATPase is vital to maintenance of normal corneal hydration. Inhibition of the pump stops sodium transport causes corneal swelling. Bicarbonate is also essential to the maintenance of corneal thickness. Removal of bicarbonate from the solution results in corneal swelling (Edelhauser and Ubels, 2002).

Early study of corneal hydration showed that the cornea swells when cooled and returns to the normal thickness at its normal temperature. This is called the corneal fluid pump. A pH range of to 6.8 to 8.2 must be maintained to prevent corneal edema. An intact endothelial barrier requires the presence of calcium, because this ion is involved in the maintenance of junctional complexes. Loss of inter cellular junctions not only disrupts the barrier, it also causes a loss of pump polarity so that the cells can no longer transport ions and set up the osmotic and ionic gradients required for movement of fluid into the aqueous humor. Corneal metabolism is dependent on oxygen derived from the atmosphere, with minor amounts supplied by the aqueous humor and limbal vasculature. The amount of oxygen in the aqueous humor is low (around 40 mmHg) in comparison to tears (155 mm Hg). During sleep or under closed-eye conditions, oxygen is delivered to the cornea via the highly vascularized peripheral conjunctivae. The corneal epithelium consumes oxygen ten times faster than the stroma (Freeman, 1972).

2.3 Limbus

The limbus, approximately 1 mm in width, is the transition zone where the cornea meets with the conjunctiva and the sclera. The limbus structure differs from the cornea. The cornea receives parts of its nutrients from limbal region and its function depends on this region which contains blood vessels and lymphatics (Edelhauser and Ubels, 2002).

The limbus is also important because the trabecular meshwork lies in the corneoscleral arm of the anterior chamber. The trabecular meshwork is an approximately triangular structure. Anteriorly its apex at the corneal periphery, bounded by Schawalbe's ring, lies between the endothelium and the deeper stroma of the cornea (Michael, 2001 and Buskrik 1997).

2.4 Stem cells

Stem cells (SCs) and are responsible for regeneration of cell populations even in adult tissues such as blood, brain, heart, muscle, skin and cornea. SCs have the ability to regenerate and provide the therapeutic potential to the tissue for treating disease and damage (Hall & Watt, 1989; Potten & Loeffler, 1990).

The concept that the limbal cornea may be involved in the renewal of the cornea first was suggested by Davanger and Evensen (Davanger & Evensen, 1971) when they observed that pigmented cells migrate from the limbus toward the central cornea. It needs to be mentioned that melanin pigmentation protects the corneal limbus which is a highly vascularized, innervated region from potential damage by UV-light. The stem cells lie in a specific region known as the ST niche, forming the limbal "palisades of Vogt" (Lavker, Tseng, & Sun, 2004; Schermer, Galvin, & Sun, 1986; Schlotzer-Schrehardt & Kruse, 2005). It is believed that SCs have the ability to divide

symmetrically or asymmetrically to produce new cells. During asymmetrical division one daughter SC and one transit amplifying cells (TA) are produced (Bentley et al., 2007). It seems that TA cells form differentiated cells (TD) which have no prolifrative capacity and are sloughed from the corneal surface (figure 2-1).

"The stem cells (red) are the basal cell layer located at the limbus; these stem cells proliferate and produce transit amplifying cells (blue) which migrate away from the limbus onto the cornea."(Bentley et al., 2007)
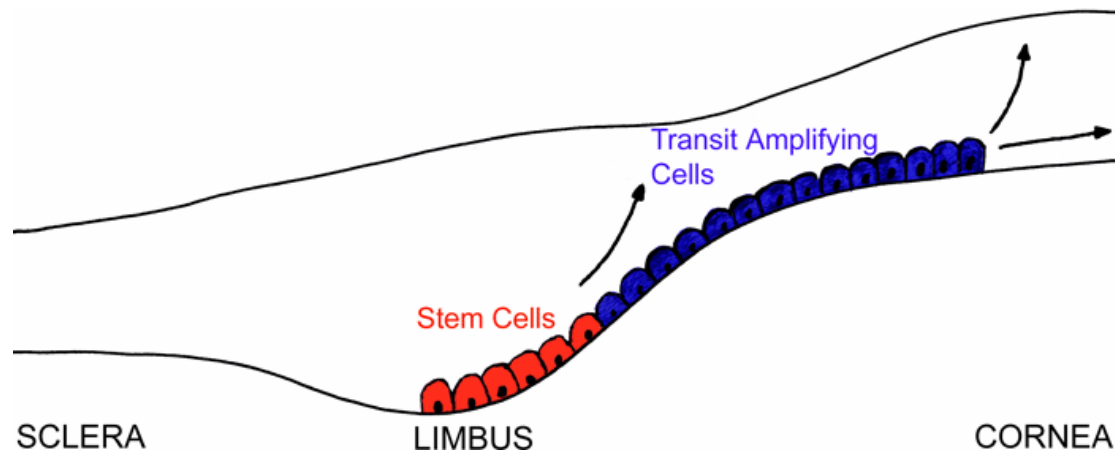
Figure 2-1 "Schematic diagram the corneal epithelium showing the location of the SC and TA cell populations." (Bentley et al., 2007)

Bentley, Mol Vis 2007; 13:237-242 <**http://www.molvis.org/molvis/v13/a27/**>

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2.5 Corneal thickness

2.5.1 Central corneal thickness Correlation with gender and refractive error

Using different instruments, studies showed no correlation between central corneal thickness with gender and refractive error. In a study by Rosa (Rosa et al., 2007), using Orbscan and Pentacam, central corneal thickness was measured in 51 subjects with a refraction ranging from -17.00 to +5.50 diopters. There was no correlation between central corneal thickness and refractive error. The same result was obtained by Pedersen (Pedersen, Hjortdal, & Ehlers, 2005) in a study in which 48 high mypoes (more than -6.00) were evaluated as well as 57 emmetropes using a Low-coherence reflectometry pachymeter. No relationship between central corneal thickness and gender and refractive error was found by Altinok (Altinok et al., 2007) as well.

2.5.2 Corneal thickness evaluation

Studies have been done to demonstrate the agreement in central corneal thickness measurements between different instruments. Some of these measurements are summarized in Table 2-1.

Table 2-1 Central corneal measurements using different instruments

Study	Instrument	Results Thickness in μm
(Hashemi & Mehravaran, 2007)	Ultrasound pachymetry	555.0 ± 30
	Pentacan	548.0 ± 32
	Orbscan II	580.0 ± 40
(Li et al., 2007)	Ultrasonic pachymetry	553.5 ± 30.26
	Orbscan II	553.22 ± 25.47
	ASOCT	538.79 ± 26.22
(Basmak, Sahin, & Yildirim, 2006)	Orbscan II	580.39 ± 37.0 (before acoustic correction factor)
	Ultrasound pachymetry	562.95 ± 32.0
	Orbscan II	533.96 ± 34.0 (after acoustic correction factor)
(Chaidaroon, 2003)	Ultrasound pachymetry	554.4 ± 27.50
	Optical pachymetry	581.1 ± 22.62
(Netto, Malta, Barros, Giovedi Filho, & Alves, 2005)	Orbscan II	534.81 ± 34.45
	Ultrasound pachymetry	535.00 ± 29.53

The results show that corneal thickness measurements are influenced by the method of measurement.

2.6 Hypoxia

The cornea needs energy for metabolic function to maintain its transparency and dehydration, and oxygen is one of the most important elements. The epithelium and endothelium provide the most oxygen consumed by the cornea through the aqueous humor (by endothelium) and the capillaries at the limbus, as well as oxygen dissolved in tear film (by epithelium).

Although the corneal stroma is a markedly hydrophilic tissue, the epithelium and endothelium help the cornea keep its water content at a steady level of about 75% to 80% of its weight by acting as barriers and metabolic pumps (Edelhauser and Ubels, 2002). As the metabolic fluid pump, the endothelium acts thirty times more effectively than the epithelium. Also epithelium permeability is seven times less to water and four hundred times less to ions than the endothelium permeability to these materials (Edelhauser and Ubels, 2002).

Hypoxia is one of the factors that can affect the function of the epithelium and endothelium, causing corneal thickness and morphologic changes. Under this condition, which induces anaerobic metabolism, lactic acid is produced (Edelhauser and Ubels, 2002). The hypothesis that an increase in stromal lactate concentration exceeds osmotic load and dilution on NaCl leads to stromal edema was suggested by Klyce (Klyce, 1981). Later, the osmotic theory was supported by Huff (Huff, 1990a; Huff, 1990b).

In 1955, Smelser & Chen proposed that lactate accumulation due to hypoxia would inhibit corneal dehydrating function (Smelser & Chen, 1955). But another study which modeled this situation with isolated rabbit corneas suggested that solute permeability might be increased instead of epithelial hydraulic conductivity changes due to hypoxia (Wilson, Fatt, & Freeman, 1973). Thoft & Friend expressed that

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"clinical epithelial edema results from the inability of anaerobic glycolysis to keep pace with epithelial ATP requirements" (Thoft & Friend, 1975).

It seems that both experimental and theoretical data obtained by Klyce provided firm support for the osmotic theory consequence of stromal lactate accumulation as the important factor involved in stromal edema due to hypoxia (Klyce, 1981).

Endothelial pH changes can be considered as another possible cause of corneal swelling under the hypoxic condition. Corneal acidosis that lowers endothelial pH, can affect fluid pumping, causing corneal hydration control reduction (Cohen, Polse, Brand, & Bonanno, 1992). Based on the assumption by Hodson & Miller that the endothelial fluid pump is based on bicarbonate carbon (Hodson & Miller, 1976), Klyce suggest that metabolic acidosis could have an effect on endothelial function (Klyce, 1981). In another study by Bonanno, it has been shown the high amounts of carbon dioxide induce significant acidosis in the epithelial, stromal and endothelial cornea as well as in the aqueous humour (Bonanno, 1996).

Baum postulated that "The limbal stem cells under the upper eyelid are subjected to continuous hypoxic stress and are at special risk to other insults" (Baum, 1997).

Hence it has been difficult to evaluate human corneal function accurately and the exact causes of corneal swelling due to hypoxia still are unclear (Polse, Brand, Cohen, & Guillon, 1990). But it appears that corneal swelling depends on a metabolic mechanism.

2.6.1 Hypoxia effect on the stem cells (SCs)

The effect of hypoxia was evaluated in the bone marrow cell liquid (Cipolleschi, Dello Sbarba, & Olivotto, 1993), neural cells of the brain (Studer et al., 2000), and epidermal keratinocytes (Kino-oka, Agatahama, Haga, Inoie, & Taya, 2005; Ngo, Sinitsyna, Qin, & Rice, 2007).

Cipolleschi, suggested that in the anaerobic condition, the lack of active mitochondria would reduce stem cells sensitivity to differentiation stage. He reported highly concentrated cells in low oxygen areas of the niches.

In the study on the neural cells of the brain, increased proliferation, reduced cell death of the stem cells as well as increasing clonal densities was reported (Studer et al., 2000).

Recently Miyashita and et al. performed an experiment to demonstrate the effect of hypoxia on the human corneal limbus *in vitro*. They showed an increase in cell proliferation rate and cell density per colony during hypoxia. Also the hypothesis that differentiation of the stem cells decrease in lower oxygen condition was supported (Miyashita et al., 2007).

2.7 Eyelid

The human eyelid is a thin fold of skin and muscle that covers and protects the anterior part of the eye from injury and excess light. It functions effectively in moisturizing the cornea, and in drainage and distribution of tears. The upper lid is responsible for blinking which has a pumping effect on the lachrymal sac and rectories the preocular tear film at each blink. The upper eyelid, which is the most mobile and is raised in vertical direction by the levator palpebral muscle, overlaps about two mm of the superior cornea in the primary position of gaze. In contrast, the lower eyelid margin lies just below the cornea at the inferior limbus.

The eyelid is divided into anterior and posterior lamellae. The anterior lamella consists of muscle, skin and associated glands and the posterior lamella consists of the tarsal plate, associated glands and conjunctiva. The meibomian glands, which lie within the tarsal plates in the upper and lower eyelids, secrete the lipid part of the tear

films. The lipid is a thin anterior tear layer that limits tear evaporation. The eyelid is supplied by lateral and medial palpebral arteries which are branches of the ophthalmic and lacrimal artery.

The conjunctiva is a thin, transparent mucous membrane that covers the sclera and lines inside of the eyelids. It is continuous with the corneal epithelium (Edelhauser and Ubels, 2002). The conjunctiva can be divided into three parts:

1- Palpebral or tarsal conjunctiva: The palpebral conjunctiva which lines in the eyelid is thin, adherent and highly vascular and contains immune cells.

2- Fornix conjunctiva: Where the inner part of the eyelids and the eyeball meet, the palpebral portions at the superior fornix and inferior fornix forms the conjunctival fornix.

3- Bulbar or ocular conjunctiva: The bulbar conjunctiva which covers the surface of the globe is attached to subjacent structures by areolar tissue (fibrous connective tissue with the fibers arranged in a mesh or net) and moves with the globe.

The conjunctiva produces mucus for the tear film and protects the ocular surface from infection. Hence it serves two purposes, as a physical barrier and as a source of the immune cells (Edelhauser and Ubels, 2002).

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2.8 Scope of the study

Considering what has been mentioned above, understanding the structure of the cornea is of particular importance because it is easily altered when function changes. For example, because the cornea is oxygenated from the atmosphere, when the eye is closed it blocks the availability of oxygen and the cornea swells. Therefore, something as simple as corneal thickness is an index of state of swelling of the cornea. Under the eye lids, particularly the upper lid, the available oxygen is also blocked and

corneal structure should be affected. However we do not know if this is the case and there is limited experimental evidence of how the corneal structure is influenced by being chronically covered by the lids. The experiments described here address this gap in our understanding by using imaging techniques that allow high resolution examination of corneal structure. Corneal and corneal epithelial thickness and other morphometric characteristics of the epithelium and endothelium will be measured.

Chapter Three

3.0 Methods and Materials

3.1 Calibration of the OCT

Thirteen rigid contact lenses which were mounted in a plastic wheel in a random manner with the refractive index of 1.376 (same as the cornea (Pepose & Ubels, 1992)) were used to calibrate the OCT. The power of all lenses was plano with a base curve of 8.6 mm and no prism. Central thicknesses of the lenses were measured using a mechanical gauge and using OCT. The equation (figure 3-1) relating physical (gauge) thickness to the OCT (optical) thickness was used to correct the OCT measurements. Measurements by OCT were about 15 μm thinner than mechanical measures of the periphery and about 5 μm of the centre of the cornea. The equation was not applied for the epithelial measurements.

Calibration

$y = 1.0596x - 9.298$
$R^2 = 0.9993$

◆ OCT
— Linear (OCT)
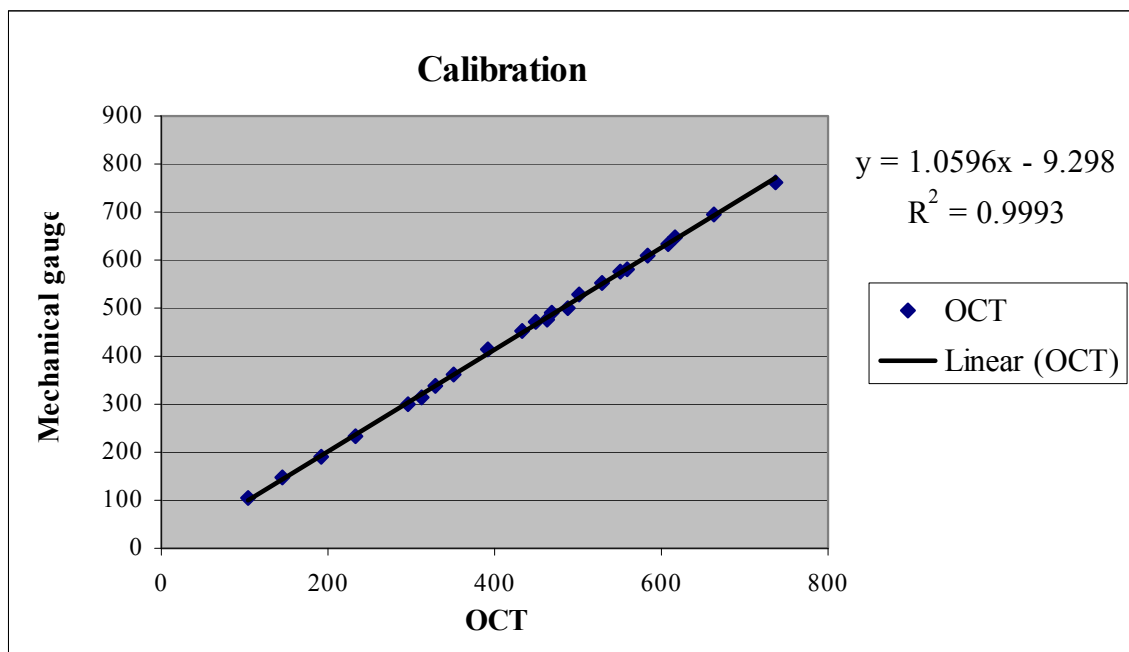
Figure 3-1 OCT Calibration

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3.2 Subjects

Thirty healthy participants from 18 to 55 years old, male and female were involved in the first study and twelve participants in the second study. All participants met the inclusion criteria and did not exhibit any of the exclusion criteria, listed below.

3.2.1 Inclusion criteria and exclusion criteria

Inclusion criteria

The subject was eligible for entry into the study if he or she:

1. Was 16 to 60 years age,

2. Was free of any eye diseases,

3. Had no history of eye surgery,

4. Understood and signed a statement of informed consent,

5. Had a refractive error $\leq \pm 4$ D and astigmatism $\leq$ 2 D.

Exclusion criteria

1. Had any abnormality in the lids,

2. Had any systemic disease that affected ocular health,

3. Had corneal vascularization.

3.3 Study procedures

Study procedures were explained and each participant was required to sign an informed consent form before participation. One eye of each subject was used for this study. Subjects were screened to determine their eligibility for the study and a slit lamp examination and automated refraction were conducted at the initial screening visit. All subjects were asked to have been awake for at least four hours before testing.

The epithelial and corneal thickness and the cell density and corneal layer characteristics were measured and at five positions; the centre, superior, inferior, nasal and temporal edges (the limbus) using optical coherence topomography and confocal microscopy.

3.3.1 OCT

Testing was performed at noon to avoid the possible effect of diurnal variation (Feng, Varikooty, & Simpson, 2001). The epithelial and corneal thickness was measured at five positions; the central, superior, inferior, nasal and temporal cornea. To evaluate the patient's cornea with OCT, when measuring central corneal thickness, subjects viewed a fixation target that was aligned with the source. Only images with evident specular reflection (present only when the corneal surface and scanning beam are orthogonal) were analyzed. A TV monitor was used to monitor eye position. In this study I used the scan length at 180˚ for the horizontal measurements and at 90˚ for the vertical meridian measurements. For limbal measurements, the subjects viewed a peripheral fixation target (Figure 3-2) and the images were taken only if the end of the scan line included the anterior chamber angle and the incident light was at a right angle to the ocular surface. At the limbus, with the smallest scan length, when the incident light was at a right angle to the ocular surface, the OCT image appeared horizontal and the specular reflection was present primarily at the front surface.
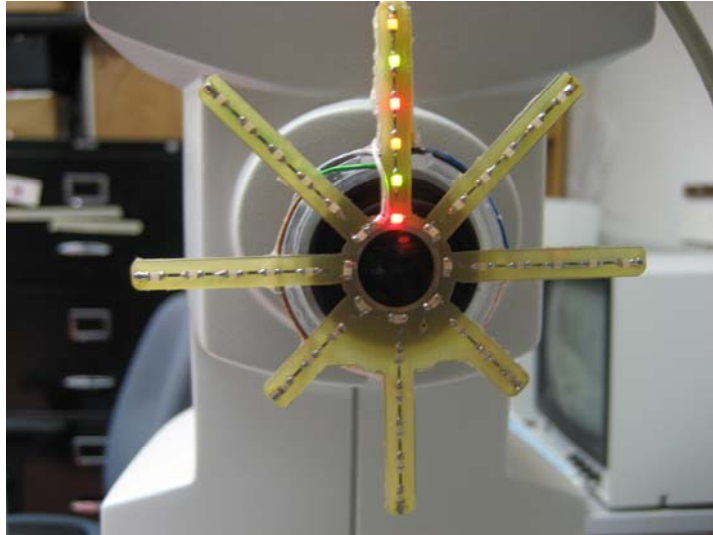
Figure 3-2 Fixation target used in OCT
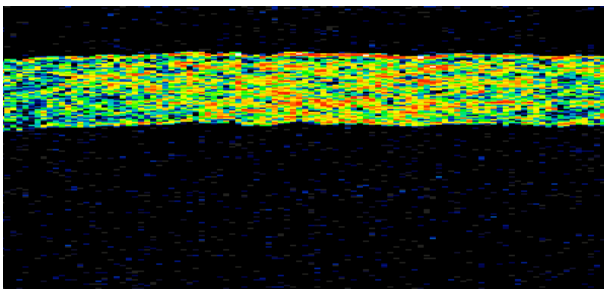
Figure 3-3 Left panel is a cross section image of the central cornea from the OCT and right panel shows the image from the OCT video camera to illustrate the positioning of the scan

Temporal cornea
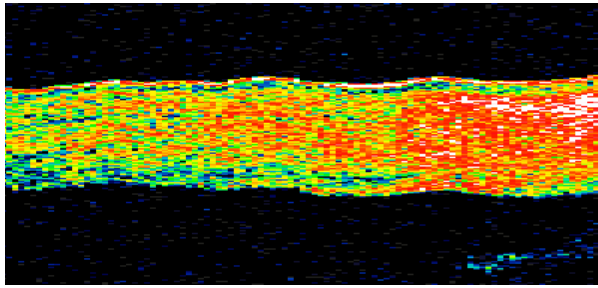
Nasal cornea
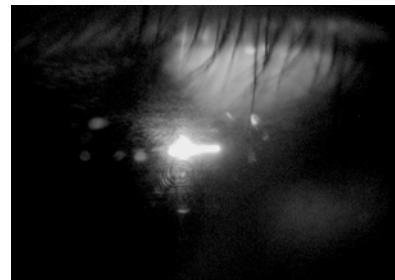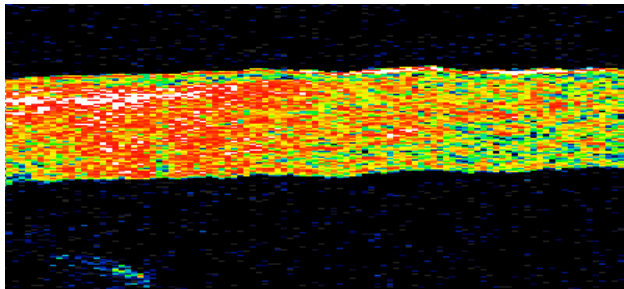
Superior cornea
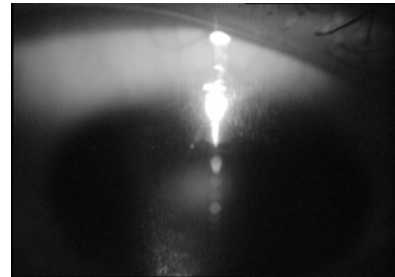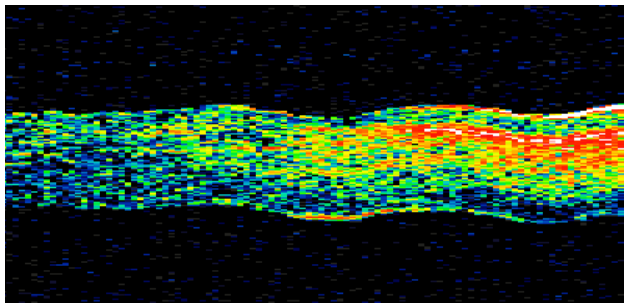
Inferior cornea
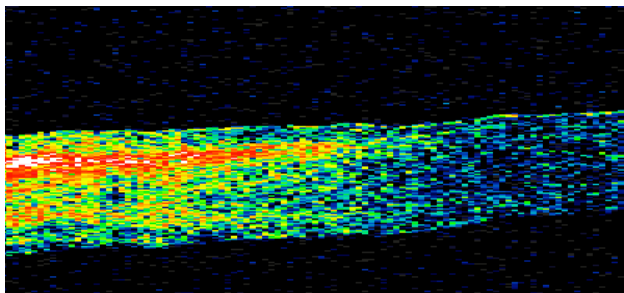
Figure 3-4 Left panels are cross sections in the 4 limbal positions from the OCT and right panels show the images from the OCT video camera to illustrate the positioning of the scan

3.3.2 Confocal microscope

The right eye was selected to undergo confocal microscopy. The chin rest, forehead bar and objective lens of the machine then were sterilized using 70% isopropyl alcohol. One drop of the anaesthetic proparacaine hydrochloride 0.5 % was instilled onto the both eyes. The time of this instillation was noted. With their chin in the chin rest, the participant's forehead was positioned snugly against the forehead rest.

The participant was instructed to look at a centrally positioned light inside the confocal objective. The controls of the confocal microscope were set to image the corneal epithelium. A drop of GenTeal® gel was placed on the tip of the confocal microscope's objective lens. The lens was slowly advanced towards the participant's eye. Images of the structure were collected automatically, once the focal distance to the cornea was correct.

On completion of this step, the objective lens was withdrawn to a safe distance from the participant's cornea. The same procedure was repeated for the limbal corneal measurement but each next limbal position was chosen randomly and participants were instructed to look at an eccentric fixation target. This target was in form of a cross with colored lights used as reference points, as seen in the (Figure 3-5) and was attached to the confocal around the objective lens. Once five sets of images were obtained, the participant's cornea was re-inspected using slit lamp biomicroscopy. Participants waited in the lab for thirty minutes from the time of instillation, a period sufficient to allow the anaesthetic to wear off.
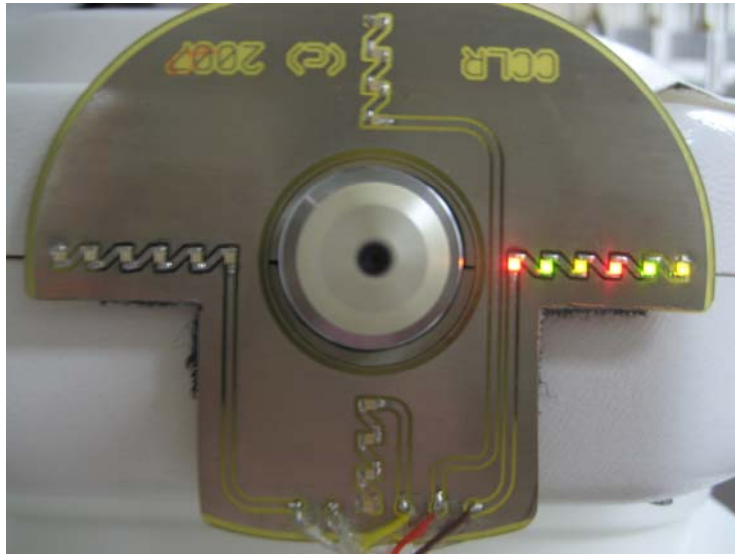
**3-5 Fixation target used in confocal**
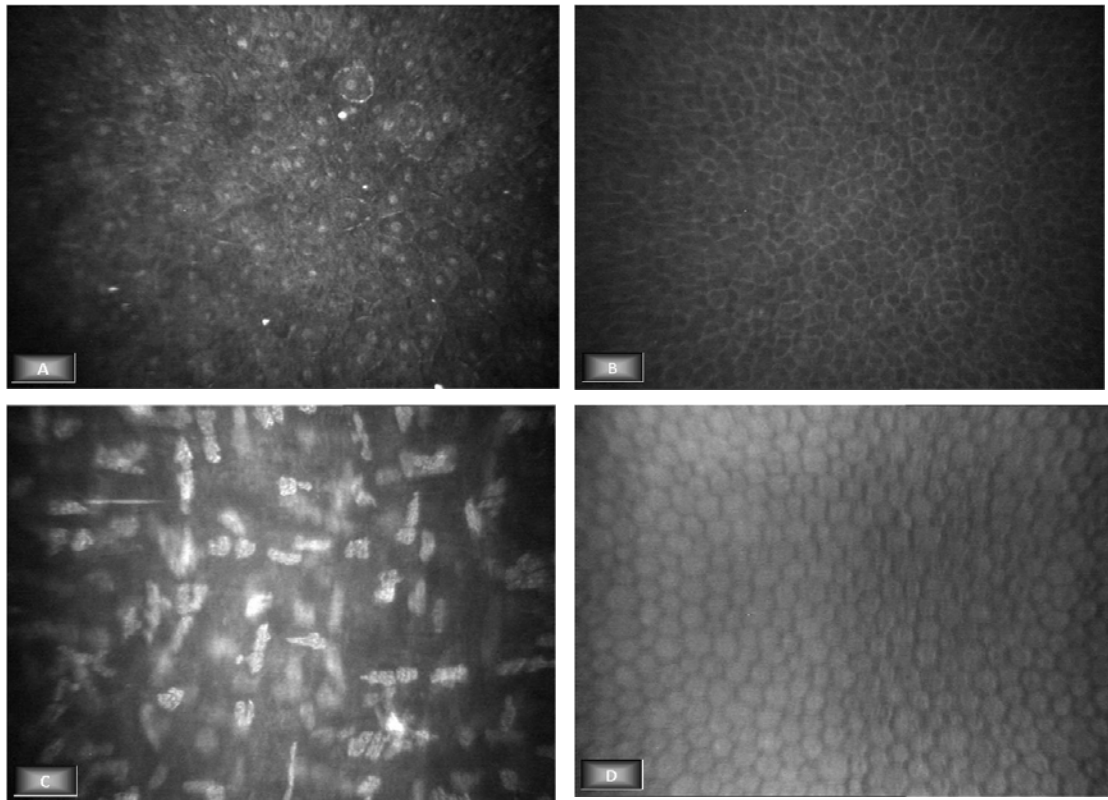
Figure 3-6 Central corneal pictures by confocal microscopy

Central corneal image layers obtained by confocal microscopy are shown in figure

3-6:

A- Superficial epithelial cornea, the outermost epithelial layer.

B- Basal epithelial cornea, the innermost epithelial layer.

C- Stromal cornea.

D- Endothelial cornea which is a single layer in the posterior cornea.
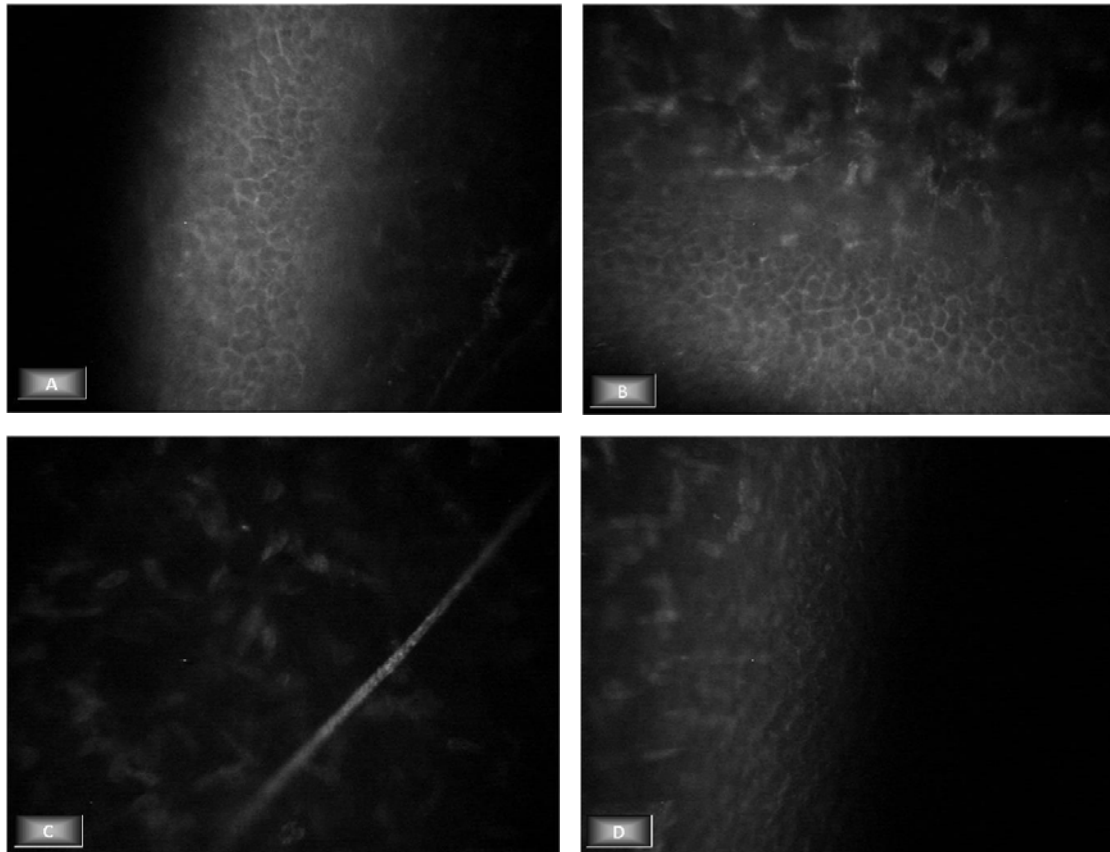
Figure 3-7 Limbal corneal pictures by confocal microscopy

Limbal corneal image layers obtained by confocal microscopy are shown in figure 3-7:

A and B - Epithelial limbal layers.

C- stromal limbal cornea.

D- Endothelial limbal cornea.

As shown in figure 3-7, D, in some cases, endothelial limbal images did not contain clearly discernable cells. A variety of conditions might have affected image acquisition. One of the most important conditions is the location of the endothelium at

the limbus. The corneal endothelium in the limbus covers the trabecular meshwork, which is a tissue, located in the anterior chamber angle of the eye.

Also as depicted in limbal corneal imaging with OCT in figure 3-4, the sclera runs into the cornea at the limbal transition zone in such a way that might prevent light reaching the endothelium.

Chapter Four

4.0 Results

4.1 OCT

I measured 30 eyes (chosen randomly) of 30 healthy subjects aged from 18 to 55 years, with refractive error $\leq \pm 4$ D and astigmatism ≤ 2 D.

Ten measurements of epithelial and total corneal thickness of each eye were taken. Each OCT measure consists of 100 adjacent sagital scans (over 1.13 mm). For the measurement of the epithelial and corneal thickness, 90 adjacent sagittal scans were used for the center and 50 adjacent sagittal scans were used for the limbal region. At least 3 images of each position for each subject were analyzed. Figure 4-1 is a sagittal scan from the OCT.
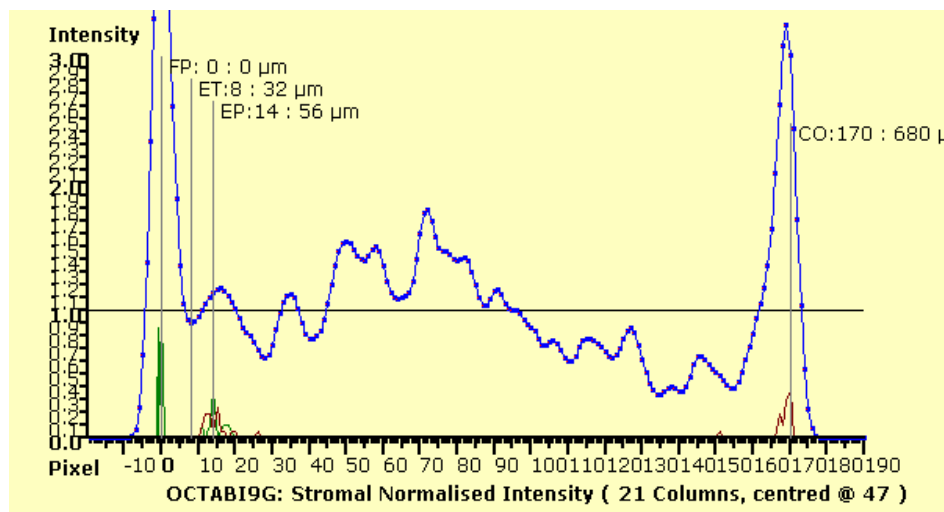
Figure4-1 a single sagittal scan from the OCT

The first peak shows the air-interface and second one the epithelial-stroma interface and major peak on the right hand side was at the endothelium-aqueous interface. The distance between the first peak and the second peak was defined as the epithelial thickness and the distance between the first peak and the last peak was the corneal thickness.

Table 4-1 Epitheilal and corneal thickness in the five positions (mean ± SD) in the first study

	Thickness in µm				
	Centre	Temporal	Nasal	Superior	Inferior
Total thickness	525.8± 31.9	765.5 ± 30.2	728 ± 27.9	714.9 ± 32.5	721.3 ± 31.1
Epithelial thickness	53.5 ± 1.9	65.1 ± 3.6	63.7 ± 4.0	63.9 ± 4.2	61.3 ± 4.1

The epithelial and total corneal thickness at the centre was significantly thinner than the limbus (Table 4-1). The differences between central and limbal epithelial cornea were significant ($p < 0.05$).

At the limbus, the inferior limbal epithelium was significantly thinner than the three other meridians ($p < 0.05$). (Figure 4-2)
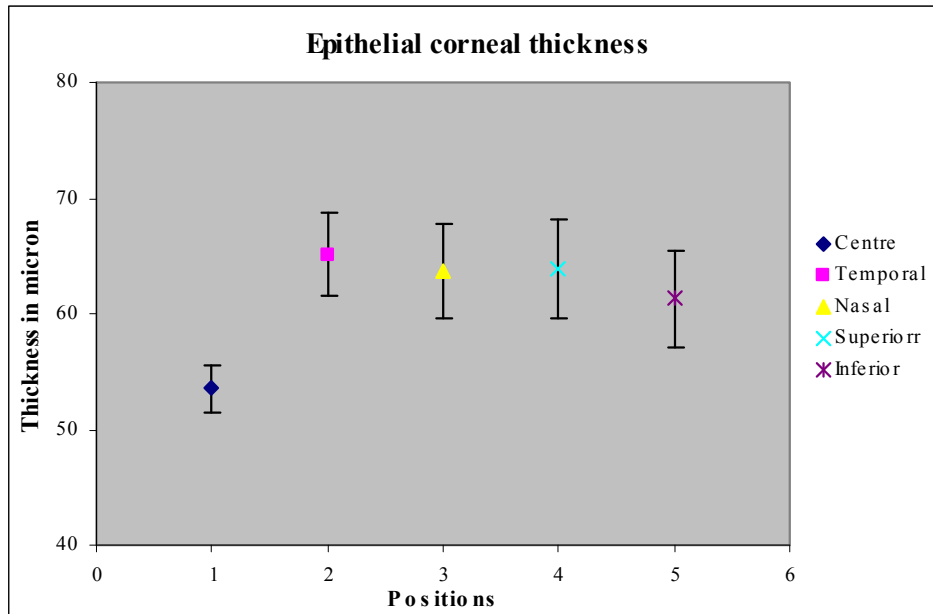
Figure 4-2 Corneal epithelial thickness in μm in the five positions (mean ± SD)

For the total corneal thickness, the central cornea was significantly thinner than limbal cornea and the temporal cornea was significantly thicker than the three other meridians (p<0.50). Figure 4-3
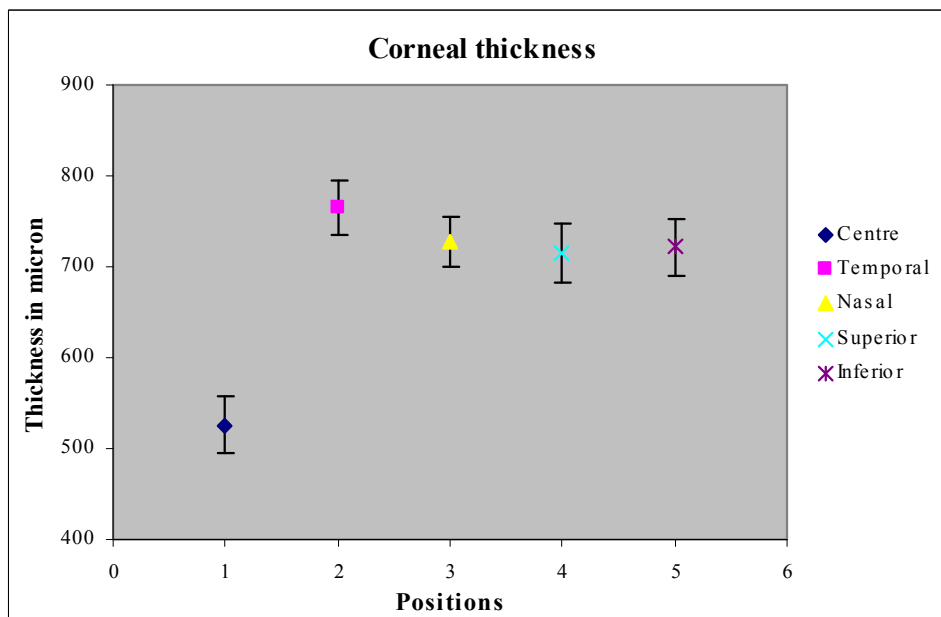
Figure 4-3 Total corneal thickness in μm in the five positions (mean ± SD)

4.2 OCT and confocal

In this part of the study, OCT and confocal microscopy measurement were performed for each subject.

4.2.1 OCT measurement

In this second study with OCT, measurements on the right eyes of 12 healthy subjects (6 males and 6 females) aged from 18 to 55 years, with refractive error $\leq \pm 4$ D and astigmatism ≤ 2 D were obtained.

The thickness of the five positions of the cornea was determined with OCT. At least three scans of each position were taken and 90 of 100 adjacent sagittal scans of the central corneal image and 40 of 100 adjacent sagittal scans of each limbal image were measured using custom OCT analysis software. These limbal measurements were unlike those from the first study in which the maximum number of sagittal scans was used from each image and an average obtained.

Similar results were obtained in the second part, in which the epithelial and corneal thickness were significantly thicker at the limbus than the epithelial and corneal thickness at the centre ($p<0.05$). (Table 4-2)

Table 4-2 Epithelial and corneal thickness in the five positions (mean ± SD) in the second study

	Thickness in μm				
	Centre	Temporal	Nasal	Superior	Inferior
Total thickness	536.0 ± 30.9	751.7 ± 36.2	715.2 ± 30.6	697.3 ± 34.8	704.7 ± 35.0
Epithelial thickness	52.3 ± 1.4	64.2 ± 2.8	63.3 ± 2.9	61.3 ± 3.6	60.1 ± 2.5

The inferior limbal corneal epithelium was significantly thinner than the temporal and nasal (p<0.05) (Figure 4-4). The thinnest was at the inferior limbus in both studies, but unlike the first study there was no significant difference between inferior and superior limbal epithelial thickness in the second study.
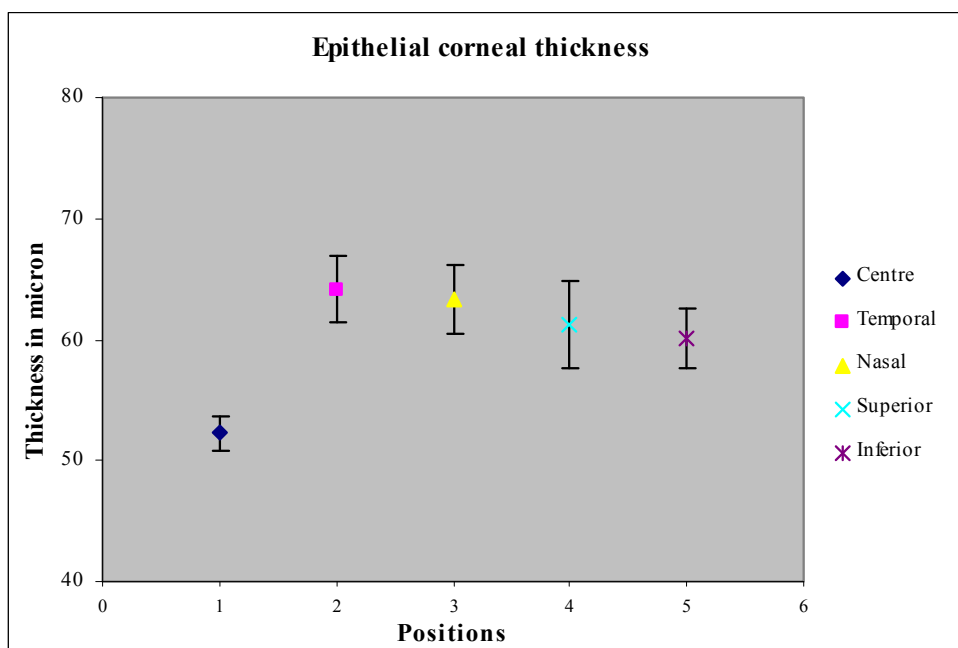
Figure 4-4 Epithelial corneal thickness in μm (mean ± SD)

As for the limbal corneal thickness, the superior cornea was thinner than the three other positions and the temporal region of the cornea is the thickest meridian in total corneal thickness (Figure 4-5).

Therefore, the results obtained from the second study supported those from the first one.
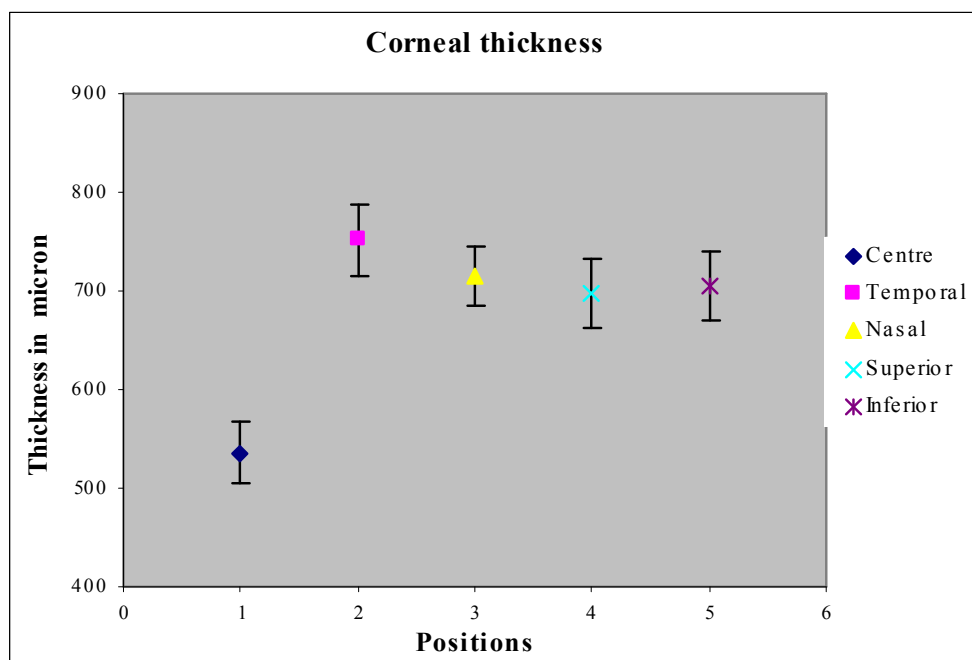
Corneal thickness

Figure 4-5 Corneal thickness in µm (mean ± SD)

4.2.1.1 Correlation between epithelial corneal thickness in five positions

To investigate the relationship between the various measures, linear correlation was performed. Nasal limbal epithelial thickness was correlated with temporal and superior epithelial thickness.

There was not a strong linear relation between temporal, superior and inferior epithelial limbal and the correlation coefficient was similar.

There was not a linear correlation between centre and nasal, temporal limbal epithelial thickness (Table 4-3).

Table 4-3 Correlation between epithelial thickness (bold correlations are statistically significant)

	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Centre		0.40	0.18	0.39	-0.11
Temporal limbus			**0.60**	0.47	0.47
Nasal limbus				**0.80**	0.18
Superior limbus					0.01

4.2.1.2 Correlation between corneal thickness

Other than centre and inferior limbal cornea, in which there was not strong linear relation, a significant association was identified between total corneal thicknesses in five measured positions. (Table 4-4)

Table4-4 Correlation between total corneal thickness (bold correlations are statistically significant)

	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Centre		**0.67**	**0.75**	**0.68**	0.52
Temporal limbus			**0.87**	**0.59**	**0.73**
Nasal limbus				**0.76**	**0.84**
Superior limbus					**0.83**

4.2.1.3   Correlation between epithelial and total corneal thickness

No significant correlations were found between epithelial corneal thickness and total corneal thickness, except between superior limbal epithelial and corneal thickness (Table 4-5).

Table 4-5 Correlation between epithelial and total corneal thickness (bold correlations are statistically significant)

	Total				
	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Epithelial					
Centre	-0.28	-0.19	-0.13	0.03	0.04
Temporal limbus	0.09	0.48	0.56	0.46	0.56
Nasal limbus	-0.00	-0.03	0.20	0.48	0.28
Superior limbus	0.08	-0.04	0.15	**0.60**	0.32
Inferior limbus	0.09	0.28	0.48	0.26	0.29

4.2.2 Confocal measurement

The confocal measurement was done on the right eyes of the 12 subjects that participated in the second OCT study. OCT measurements were done before confocal measurement. Both measurements were typically on the same day, although as much as four days separated them.

About 350 images were taken for each position in approximately one minute utilizing the confocal instrument. Each image frame size is 768 x 576 pixels (440 x 355 μm). Endothelial and basal epithelial cell density was acquired by automatically counting the number of the cells using Image J software (http://rsb.info.nih.gov/ij/).

4.2.2.1 Cell counting process

The original image, Figure 4-6 (A), was first cropped to select an area that contained a sufficient number of clearly discernable cells that could be counted. The 'Process', 'Subtract Background' commands were then applied to obtain the image shown in Figure 4-6 (B). Lastly the plug-in ITCN, was applied to obtain the cell count that was indicated by a pink dot in the nucleus of each cell. The software in this instance gave final results of:

Number of Cells: 363 in 442368.0000 square pixels

Density: 0.0008 cells per square pixel

Based on the above results, this per-square-pixel measure can be converted to a mm^2 measure.
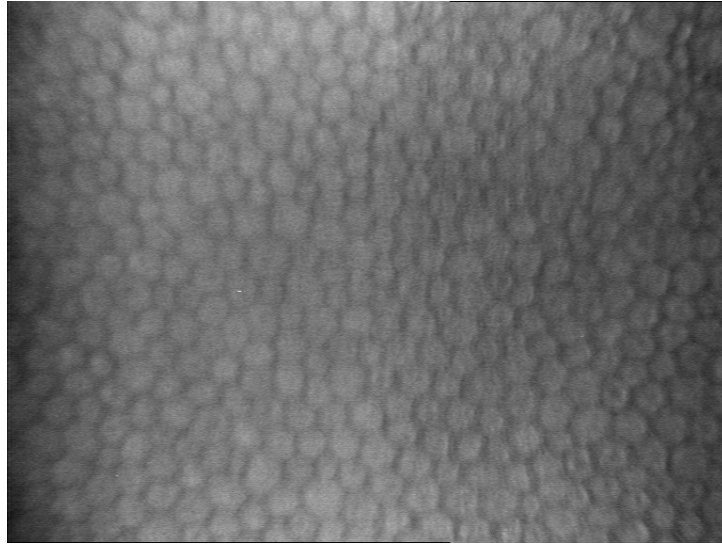
(A)

(B)

Figure 4-6 (A) and (B) Endothelium cell layer

In the endothelium case as shown above the 'Subtract Background' command was executed in such a way as to have a cell that had white background with a black membrane, whereas in the case of the epithelium the case was reversed so that the cell had a black background with a white membrane. This was achieved by performing the 'Subtract Background' in two stages; in the first stage the 'Light Background' option

was checked, and in the second stage that option was left unchecked so that final result is shown in Figure 4-7.
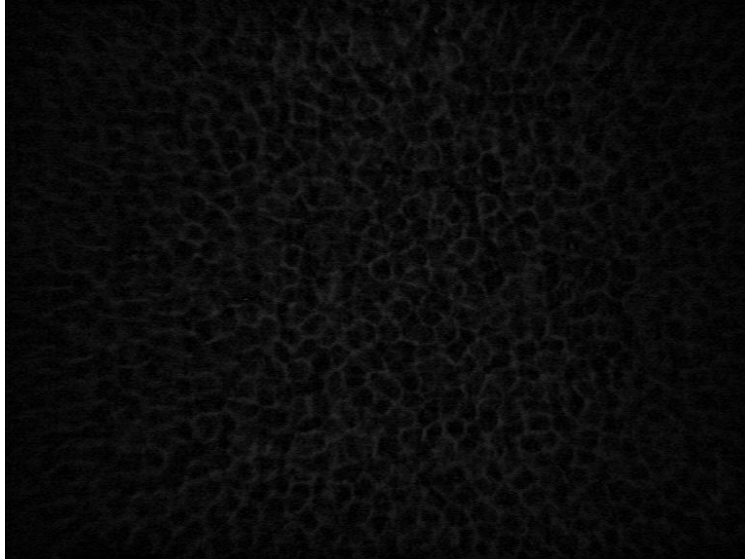
Figure 4-7 Epithelium cell layer

Figure 4-8 Limbal endothelium cell layer

However, not all of the images obtained were of the same nature as shown in A and B (Figure 4-6). Some of the images as is shown in Figure 4-8, did not have a central

field from which a single large area could be cropped to allow for cell counting but rather had an oblique field of visible cells. In this situation the field of visible cells was divided into two or three smaller areas, and each area was individually counted, with the final result being the average of the different counts.

4.2.2.2 The epithelial corneal cell density

The mean epithelial cell density at the five positions is shown in table 4-6 & figure 4-6. The cell density at the superior cornea was significantly lower than the other four corneal positions. The least mean density was at the superior limbal cornea and the highest mean value was obtained for the temporal limbal cornea.

Table 4-6 Corneal cell density in the five positions (mean ± SD)

	Cells / mm^2	
	Epithelium	*Endothelium*
Centre	5247.96 ± 136.17	2368.82 ± 177.82
Superior limbus	4824.62 ± 278.69	2400.37 ± 170.14
Inferior limbus	5117.93 ± 271.91	2331.79 ± 141.54
Temporal limbus	5279.13 ± 200.56	2301.32 ± 201.65
Nasal limbus	5225.07 ± 390.61	2335.21 ± 161.78
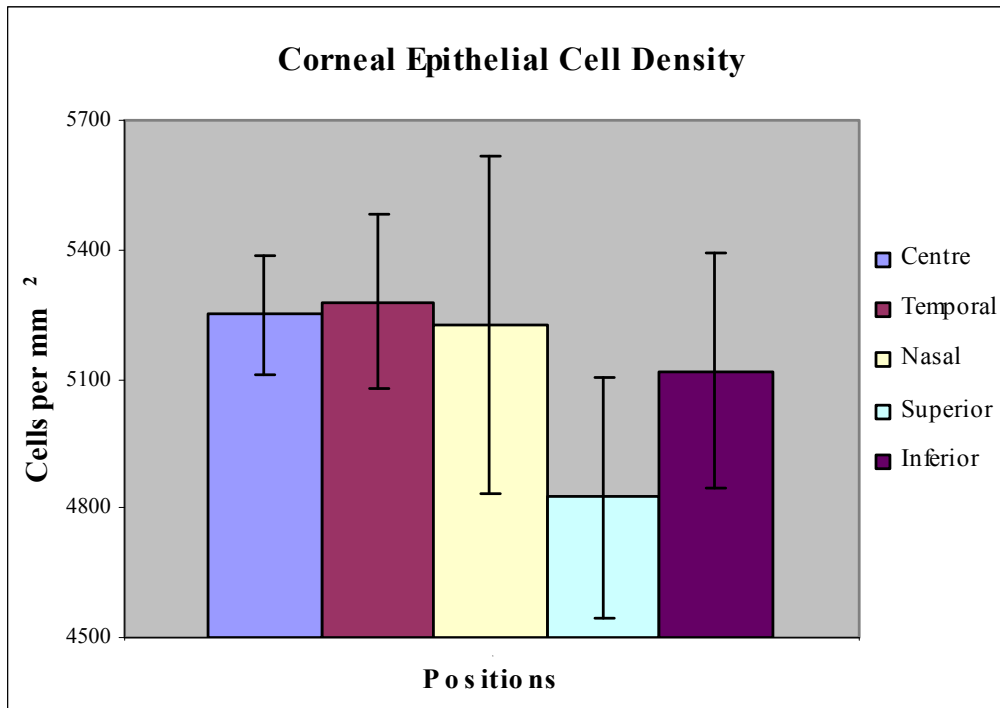
Figure4-7 Corneal epithelial cell density (cells / mm^2) in the five positions

(mean ± SD)

4.2.2.3   Correlation between epithelial cell densities

There were no significant correlations between epithelial cell densities in the five

measured positions except between temporal and superior limbus.

**Table 4-7 Correlation between epithelial cell densities (bold correlations are
statistically significant)**

	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Centre		0.04	-0.13	0.38	0.13
Temporal limbus			0.53	**-0.58**	0.05
Nasal limbus				-0.23	0.37
Superior limbus					0.35

4.2.2.4 The endothelial cell density

The endothelial cell density was measured at the five positions. The mean values were:

Central; 2363.13 ± 177.82, Temporal limbus; 2301.32 ±201.65, Nasal limbus; 2335.21 ± 161.78, Superior limbus; 2400.37 ± 170.14, Inferior limbus; 2331.79 ± 141.54 (Table 4-6).

There was no significant difference between cell densities in the five positions (p > 0.05).
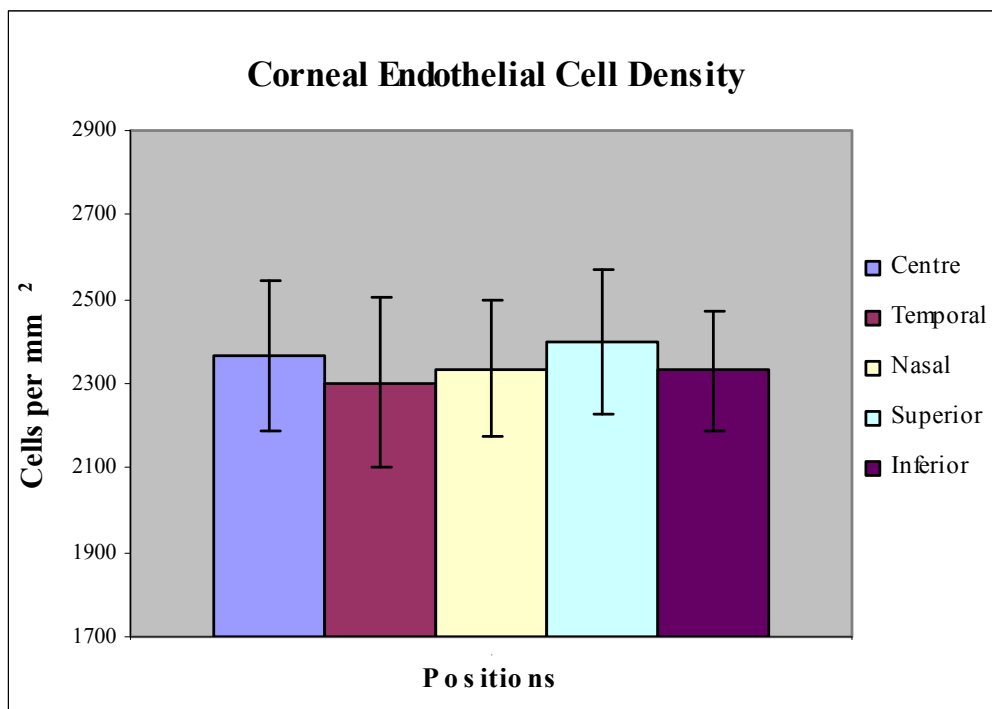
Figure 4-8 Corneal endothelial cell density (cells / mm^2) in the five positions

(mean ± SD)

4.2.2.5   Correlation between endothelial cell density

There were no significant associations between endothelial cell densities except between central and nasal positions (Table 4-8).

Table 4-8 Correlation between endothelial cell densities (bold correlations are statistically significant)

	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Centre		0.56	**0.59**	-0.19	-0.45
Temporal limbus			0.32	-0.18	0.02
Nasal limbus				0.30	-0.13
Superior limbus					0.19

4.2.2.6 Correlation between epithelial and endothelial cell densities

Pearson's correlation coefficient was derived to assess the association epithelial and endothelial cell densities. There was no significant correlation between the epithelial and endothelial cell density in the same position. However a significant correlation was found between temporal, nasal and superior limbal epithelial and endothelial corneal cell densities. Also the centre and inferior, nasal limbal corneas were correlated (Table 4-9).

Table 4-9 Correlation between epithelial and endothelial cell densities (bold correlations are statistically significant)

| | Endothelium | | | | |
	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Epithelium					
Centre	-0.24	0.25	0.00	0.29	0.24
Temporal limbus	0.04	0.26	**-0.64**	**-0.59**	-0.15
Nasal limbus	-0.08	0.13	-0.51	-0.11	-0.22
Superior limbus	-0.44	-0.25	0.16	0.35	-0.11
Inferior limbus	**-0.67**	-0.45	**-0.70**	0.05	0.20

Chapter Five

5.0 Discussion

5.1 OCT

Most studies have shown that the central cornea is significantly thinner than the peripheral cornea. However there is little agreement about different region of the peripheral cornea. Different reports have shown: 1) the superior peripheral cornea is the thickest position (Liu et al., 1999) using Orbscan, 2) superior cornea is thicker than the inferior along a 7.1 mm chord (Rapuano, Fishbaugh, & Strike, 1993) using an ultrasound pachymeter, 3) no difference in the thickness in the four meridians (Steinberg, Waring, & Lynn, 1986) using ultrasonic pachymetry, and 4) no difference between nasal and temporal limbal cornea (Feng & Simpson, 2005) using OCT. The superior limbal cornea was found to be the thinnest and temporal limbal cornea thickest in four meridians. The study done by Remon and et al. (Remon, Cristobal, Castillo, Palomar, Palomar, & Perez, 1992b) revealed a similar result. There is agreement between these two studies that the temporal cornea is thickest and the superior cornea is thinnest.

Corneal thickness measurements in different studies are shown in table 5-1. Sample size, different devices and different techniques could have accounted for the discrepancy seen in the studies. For example the mechanical pressure and mucous gel that used in the systems requiring corneal contact (e.g. confocal microscopy) and failure to avoid measuring the tear film thickness in the non- contact (e.g. OCT) systems may affect the data.

Table 5-1 Corneal Thickness Measurement Results In Different Studies

Study	Instrument	Results Thickness in μm	
Nine point corneal thickness measurements and keratometry readings in normal corneas (Rapuano et al. 1993)(Rapuano et al., 1993)	Ultrasound pachymetry	Mean CCT; 515 ± 34, Mean paracental; ranged from 522 ± 40 inferiorly to 574 ± 41 superiorly Mean peripherally; ranged from 633 ±50 inferiorly to 673 ± 49 superiorly	
Evaluation of corneal thickness and topography in normal eyes (Liu,Z et al. 1999)(Liu et al., 1999)	Orbscan II	CCT; 560 ± 30 TCT; 590 ± 30 NCT; 610 ± 30 SCT; 640 ± 30 ICT; 630 ± 30	
Comparison of human central cornea and limbus in vivo (Feng and Simpson 2005)(Feng & Simpson, 2005)	Optical coherence tomography	Epithelial thickness	Total thickness
		CET; 58.4 ± 2.5	CCT; 507.9 ± 35.8
		TET; 77.5 ± 2.8	TCT; 704.4 ± 31.8
		NET; 76.8 ± 3.5	NCT; 703.8 ± 32.1
Central and peripheral corneal thickness in full-term newborns (Remon et al. 1992)(Remon, Cristobal, Castillo, Palomar, Palomar, & Perez, 1992b)	Ultrasonic pachymetry	CCT; 585 ± 52 TCT; 748 ± 55 NCT; 742 ± 58 SCT; 696 ± 55 ICT; 744 ± 62	

In the current study, OCT was used for corneal thickness measurements twice. During the first study thirty subjects were measured and at the second one (almost one year later) twelve subjects participated. About 30% of the second study participants were from the first study. The results in the both studies were similar. Both epithelial and total corneal thickness was significantly thinner at the centre than at the limbus in both studies. The limbal epithelium was the thickest whereas the epithelial thickness of the other three limbal positions was similar to each other. The total thickness in the limbal region was greatest temporally whereas the thickness in the three other limbal regions were similar to each other. However the difference between inferior and superior epithelial thickness was not as large as it was in the first study.

The mean values for the both studies are shown in Table 5-2 and Figure 5-1 & 5-2.

Table 5-2 Comparing epithelial and total corneal thickness in two studies

Position	Epithelial corneal thickness in μm		Total corneal thickness in μm	
	First study	Second study	First study	Second study
Centre	53.5 ± 1.9	52.3 ± 1.4	525.8 ± 31.9	536.0 ± 30.9
Temporal	65.1 ± 3.6	64.2 ± 2.8	765.5 ± 30.2	751.7 ± 36.2
Nasal	63.7 ± 4.0	63.3 ± 2.9	728 ± 27.9	715.2 ± 30.6
Superior	63.9 ± 4.2	61.3 ± 3.6	714.9 ± 32.5	697.3 ± 34.8
Inferior	61.3 ± 4.1	60.1 ± 2.5	721.3 ± 31.1	704.7 ± 35.0
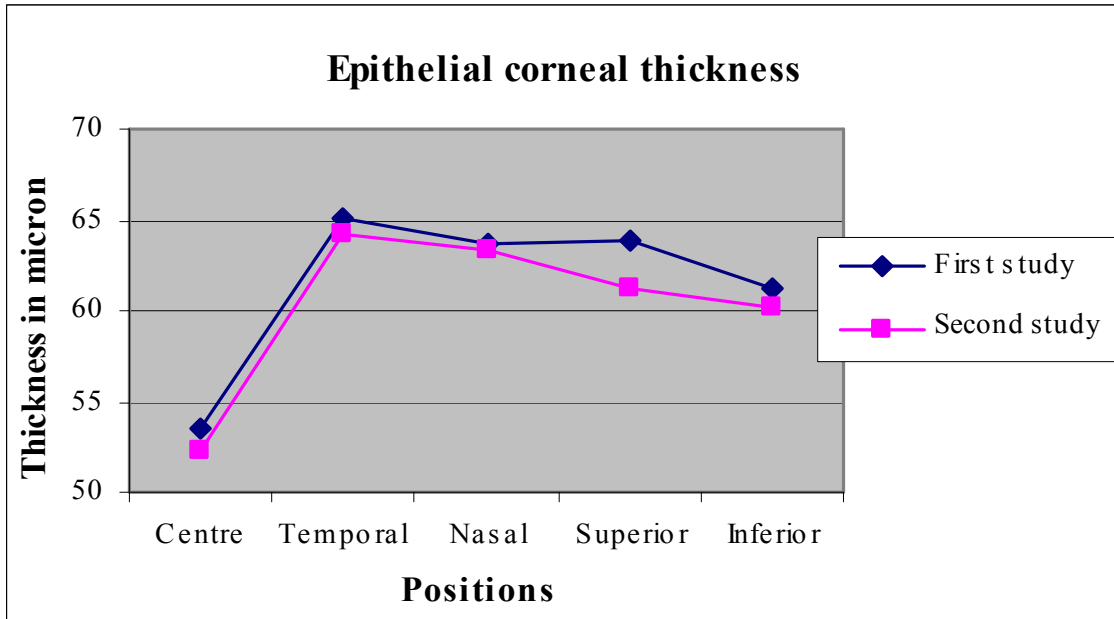
Figure 5-1 Epithelial thickness in two studies
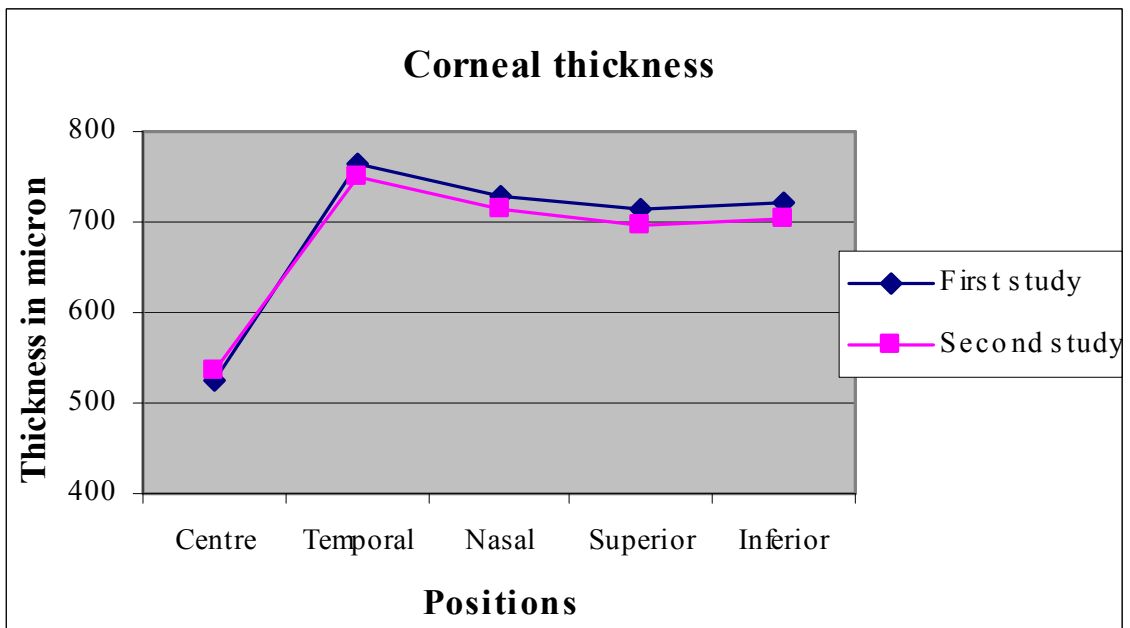
Figure 5-2 Total corneal thickness in two studies

5.2 Confocal measurement

5.2.1 Epithelium evaluation

The epithelial cornea consists of three types of cells. Basal cells that are innermost cells arranged in a single layer on the basal membrane become large and flat as they move toward the surface during cell development (Edelhauser and Ubels, 2002).

So we expect the cell density to decrease from basal cells to superficial corneal cells (Mustonen et al., 1998). It is very important to distinguish the basal epithelial layer from other corneal epithelial layers, especially in the case of poor image quality or when the image is taken at an angle (as when imaging limbal cornea), which makes quantitative analysis more difficult.

The goal of this study was to investigate superior corneal morphology under the eyelid and compare it with the other similar limbal regions (inferior, nasal and temporal limbal) as well as the central cornea. In my experiment using confocal microscope to image the corneal limbus, I tried to be consistent in counting the same limbal position in all the subjects but measurements at the exact limbal location in all the subjects were impossible to achieve with certainty.

To my knowledge this is the first study of its kind to measure limbal cornea at four positions as well as the central cornea. Results in the previous studies measuring of the basal epithelial central cell density measurements vary from 3601.38 ± 408.19 cells / mm^2 (Vanathi et al., 2003) to 8996 ± 1532 cells / mm^2 (Eckard, Stave, & Guthoff, 2006) in different studies using different models of confocal microscope (Table 5-3).

In the case of central basal epithelial density, the mean value in my study (5247.96 ± 136.17 cells / mm^2) is similar to the results of Hitzenberger, Baumgartner, Drexler, &

Fercher, 1994 and to Mustonen et al., 1998. However some studies reported higher (Eckard et al., 2006) or lower (Vanathi et al., 2003) epithelial cell density.

In the limbal cornea, most of the O_2 consumed by the cornea is provided by the epithelium and the endothelium. Since the oxygen is taken from the aqueous humor and from the capillaries at the limbus and tear film (Edelhauser and Ubels, 2002), we expect cell morphologic changes in the superior limbal cornea covered by the lid due to assumed hypoxia. Previous experiments have observed basal epithelial cell density of the peripheral cornea, but either the position of the measured area relative to the limbal cornea is unclear (Eckard et al., 2006) or only two limbal positions were investigated (Patel, Sherwin, & McGhee, 2006). In addition, the comparison between superior limbal cornea with the other limbal positions was not done. In my study, a significant difference between superior epithelial density with three other limbal regions as well as with central cornea was obtained. The mean value for the superior limbal epithelial density was lower than that of the four other regions which raise a question about the effects of probable chronic hypoxia on the cell density.

Recently, Miyashita and et al. suggested that hypoxia could increase the cell proliferation rate and decrease the number of differentiated human corneal limbal stem cells *in vitro*. The differentiated cells later form basal cells that move to the centre (Miyashita et al., 2007).

5.2.2 Endothelium

Endothelial cell density varied from 2488 ± 301 cells / mm^2 to 2818.1 ± 361.03 cells / mm^2 (Table 5-3). No significant difference was found between the five positions. The result is similar to a previous study by Amann, Holley, Lee, & Edelhauser, 2003 in which the highest mean value was obtained for the superior position.

The same result was obtained by Muller, Craig, Grupcheva, & McGhee, 2004 using confocal microscopy and Blackwell, Gravenstein, & Kaufman, 1977 using contact specular microscopy in which there was no significant difference between central and peripheral cornea. However none of these studies were performed in the four limbal positions.

In a recent study Wiffen, Hodge, & Bourne, 2000 found significant differences between central and peripheral endothelial cell density using specular microscopy. They reported higher cell densities in the central cornea versus the peripheral region. However other recent studies (Cheung & Cho, 2000 and Amann, Holley, Lee, & Edelhauser, 2003b) reported opposite results in that they found higher cell densities in the periphery. In the investigation by Amann, Holley, Lee, & Edelhauser, 2003 higher endothelial cell density in the superior cornea were reported. In my study the highest mean cell density (not significant) was at the superior limbal cornea as well.

In most studies, the exact peripheral locations at periphery are unclear. Amann et al. (2003) YEAR mentioned that "The area of highest cell density in the periphery appears to be a small ring of endothelial cells close to the Schwalbe line. Schimmelpfennig (1984) has previously reported a 23% increase in ECD in this area". This idea is supported by Bednarz et al. in the two studies (Bednarz, Rodokanaki-von Schrenck, & Engelmann, 1998) and (Bednarz & Engelmann, 2001). Their finding confirms the existence of an endothelial cell regeneration zone in the peripheral cornea region and they believe that the peripheral endothelial cells may function as stem cells for this layer.

A study by Bourne and Enoch (1976) showed that the magnification effect of the specular microscope can cause about 1% corneal endothelial cell density variation over the corneal thickness.

Table 5-3 Epithelial and endothelial corneal cell densities (cells /mm^2)

Study	Basal epithelium		Endothelium	
(Mustonen et al., 1998)	Central cornea 5,699 ± 604			
(Harrison, Joos, & Ambrosio Jr, 2003)	Central cornea 5,274 ± 575			
(Eckard et al., 2006)	Central cornea 8996 ± 1532 Peripheral 10,139 ± 1,479			
(Vanathi et al., 2003)	Central cornea 3601.38 ± 408.19		Central cornea 2818.1 ±361.03	
(Niederer, Perumal, Sherwin, & McGhee, 2007)	Central cornea 6000 ± 1080		Central cornea 2720 ± 367	
(Patel et al., 2006)	age < 45	age≥ 45		
	Central cornea 6162 ±503	Central cornea 6362±614		
	Inferior limbus 7253 ±1077	Inferior limbus 6614± 987		
(Amann, Holley, Lee, & Edelhauser, 2003)		Peripheral	Central cornea 2,730 ±301	
			Paracentral cornea 2,887± 213	
			Superior cornea	3,166 ±272
			Nasal cornea	2,944 ± 292
			Inferior cornea	2,912 ± 276
			Temporal corne	2,992 ± 226
(Muller et al., 2004)			Central cornea	2488 ± 301
			Temporal cornea	2525 ± 505
			Superior cornea	2639 ± 398

5.3 Correlation between epithelial thickness and cell densities

To investigate the association between epithelial thickness and cell density, Pearson's correlation coefficient was obtained. There was no significant association between cell density and thickness of corneal epithelium (Table 5-4).

Table 5-4 Correlation between epithelial thickness and cell densities (bold correlations are statistically significant)

Thickness	*Cell density*				
	Centre	Temporal limbus	Nasal limbus	Superior limbus	Inferior limbus
Centre	-0.19	0.34	0.25	-0.39	0.04
Temporal limbus	-0.38	**0.63**	0.53	-0.44	-0.25
Nasal limbus	-0.14	0.38	0.46	-0.24	-0.13
Superior limbus	-0.29	0.07	0.41	-0.18	-0.23
Inferior limbus	0.15	0.55	0.37	-0.44	-0.48

Chapter six

6.0 Conclusions

The goal of this study was to initially determine epithelial and endothelial cell density as well as epithelial and corneal thickness in the superior region of the cornea (under the upper lid). Based on the results, a comparison was done to those of the inferior, temporal, nasal limbal and central cornea using OCT and confocal microscope to reveal the effect of probable chronic hypoxia due to the covering of the superior cornea by the upper lid during waking hours. This was the first study to do measurements in the five positions.

OCT showed that central epithelial and corneal thickness is significantly thinner than epithelial and corneal limbal thickness, a finding which is also supported by most of the studies that have been carried out.

In the limbus, the inferior epithelium was significantly thinner than the three other positions. The temporal cornea was the thickest ($p < 0.05$). The results obtained from the first study using OCT were supported by a second set of measures on a smaller sample and including a different technique; however, the difference between inferior and superior epithelial thickness was not significant in the second study.

There was no linear association between the epithelial thicknesses in any of the five positions. On the other hand, there were linear associations in corneal thickness between different locations.

In the study with confocal microscopy, epithelial cell density was lower in the superior cornea ($p < 0.05$), but there was no significant difference in endothelium cell densities ($p > 0.05$) and there were no associations in epithelial density between regions. A similar result was found for the endothelium.

In this study the hypothesis that the superior cornea might be thicker due to chronic hypoxia was not confirmed, but there was a lower epithelial cell density in the superior cornea.

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