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A Study Comparing the Regulation of Epithelial Homeostasis in Transplantable Human and Porcine Corneas

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Submitted for fulfillment of the requirements for Doctor in Philosophy

September 2005

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To my brother faramarz

*Its love not reason that is stronger
than death*

Thomas Mann

Acknowledgements

The biggest thanks go to my supervisors Dr Julie Albon and Professor Mike Boulton, for guiding me in this game of my life, and for being compassionate and understanding as I did this project. Your enthusiasm and optimistic attitude towards research have inspired me during these years. It is you two who made this all possible and I am eternally grateful.

I specially want to thank Dr Louise Carrington for all the help in the laboratory; you were irreplaceable in this work.

I also wish to thank Professor John Armitage for sharing his vision and comments.

I wish to to thank Dr Paul Murphy for his kind advices.

I do wish to thank our co-workers in the office; Susan, Steve, Phil, John.

I thank my mother and father for a lifetime support and for never ending love. For always helping me remember what is real and important. I do appreciate everything you have done for me.

Thank you to all my friends in the university (Ahiva, Andreas, Gill, katerina, marieah and Tina) even though we are individually different, we found out how united we are in our dreams and support for each other.

To Veronique, we have been together since writing up started and hope our friendship never ends.

Finally, my warmest thanks belong to my beloved Mohammad you have added something special to my life and thank you for all your understanding.

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Abbreviations:

ALDH	Aldehyde dehydrogenase
bFGF	Basic fibroblast growth factor
BA	Basement membrane
Cm	Centimetre
CKs	Cytokeratins
Cx43	Connexin 43
Cx50	Connexin 50
°C	Degrees Centigrade
CTS	Corneal Transplant Service
DH₂O	Distilled water
Dsc	Desmocollins
Dsg	Desmogleins
EGF	Epidermal growth factor
EEBA	European Eye Bank Association
g	Gram
H&E	Haematoxylin and eosin staining
HGF	Hepatocyte growth factor
IGF-1	Insulin like growth factor-1
IL-1	Interleukin-1
IMS	Industrial methylated spirits
GuK	Guanylate kinase
KDa	Kilo Dalton
KGF	Keratinocyte growth factor
LAPs	Latency-associated peptides
LC	limbo-corneal
LSC	Limbal stem cell
LRCs	Label retaining cells

MAGUK	Membrane-associated guanylate kinase homologue
Mag.	Magnification
µm	Micrometer
mg	Milligram
ml	Millilitre
PKC	Protein kinase C
PDGF	Platelet-derived growth factor
SCs	Stem cells
TACs	Transient amplifying cells
TGF-	Transforming growth factor-β
TJs	Tight junctions
TKT	Transketolase
VEGF	Vascular endothelial growth factor
%	Percentage

ABSTRACT

PURPOSE

To obtain a better understanding of corneal epithelial cell homeostasis during organ culture storage.

METHODS

Porcine corneas were used in this study as a model for organ culture storage of human cornea which is carried out routinely for storage of transplant corneas. Porcine corneas were maintained in immersion organ culture for varying periods of time. For proliferation studies BrdU at 1/500 dilution was added. Corneas were then stored in an incubator at 34° C for up to 14 days. At each time point the cornea was bisected: one half was fixed for wax sectioning and the other half snap frozen for cryosectioning. General morphology, epithelial stratification and cell density were assessed by light and fluorescence microscopy using Leica QFluoro image analysis software. Keratocyte number and density was quantified in the anterior, posterior and middle regions of the corneal stroma. The proliferative status of the epithelium was assessed as a function of BrdU incorporation and quantified in central, peripheral and limbal regions. The TUNEL assay was also performed to identify epithelial cell death. Changes in porcine corneas were compared to those in human organ cultured corneas.

RESULTS

A significant decrease in corneal epithelial thickness was demonstrated with increasing time of organ culture from a maximum value of $45\mu\text{m} \pm 3.6$ at 0 day to a minimum of $7.7\mu\text{m} \pm 0.6$ at 14 days ($P < 0.0001$). Stromal thickness increased from $691\mu\text{m} \pm 53$ at 0 day to a maximum of $1569\mu\text{m} \pm 126$ at 14 days organ culture storage ($P < 0.0001$). There was also an apparent decrease in keratocyte density in anterior, posterior and middle stromal ($P < 0.0001$). The BrdU labelling index as a measure of the proliferation rate was not equally distributed across the cornea. Proliferation was greatest in the peripheral epithelium at 12 days ($27.7\% \pm 8.6\%$) and the labelling index appeared to increase in all regions: peripheral ($r=0.79$), central ($r=0.62$) and limbal ($r=0.67$) as a function of organ culture storage time ($P < 0.05$).

The cytoskeletal properties of epithelial cornea seemed to remain stable during storage. Lack of ZO-1, Cx43 and 50 expression were observed in cornea at some time points of culture (especially day 6). An increase in percentage of TUNEL-positive corneal epithelial cells was also demonstrated in the central, peripheral and limbal regions (58% at day 12 in peripheral region) with organ culture storage time ($p < 0.05$). The lack of positive activated caspase 9 staining with the high intensity of immunorelated caspase-8 detected in the corneal organ storage model cornea suggests that the epithelial apoptotic pathway during storage is initiated by an extrinsic death receptor-mediated pathway

CONCLUSIONS

Porcine cornea is a good model for organ culture storage. Epithelial loss occurs during organ culture and is likely to cause barrier dysfunction, therefore contribute to stromal swelling and thus indirectly endothelial cell death. The lack of ZO-1, Cx43 and 50 expression in corneal epithelium is likely to contribute towards deregulation of the corneal epithelium. Epithelial cell loss during organ culture storage appears to be a consequence of accelerated cell death during storage. It is important for the injured epithelium to be replaced with new cells rapidly. However the proliferative activity of corneal epithelial cells during organ culture does not appear to be sufficient to replace the shed cells. This may result in barrier dysfunction and therefore indirectly endothelial cell death that is responsible for the 30% discard rate of corneas stored for transplantation. Changes in porcine corneas on the whole correlated with those in human corneas. Thus the porcine corneas provide a good model by which to study corneal integrity and cell homeostasis during organ culture.

CHAPTER ONE

1. Chapter 1 - Introduction

When a cornea is damaged to the point that it can no longer function, a corneal transplant can help to restore sight and ocular function. Donated human eyes and corneal tissue are used for transplantation. When consent for donation is given, eyes must be surgically removed within 24 hours of the donor's death. Some conditions, can not be accepted as corneal donors such as infectious disease, exclude individuals from donation although cataracts, poor eyesight and age, tissue from donors with viral disease or disease of unknown etiology, dementia, the Reye's syndrome, active septicaemia (bacteraemia, fungaemia, viraemia), or who have had previous intrinsic eye disease, or intraocular surgery (European Eye Bank Association (EEBA) standards 2003). Corneal transplantation would not be possible without donors who donate corneal tissue. The transplantation process depends upon the priceless gift of corneal donation from one human to the next. The UK Corneal Transplant Service (CTS) Eye Banks provide organ-cultured donor corneas for over 2500 corneal transplantation per annum.

Corneal transplantation is found in 18th century literature and was performed experimentally on animals in the 19th century. For the next 30 years, all human penetrating keratoplasties were performed using fresh tissue from living donors whose eyes were enucleated because of pathologic conditions of the posterior segment. Advances in surgical techniques and instrumentation as well as introduction of antibiotics in the 1930s and steroids in the 1940s improved the prognosis and increased the success of ophthalmic surgical procedures, including corneal transplantation. A seminal event for eye bank tissue preservation occurred in 1935 with the report by Filatov, showing that cadaver tissue stored at 4°C could be used as donor material. This development vastly increased the number of eyes available for transplantation and led to the establishment of eye "banks" to collect, store, and distribute tissue.

The modern eye bank is a technically demanding, scientifically sophisticated laboratory that has evolved procedures and standards that ensure that the patient receives the safest and highest quality donor tissue.

1.1. Various methods of corneal preservation

Corneal preservation can be classified according to length of storage time: A) short-term, up to 48 hours, B) intermediate-term, up to 10 days C) and long-term (up to 28 days).

1.1.1 Short-term storage (0 to 48 hours)

1.1.1.1 4°C moist chamber storage

Until approximately 15 years ago, whole globe storage in a sealed container at 4°C was the method used for corneal preservation by most transplant surgeons. It is the standard storage method used successfully since Filatov first described it in 1935. Eyes were readily transportable in an insulated container with ice (frozen water, not dry ice). Its major shortcoming was limited duration of storage. Although the exact limit of this time is not known, most surgeons will not use corneas stored in this manner beyond 24 hours.

It was assumed that endothelial cell death occurs in a linear fashion proportional to postmortem time at 4°C (Komuro et al., 1999). However, Saleeby et al. (1972) has reported an 83% transplant success rate using 4°C stored corneas between 50 to 80 hours postmortem. This indicated that if cell death was proportional to postmortem time, the rate was much slower than previously assumed and also meant that although a large number of cells perished at 4°C, the remaining viable cells were able to maintain graft function. The major disadvantage was the inability to assess endothelial viability pre-operatively. Accelerated endothelial cell loss has also been observed with the short term storage of cornea, 48% for moist chamber (Obata et al., 1991). This method is currently only used in the UK for transportation of donated eyes to an eye bank where the cornea is removed and is stored until transplantation and in very few cases to supply tissue for limbal stem cell transplants.

1.1.2 Intermediate-term storage (0 to 10 days)

This method of corneal preservation, allows up to 10 days storage, and therefore allows more efficient use of all resources, including donor tissue, operating rooms, surgeon time,

and patient time. The ingredients that allow extended storage at 4°C are chondroitin sulphate and dextran. Both have been reported as an essential medium component for the repeated success of establishing primary cultures of human corneal endothelial cells (Lindstrom 1990a). They may play a role in the intracellular redox system, act as an antioxidant, contribute to the formation of an extracellular matrix act as a membrane and growth factor stabilizer (Lindstrom et al., 1990b). The addition of chondroitin sulphate added to tissue culture media led to the development of several intermediate storage solutions including K-Sol, CSM, Dexsol, and Optisol (see below).

1.1.2.1 M-K medium

M-K medium was developed by Bernard McCarey and Herbert Kaufman (McCarey and Kaufman et al., 1974). The corneoscleral segment is removed from the globe and placed in TC-199 medium. Dextran is added to a final 5% concentration as an osmotic agent. An antibiotic solution, usually gentamicin, is added to control bacterial contamination, and the cornea in preservation medium is stored at 4°C. Laboratory studies have shown that rabbit endothelium remains viable for up to 14 days, and human endothelium remains viable for at least 4 days when stored in M-K medium. (Van et al., 1975). However, other studies show no superiority over moist chamber storage (Friedland et al., 1976). It has been reported that the quality of M-K medium stored donor corneas is as good as that from 4°C moist chamber refrigeration or cryopreservation when storage lasts up to 96 hours (Brightbill et al., 1973).

The main disadvantage of this method is the limited storage duration. Most surgeons prefer to use M-K medium-stored corneas within 48 hours, which, as with 4°C moist chamber storage, does not always allow for transport or elective scheduling of surgery. Transplantation of the human cornea results in about 25% accelerated loss of endothelial cells compared to 33.6% endothelial loss after 12 months of transplantation (Bourne 1983).

1.1.2.2 K-Sol

Kaufman et al. (1984) first used chondroitin sulphate in TC-199 at 4°C and named it K-Sol medium. K-Sol contains 2.5% chondroitin sulfate in TC-199, 0.025m HEPES, and 100 µg/ml gentamicin sulfate (Wilson and Bourne 1989). Bourne reported average storage duration of 6 days (range 1 to 13) in 37 patients (Bourne et al., 1986). Significant endothelial cell loss appeared after 7 days storage. This medium is no longer commercially available due to its removal in 1988, because of contamination detected at the time of manufacture (Sieck et al., 1988).

1.1.2.3 CSM

In 1985, Minnesota Lions Eye Bank developed corneal storage media (CSM) with chondroitin sulphate for intermediate at 4°C. CSM is composed of minimum essential medium, 1.35% chondroitin sulfate, mercaptoethanol, and nonessential aminoacids (Wilson and Bourne 1989). Greiner et al. (1982) reported that CSM was more effective than K-Sol in maintaining ATP and the P spectral modulus (high/low energy phosphatic metabolites) over the same storage interval. Excellent endothelial cell preservation was maintained up to 12 months after keratoplasty in 83 donor corneas that had been stored for 1-7 days (average 3.6 days) in CSM (Lindstrom et al., 1987). Serum was not needed to support corneal viability in CSM at 4°C. CSM is no longer commercially available.

1.1.2.4 Dexsol

During storage at 4°C, chondroitin sulphate in CSM keeps the tissue more deturgesced than minimal essential media (MEM: a medium suitable for culturing a broad spectrum of mammalian cells, its amino acid concentrations conform closely to the protein composition of human cells) without chondroitin sulphate. However, CSM stored corneas still swell during storage. In addition, there is swelling of the CSM-stored donor cornea at the time of surgery (i.e. rebound swelling). Studies have shown that dextran is a clinically safe and effective osmotic agent (Bigar et al., 1975; Stark et al., 1975). In addition dextran-supplemented CSM has been shown to be safe and effective, reducing corneal thickness during storage, as well as rebound swelling at the time of surgery (Lindstrom 1990b).

Thus the FDA approved the manufacture and distribution of Dextsol in 1989. Endothelial viability was reported to be preserved for up to 2 weeks of storage (Lindstrom 1990b) although most surgeons use Dextsol-stored corneas within 5 days.

1.1.2.5 Optisol

Optisol is a hybrid of K-Sol, Dextsol, and CSM with additional constituents, including 14 additional vitamins, amino acids, cell metabolites, antioxidants, and precursors of adenine triphosphate. A prospective, multicentre, randomised, double masked clinical trial showed that Optisol was no more effective than Dextsol in preserving human corneas. However, because of its increased colloidal osmotic pressure, Optisol keeps the cornea thinner than Dextsol, which is preferred by most surgeons. Optisol GS at 4°C has been reported to preserve endothelial function up to 21 days (Means et al., 1995) and is widely used especially in the US (Kaufman et al., 1991; Lindstrom et al., 1992).

1.1.3 Long- term corneal storage methods

1.1.3.1 Cryopreservation

Cryopreservation is the only available method permitting corneal storage for an indefinite period (Ehlers et al., 1982; Kaufman et al., 1966) The transplantation of human corneas that have been cryopreserved in the presence of dimethyl sulfoxide (DMSO) has been successful, in both the short (Ehlers et al., 1982; Kaufman et al., 1966) and long-term. However, with the introduction of short-term storage at 4°C, cryopreservation failed to gain wide acceptance, owing to its technically demanding nature, its costliness, and the variable clinical (Brunette et al., 2001; Muller et al., 1967) and experimental (Bourne et al., 1999; Kaufman et al., 1966; Van et al., 1970) results achieved.

1.1.3.2 34°C Organ Culture

In Europe, long-term preservation in organ culture at 34°C is preferred (Armitage and Easty 1997; Bohnke 1991; Pels and Schuchard 1986, Pels et al 2000). Organ culture was first used successfully for corneal storage in the early 1970s, (Doughman et al., 1976). More than 14000 human donor corneas are processed annually in European Eye Banks (Pels et al., 2000). The cornea can be kept at 34°C for a current maximum of 28 days and

grafted successfully afterwards (Ehlers et al., 1999; Frueh et al., 1995; Pels et al., 2000 Redbrake et al., 1998). The organ culture medium consisted of Eagle's minimum essential medium with Earle's salts and 25mM Hepes buffer, containing 2% fetal calf serum, 2mM glutamine, 100U/ml, penicillin 0.1mg/ml streptomycin, and 0.25 µg/ml amphotericin B (Albon et al., 2000).

During the initial organ culture period, the donor tissue undergoes screening for donor history, microbial contamination and positive serology (hepatitis B&C, HIV, CMV) as well as HLA typing in rare cases if recipients are to be tissue matched (Pels et al., 2000).

1.2 Endothelial assessment of organ cultured corneas following storage

As well as the long term storage time, a major advantage to this preservation method is the endothelial assessment, carried out after storage, prior to transplantation. This ensures the transplantation of only high quality corneas. Corneas with an endothelial cell density below 2200 cells/mm² are deemed unfit for penetrating keratoplasty. This is a key issue for eye banks and also patients, because endothelial density is known to influence the long term survival of corneal grafts (Thuret et al., 2003b). Two days before the scheduled date for graft, endothelial quality is assessed by light microscopy. Endothelial cell density, the number and distribution of dead cells, loss of cells, and variation in cell size, shape and degree of folding are considered. On this basis, corneas are deemed suitable or unsuitable for penetrating keratoplasty (Armitage et al., 1997). However, this assessment results in a 30% discard of corneas at this stage due to endothelial deficiencies. Deterioration of the corneal endothelium has been shown to increase with increasing time of storage (Armitage et al. 1997; Pels et al., 1983; Borderie et al., 1998).

1.3 Summary

The knowledge and understanding of corneal preservation has led to improvements in the management of corneas for transplantation. The advantage of organ culture to other methods of preservation is the improved ability to diagnose infections before transplantation (Frueh et al., 1995; Redbrake et al., 1998; Doughman et al., 1980, 1982). More than 85% of infected corneas will be detected and discarded at this stage, and the

rest are detected by direct growth of contaminants in the organ culture media (Armitage et al., 1997). The disadvantages are the need for trained personnel, the risk of possible alterations in the components of the fetal calf serum used (Engelmann et al., 1998) and changes in the endothelium (Albon et al., 2000; Armitage et al., 1997, 2001).

1.4 Clinical use of corneal tissue

The corneal epithelium is a stratified cell layer whose integrity is important to the function of the cornea. The corneal epithelium provides an important barrier function against toxicant penetration of the eye. Disease or injury to the corneal epithelium through viral infection, penetration of chemicals applied topically, or exposure to toxic radiation results in toxicity, irritation, or inflammation. Such reactions in the cornea can cause loss of visual acuity (Offord et al., 1999).

Corneal transplant procedures may restore vision to otherwise blind eyes in some cases. There are many conditions in which corneal transplantation may be considered. The most frequent indications are: Corneal dystrophies, infection, mechanical trauma, chemical and thermal burns, and refractive surgery (Coster et al., 1995; Ang et al., 2004; Thoft et al., 1989; Dua et al., 2000).

Von Hippel, in 1886, was the first person to perform a successful human corneal transplant. **Lamellar (partial thickness) keratoplasty** was the technique of choice. Later, **penetrating keratoplasty** became more common as the techniques were improved and as the realisation of the importance of the endothelium became more widespread (Smith 1980). In a corneal transplant, a circular disc of tissue is removed from the centre of the cornea and then replaced by a disc from the donor eye using an instrument called a trephine. In penetrating keratoplasty, the disc removed as well as the replacement disc comprise the entire thickness of the cornea (Brady 1989). Lamellar keratoplasty however involves replacement of the patient's diseased anterior corneal stroma and Bowman's membrane with donor material. Therefore host endothelium, Descemet's membrane, and a part of the deep stroma are preserved (Hippel, 1886).

Because of the historically poor outcomes with conventional therapy in patients with severe ocular surface disease, new technique was required and the concept of epithelial transplantation came to account (Holland 1996). The early procedures were developed before the concept of limbal stem cells (SCs) was understood, and involved only transplantation of conjunctiva but to date limbal SC tissue transplantation is the method

of choice (Holland 1996). The management stem cell deficiency includes keratoepithelioplasty and partial or total allo-auto limbal transplant (Holland 1996).

1.4.1 Indications for limbal transplantation

The ocular surface describes the entire epithelial surface of the external eye, encompassing the corneal epithelium as well as bulbar and palpebral conjunctival epithelium. Therefore diseases that produce keratinisation of the palpebral conjunctiva (Stevens-Johnson syndrome) can induce serious mechanical damage to the corneal epithelium (Gunderson et al., 1958; Thoft et al., 1987). For proper movement of the globe and maintenance of normal globe relationship (by protection of the ocular surface from pathogens and provision of mucus for the tear film) a flexible conjunctival globe is necessary. Thus conditions causing conjunctival scarring (ocular cicatricial pemphigoid) can cause secondary corneal erosion and ulceration (Ballen et al., 1963; Leone et al., 1974). Mucus secreted by conjunctival goblet cells is also essential for ocular surface wetting and when this function is deficient (like after chemical injury) poor wetting of the corneal surface causes epithelial damage (Shore et al., 1990). These conditions could all be treated by conjunctival autograft transplantation for those situations in which abnormalities are not complicated by extensive damage of the limbal epithelial stem cells. However limbal autografts are employed now to restore a more normal ocular surface epithelium in situations in which widespread destruction of the limbal stem cells has occurred, for example: aniridia, a heritable disease in which the eye develops without an iris. Although absence of the iris is its most striking feature, it is a panocular disorder that also affects the cornea, (poor stromal support may produce stem cell dysfunction) anterior chamber angle, lens, retina and optic nerve (Alm 1973). More commonly, stem cell deficiency occurs as a result of acquired factors such as chemical or thermal injury, contact lens keratopathy, chronic limbitis, limbal surgery or Stevens-Johnson syndrome (Ang et al., 2004; Tsubota et al., 1996). Severe conjunctivitis and keratitis are common complications of Stevens-Johnson syndrome which result in stem cell deficiency. In each of the above conditions patients can experience severe photophobia, pain, reduced visual acuity, and even blindness. When limbal stem cell deficiency occurs, the neighboring conjunctival epithelium, which

is normally prevented from encroaching on the corneal surface by the limbal cells, migrates over the corneal stroma (conjunctivalization) (Kruse et al., 1990; Dua 1998). This may be a useful strategy to protect the stroma, except that the conjunctival epithelial cells do not share the same phenotypic properties as corneal epithelial cells, are not able to fully transdifferentiate into corneal epithelial cells, and also produce mucin from goblet cells (Chen et al., 1994; Kruse et al., 1990). All of these cause corneal opacity and therefore very poor vision, vascularisation, an unstable surface prone to epithelial breakdown, and patient discomfort (Tseng et al., 1984). Therefore the transfer of limbal cells optimises the rehabilitation potential of corneas that suffer from conditions described above (Kenyon et al., 1989).

1.4.2 Limbal stem cell deficiency therapy

1.4.2.1 Limbal transplantation

Limbal transplantation is required to restore the ocular surface of patients with stem cell deficiency covering the whole cornea (Dua et al., 1995).

There are several techniques that could restore stem cell function. In the case of unilateral limbal stem cell deficiency, a limbal autograft can be harvested from the healthy fellow eye (Jenkins et al., 1993; Frucht et al., 1998).

Limbal autograft transplantation is the preferred surgical technique for treating ocular surface disorders as it is usually safe to collect the donor limbus from a normal limbus in the contralateral eye. This is possible when disease is unilateral as is usually the case with tumors and sometimes with chemical burns. However, it is important to be sure that the limbus in the donor eye is normal, and not exhibiting mild pathology. However not all patients are prepared to risk compromising their better eye, therefore in these cases and where there is extensive bilateral disease, it becomes necessary to consider other alternatives, including the use of allografts (Pfister et al., 1994; Tsai et al., 1994). For bilateral stem cell deficiency a limbal allograft can be performed using tissue from a living relative or cadaver donor (Holland et al., 1996). Oversized corneal grafts incorporating the limbus can also be used to transplant limbal stem cells. However this

has still got its own limitation such as donor tissue availability and patient rejection of the graft and the requirement of more limbal stem cells, receiving long-term immunosuppression and, repeated surgery if rejection does occur for the recipient (Pels 1984). A policy has been adapted of using corneal donor within 24 hours of collection but the precise limits of storage have not been determined.

1.4.2.2 Ex vivo expansion of stem cell therapy

Finally corneal stem cell therapy that has recently been suggested as an alternative to limbal grafting may be considered for some patients to promote re-epithelialisation of the cornea, provide stable epithelium and prevent regression of new vessels, and restore epithelial clarity (Pellegrini et al., 1997). Pellegrini et al. (1997) have shown that epithelium can be produced for grafting of corneal patients with unilateral limbal stem cell deficiency. Further results have shown clinical improvement of the corneal surface following application of cultured autologous corneal epithelial cells (Tsai et al., 2000). James et al. (2001) has also shown the potential for using limbal tissue stored in eye banks as a source of cells for producing cultured corneal epithelial allografts. Having the cultured epithelium with a basement membrane is likely to improve graft take and may even promote survival of any cultured stem cells, allowing them to establish themselves in the host stem cell niche. For this purpose Tseng et al. (1998), used an amniotic membrane with utilizing placenta from normal caesarean sections, that was peeled from the chorion, spread on filter paper and stored at 4 °C in culture medium. Tseng has developed a technique where epithelial cells from a limbal biopsy are explanted directly onto amniotic membrane in culture and after 2 to 3 weeks the composite graft is then ready for the patient. Significant improvements in corneal clarity and surface stability have been achieved using this technique.

1.5. Summary

Development of a corneal storage technique for preservation of limbal stem cells especially for bilateral grafts would enhance stem cell transplantation. Since currently organ culture stored corneas are not used for this purpose. The future research should be

directed at determining epithelial and limbal stem cell homeostasis and viability during organ culture.

1.6 Structure of cornea

The cornea forms part of the structural coat of the eye. It is a highly specialised tissue that reflects and transmits light to the lens and retina (Freegard et al., 1997). The cornea is a unique connective tissue that combines transparency, refractive power for correct vision, tensile strength, and protection against infection (Maurice 1957; Chakravarti 2001). Its multilayered protective epithelium; transparent, refractive stroma of highly organised collagen fibrils and single layered endothelium, which regulates hydration, are suited for these functions (Maurice 1972). Fibrous collagen generates the mechanical strength of both cornea and sclera, providing strong protection of the inner components of the eye mechanically and maintaining the ocular contour. The cornea also serves as a part of the ocular biodefense system and for uninterrupted vision, the cornea minimises inflammation and maintains an immune-privileged status (Streilein 2003). As a mechanical barrier, the corneal epithelium has developed intercellular binding characterised by interdigitation of the cell membranes and the formation of junctional complexes (Wang et al., 1993; Garrod et al., 1993). The corneal epithelial surface also contributes significantly to visual clarity. Maintenance of corneal shape and clarity is critical to refraction. The cornea accounts for more than two thirds of the total refractive power of the eye. Therefore any slight change in corneal contour may result in a change of refractive error. Thus many components work together to maintain the optical function of the cornea.

1.6.1 Microscopic anatomy and physiology

The cornea can be divided into five layers: the epithelium, Bowman's layer, stroma, Descemet's membrane, and the endothelium. Although the preocular tear film is not part of the cornea, it is intimately associated with the cornea anatomically and functionally (Fig 1.6.1).

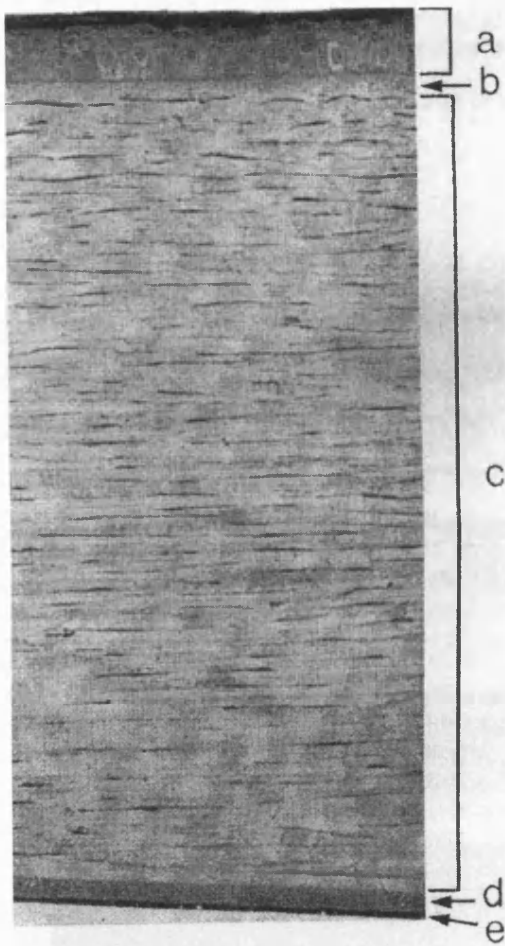


Fig 1.6.1 Transverse section of human cornea: (a) epithelium, (b) Bowman's layer, (c) stroma, (d) Descemet's membrane (e) endothelium (Copied from Hogan et al., 1971).

1.6.2 Tear film

The tear fluid covers the corneal surface, protects the cornea from dehydration and maintains the smooth epithelial surface. It consists of three layers: (Holly et al., 1977) a superficial lipid layer (about 0.1 μm), an aqueous layer (about 7 μm), and a mucin layer (0.02 to 0.05 μm). More than 98% of total volume of the tear is water. The tear film contains many biologically important factors including electrolytes, glucose, immunoglobulins, lactoferrin, lysosome, albumin, oxygen, and a range of various biologically active substances such as histamine, prostaglandins, growth factors, and interleukins (Van et al., 1994; Van et al., 1989). Therefore, the tear film serves not only as a lubricant and nutritional source for the corneal epithelium but also as the source of the

regulatory factors for the maintenance and repair of the corneal epithelium (Tsutsumi 1988; Wantanabe et al., 1987; Nishida et al., 1983).

1.6.3 Epithelium

The corneal epithelium (Fig 1.6.2) is composed of nonkeratinized, stratified squamous epithelial cells with thickness of approximately 50 μ m, that is, about 10% of the total thickness of the cornea (Hogen et al., 1971). The corneal epithelium consists of five to six layers of three different types of epithelial cells: two to three layers of superficial cells; consisting of highly flattened epithelial cells (squamous cells), two to three layers of polygonal, wing shaped cells (wing cells), and a monolayer of columnar basal cells (Hogan et al., 1971; Kenyon et al., 1979).

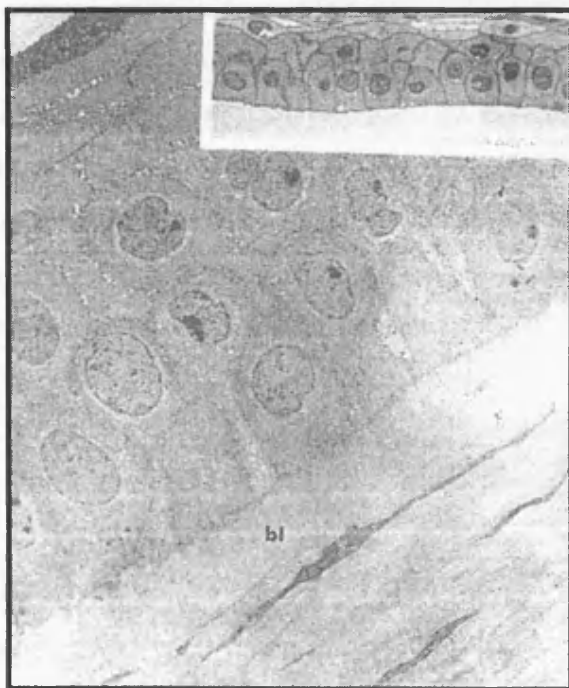


Fig 1.6.2 Corneal epithelial section as seen by light (inset) and electron microscopy showing superficial, wing, and basal cell layers and Bowman's layer (bl) (Mag. x 300, inset x 2700) (Copied from Gipson et al., 2000).

All cell layers of epithelium have a sparse accumulation of cytoplasmic organelles. Endoplasmic reticulum and mitochondria are sparsely distributed around the cytoplasm, with a golgi apparatus present in a supranuclear position, particularly in the basal cell layer. Of the three cytoplasmic filament types within all cells, keratin or intermediate filaments are the major type within the cytoplasm of cells of the corneal epithelium that provides a structural framework for the epithelial cytoplasm. The cytokeratins (CKs) are a heterogeneous class of approximately 30 structurally related polypeptides that comprise the intermediate filament system characteristic of epithelial cells (Franke et al., 1981; Moll et al., 1982; Sun et al., 1983). Based on isoelectric charge, the CKs are subcategorised into type I or acidic; and type II or neutral and basic. The intermediate filaments within ectodermally derived epithelial cells are formed by the pairing of two specific keratin proteins, one from each class (Sun et al., 1984; Cooper et al., 1985). In the corneal epithelium, as basal cells differentiate to apical cells, two keratin pairs are expressed sequentially. First K5 and K14 in basal cells, subsequently, suprabasal cells express K3 and K12 (Kurpakus et al., 1990). The other two types of cytoskeletal filaments within cells are actin filaments and microtubules. Actin filaments are prevalent as a network along the apical cell membranes, and at the junctions of the lateral membranes, where they are associated with adherens and tight junctions (Gipson et al., 1977).

1.6.3.1 Intracellular junctions of the corneal epithelium

The corneal epithelium, like all other epithelia, has intercellular junctions that function not only in cell adhesion but also in cell communication and barrier formation. Four junction types are present.

Desmosomes

Desmosomes, which are present along the lateral membranes of all corneal epithelial cells, function in cell-to-cell adherence (Fig 1.6.3.). Desmosomes are intercellular, plaque-bearing adhesive junctions (Garrod et al., 1993; Green and Janes 1996; Buxton et al., 1992).

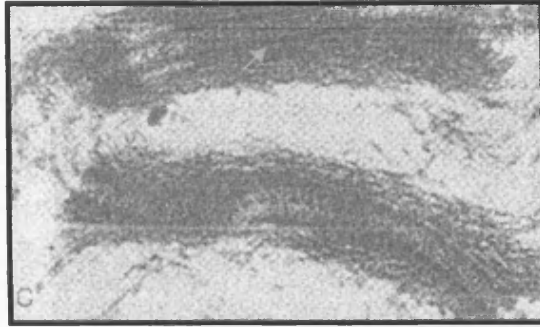


Fig 1.6.3 Cell to cell junctions of the corneal epithelium, as demonstrated by electron microscopy, Desmosomes are prominent along cell membranes (yellow arrow, Mag. x 66000, copied from Zieske et al., 1989).

The major desmosomal glycoproteins, the desmocollins (Dsc) and desmogleins (Dsg), are members of the cadherin family of cell adhesion molecules. Three distinct isoforms of both desmocollin (Dsc1 through Dsc3) and desmoglein (Dsg1 through Dsg3) have been isolated, each protein is the product of a separate gene (Koch et al., 1994; Chidgey et al., 1997). Other desmosomal proteins include plakoglobin, plakophilin, and desmoplakin, which bind to desmosomal glycoproteins in the plaque region (Korman et al., 1989; Cowin et al., 1986; Mathur et al., 1994). Desmoplakin links desmosomes to the cytoskeleton (Norvell et al., 1998).

Adherens junctions

Adherens junctions, which are present along the lateral membrane of the apical cells of the epithelium, function to maintain cell-to-cell adherence in the region of the tight junctions.

Tight junctions

Tight junctions (TJs) are present along with adherens junctions in apical cell lateral membranes, where they function to provide paracellular permeability barrier (Fig 1.6.4, McLaughlin et al., 1985; Wang et al., 1993).

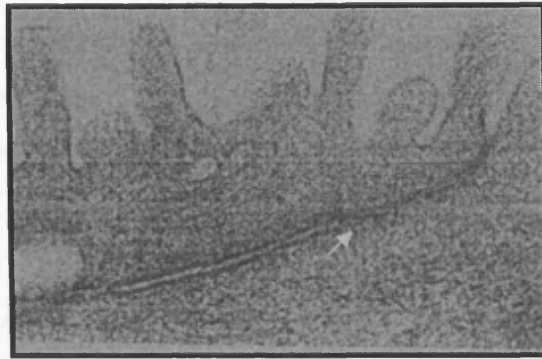


Fig 1.6.4 Cell to cell junctions of the corneal epithelium, as demonstrated by electron microscopy, areas of apparent membrane fusion at the tight junction are obvious (arrow, Mag. x 66000), (Copied from Zieske et al., 1989).

TJs appear as a series of apparent fusions, involving the outer leaflets of the plasma membranes of adjacent cells. In addition to the barrier function, TJs serve a fence function. For vectorial transport of materials across cellular sheets, plasma membranes are functionally divided into apical and basolateral domains. TJs are the morphological counterpart of a localised diffusion barrier between those domains (Anderson et al., 1993). The TJ complex includes two integral transmembrane proteins: claudin and occludin; and membrane-associated proteins such as ZO-1, ZO-2, and ZO-3 (Tsukita et al., 2001).

Occludin (~60 kDa) was the first transmembrane protein identified (Furse et al., 1993) and current data indicate that it plays a regulatory rather than a structural function in TJs, a function that is controlled partially by phosphorylation (Saitou et al., 1998 and Sakaibara et al., 1997).

Claudin (~23 kDa) comprises a family of transmembrane proteins that form the strands of the tight junction and are the only junctional proteins known to have tissue specificity. Both occludin and claudins contain four transmembrane domains, with both N and C termini oriented into the cytoplasm, but these two proteins show no sequence similarity

(Furuse et al., 1998; Morita et al., 1999; Tsukita and Furuse 1999). Different mixtures of claudins and occludin create TJ strands that are associated laterally with strands of adjacent cells, forming paired strands that eliminate the extracellular space (Furuse et al., 2001). The membrane-associated guanylate kinase homologue (MAGUK) protein family is distinguished by a core cassette of protein-binding domains, which include one or more PSD95/dlg/ZO-1 (PDZ) domains, an SH3 domain and a region of homology to guanylate kinase (GuK). The domains assemble signaling, receptor, and transport complexes at a variety of membrane junctions.

The junction scaffold in TJs is formed by three members of the MAGUK protein family: ZO-1, ZO-2 and ZO-3, which bind not only to occludin or claudin, but also to the cortical actin cytoskeleton (Balda et al., 1993; Fanning et al., 1998; Gumbiner et al., 1991; Haskins et al., 1998; Itoh et al., 1999a, b and Stevenson et al., 1986). In the rabbit cornea, ZO-1 is found in the superficial layer of epithelium where TJs are located, as well as between wing cells and basal epithelial cells that have no TJ structure (Surgue and Zieske 1997).

Gap junctions

Gap junctions (Fig 1.6.5) which function in cell-to-cell communication, allow intercellular passage of small molecules up to 2000 Da (Goodenough et al., 1996; Loewenstein et al., 1981; Beyer et al., 1990; Wolosin et al., 2000). Gap junctions are specialised appositions between two cells generated by the local aggregation of large numbers of large pore (about 2 nm) intercellular channels (Loewenstein et al., 1981; Kumar and Gilula 1992; Goodenough et al., 1996).

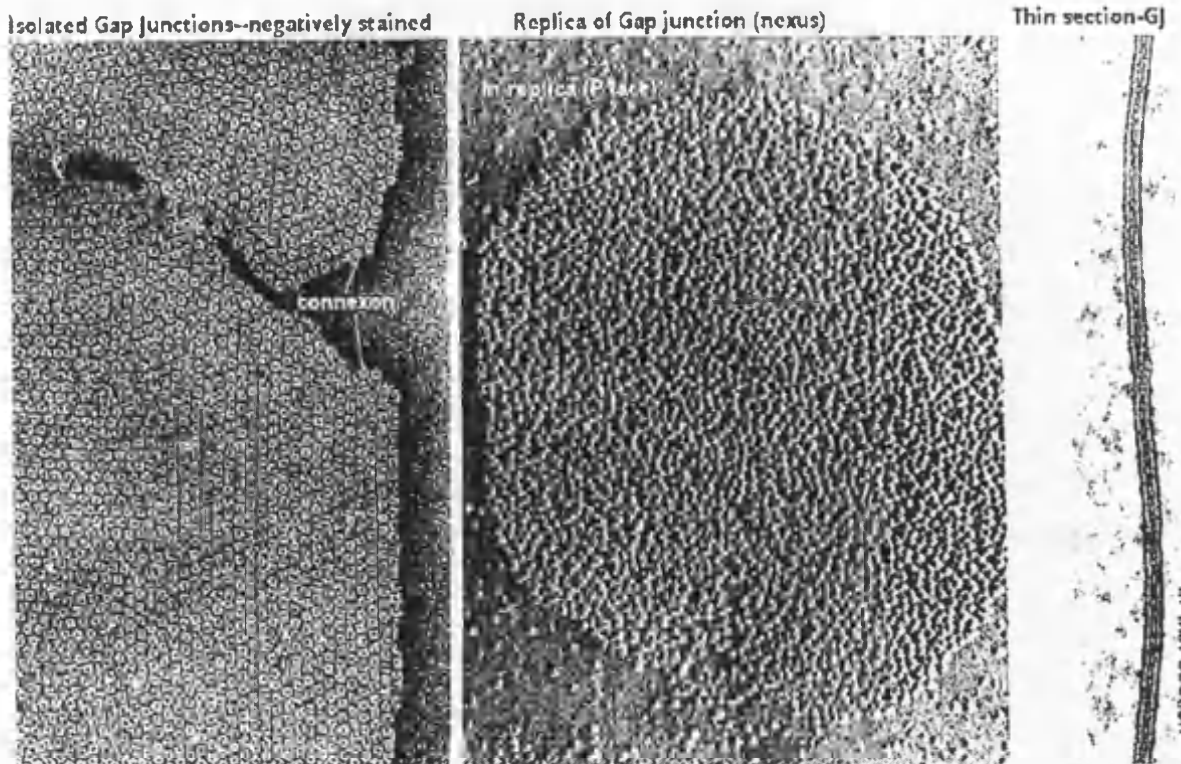


Fig 1.6.5 The above freeze-fracture /freeze etch image shows the internal view of the gap junction on the left. The proteins look like little donuts which reflects the fact that they are actually a channel. These proteins are "connexon" molecules. The side facing the cytoplasm (called the P face) is shown in the panel. The region looks like aggregated lumps. Finally the typical electron microscopic view is seen in the third panel. This shows a thin line between the two plasma membranes indicating a "gap junction". This figure was modified from Bloom and Fawcett 1994, Figure 2-14.

The channels are formed by an extended (>13 species) family of related amphipathic polypeptides called connexins, which are more commonly identified according to their molecular weight. They allow direct passive diffusion of low molecular weight solutes (< 1.2 kDa) between neighbouring cells without solute loss into the extracellular space. This molecular discrimination range permits efficient passage of ions and typical metabolites, as well as second messengers such as cAMP and $1P_3$. Two gap junction proteins have been identified in corneal epithelium, Cx43 and Cx50 (Dong et al., 1994). Cx43 is almost

completely localised to basal cells. Cx50 is present in all cell layers.

1.6.3.2 Specifications of epithelial surface

The two surfaces of the corneal epithelium, the apical and basal surfaces, have also specialisations, indicative of their roles in epithelium. The apical surface is specialised to maintain the tear film and mucous layer (Nichols et al., 1985) and with that layer provides the extraordinarily smooth refractive surface of the cornea. The basal surface is specialised to provide tight anchorage of the epithelium to the stroma (Gipson et al., 1987; Gipson et al., 1983). A series of linked structures, termed the anchoring complex, form an intertwining network and terminate distal to the basement membrane in anchoring plaques.

1.6.4 Basement membrane zone

The basement membrane zone is a complex interface between the basal cells of the epithelium and underlying Bowman's layer. Major components of the basement membrane are type IV collagen and laminin. Furthermore heparin sulphate proteoglycans, fibronectin, and fibrin are also present (Berman et al., 1983). On the stromal side of the basal lamina, anchoring fibrils of type VII collagen, terminating in anchoring plaques, secure the basal lamina to Bowman's layer. The basal lamina itself is composed of type IV collagen, the glycoproteins laminin and entactin/nidogen, and heparan sulphate proteoglycan (Sanes et al., 1990; Gipson et al., 2000). The basal plasma membrane of the basal cells is rich in hemidesmosomes, specialised adhesion plaques, which contain $\alpha 2\beta 6$ integrin one of the four cell adhesion molecule super-families are important for interactions between cells, and the extracellular matrix and for signal transduction. They are glycoproteins consisting of two non-covalently bound α - and β -subunits that have intracellular, transmembrane and extracellular domains adhesion receptors. These receptors bind to adhesive glycoproteins such as laminin and fibronectin in the underlying basement membrane. The strength of adhesion is achieved by binding between the epithelial/basement and Bowman's layer (Jain and Azar 1994).

1.6.5 Bowman's layer

A Bowman's layer is an acellular membrane like zone between the corneal epithelium and the corneal stroma in humans and in certain other mammals. It is an 8 to 10 μm acellular zone of randomly arranged collagen fibrils that forms an interface between the basal lamina of the epithelium and the subjacent lamellar stroma. Constituents of this layer are synthesised and secreted by both epithelial cells and stromal keratocytes (Rodrigues et al., 1982) it contains several collagen types, including types I, V, and VII, (Gordon et al., 1994; Nakayasu et al., 1986) and proteoglycans such as chondroitin sulphate proteoglycan (Li et al., 1991).

The specific function of this layer is not clearly understood, but its feltwork of collagen fibrils may stabilize the transition between the epithelial and stromal layers, ensure adhesion of the overlying epithelial cells to the stromal matrix, and contribute to the smooth curvature of the corneal surface (Klyce et al., 1988a).

1.6.6 Stroma

The majority of the cornea is composed of stroma (90%). It is the thick collagenous layer posterior to Bowman's membrane (Fig 1.6.6).

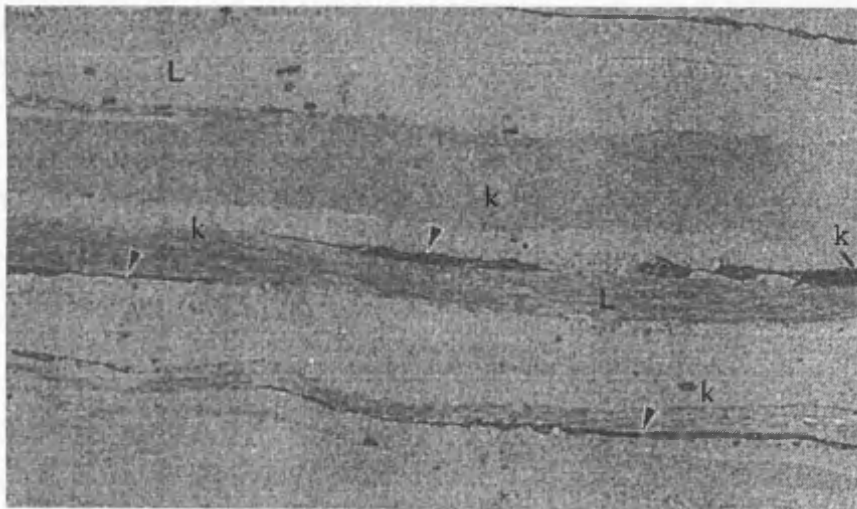


Fig 1.6.6 Corneal stroma showing collagen bundles arranged in lamellae (L), and long attenuated processes (arrow heads) of the stromal keratocytes (K) (Mag. x 4800) (Copied from Gipson et al., 2000).

The corneal stroma consists of extracellular matrix, keratocytes and nerve fibers. Cellular components occupy only 2% to 3% of the total volume of the stroma and keratocytes are the major cell-type in the corneal stroma (Chen and Tseng 1991). Various extracellular matrices, mainly collagen and glycosaminoglycans, occupy the rest. Collagen occupies more than 70% of the dry weight of the cornea. The collagens in the corneal stroma are primarily collagen type I with a lesser amount of collagen type III, V and VI (Komai et al., 1991). Keratan sulphate proteoglycan and dermatan sulphate proteoglycan are the predominant proteoglycans within the corneal stroma (Hassel et al., 1992). Lumican, keratocan, and mimecan are the core protein of keratan sulphate proteoglycan, whereas decorin is the core protein for dermatan sulphate proteoglycan (Chakravarti and Magnuson 1995; Corpuz et al., 1996; Funderburgh et al., 1997). The basic structural unit of the fibrillar collagens is tropocollagen, an asymmetric molecule about 300nm long and 1.5nm in diameter. Fibrillar collagens are composed of three polypeptide chains coiled in a triple helix. These molecules polymerise to form elongated collagen fibrils with diameters of 25 to 30nm. The uniformity of collagen fibril diameter appears to result from specific interactions between type V collagen, located toward the centre of the fibril, and type I collagen, on the fibril exterior. The relative ratio of type V to type I collagen appears to regulate fibril diameter (Hogen et al., 1971; Rodrigues et al., 1982). The interfibrillar distance also is highly uniform and may be maintained by apposing interactions at the fibril surface.

Proteoglycans bind to the exterior surfaces of collagen fibrils. The polyanionic nature of the glycosaminoglycan side chains attracts cations and water molecules and may exert a swelling pressure on the collagen fibrils, which is balanced by the interactions between collagen types XII and I. Microfibrils composed of type VI collagen also associate with type I collagen (Nakamura et al., 1992) but the specific function of these fibrils is not known. Collagen fibrils are packed in parallel bundles extending from limbus to limbus, and the bundles are arranged in layers, or lamellae (Meek and Leonard 1993, Olsen and McCarthy 1994).

The stroma of the human cornea contains 200 to 250 lamellae (Pratt 1985). Lamellae in the middle and posterior regions of the stroma are arranged at approximate right angles, whereas those in the anterior stroma are arranged randomly. The small diameter of collagen fibrils and their close, regular packing creates a lattice or three-dimensional diffraction grating (Berman et al., 1991). The lamellar organisation of the stroma also produces a uniform tensile strength across the cornea, withstanding intraocular pressure and maintaining appropriate corneal curvature.

The matrix components of the lamellar stroma are secreted and maintained by stromal fibroblasts, also known as keratocytes. These long, attenuated cells are arranged parallel to the corneal surface and are located between the collagen lamellae. The keratocyte cell body contains an elaborate rough endoplasmic reticulum and golgi apparatus, reflecting its active synthetic function. Using ultrastructural techniques, Smith et al. (1969) found evidence of desmosomes, whereas Hogan and co-workers (1971) found tight junctions, both junctions being associated with structural support rather than intercellular communication. Gap junctions (Cx 43) have also been identified at the point of contact between keratocyte processes (Ueda et al., 1987). Such junctions are known to provide metabolic contact between adjacent cells, and their presence in keratocyte implies transcellular communication. Nishida et al. (1988) confirmed the presence of an extensive network of cell processes and suggested that keratocytes were arranged in this manner to maintain synchronised metabolic homeostasis of the corneal stroma. Birk et al. (1990) have shown that keratocytes can synthesise and secrete collagen types I, III, V, VI, The glycosaminoglycans hyaluronan, keratan sulphate, chondroitin 4-sulphate, chondroitin 6 glycoproteins such as fibronectin, laminin and nidogen (Khohno et al., 1987; Schittny et al., 1988). The interaction between these extracellular matrix macromolecules and water which makes up almost 78% of the stromal volume, determines the transparency and refractile properties of the cornea (Maurice 1957).

Nerve fibers were found to invaginate stromal keratocytes as well as corneal epithelial cells. This finding suggests that nerves may mediate information exchange between the

epithelium and stroma under certain conditions, such as corneal wounding (Muller et al., 1996).

1.6.7 Descemet's membrane

Descemet's membrane is the thick extracellular matrix synthesised and secreted by the corneal endothelium (Iwamoto et al., 1965). In adult the matrix has two layers: the anterior banded layer that is formed during fetal development and consists of highly organised collagen lamellae and proteoglycans and a posterior amorphous layer that is synthesised after birth and is less organised than the fetal layer. It contains fibronectin, laminin, and types IV and VIII collagen, heparan sulphate, and dermatan sulphate proteoglycan (Suda et al., 1981). It gradually increases in thickness from birth (3 μm) to adulthood (8 to 10 μm). Clinically Descemet's membrane is tough and resistant to enzymatic degradation by MMPs. It is tightly adherent to the posterior surface of the corneal stroma and reflects any change in the shape of the stroma. If the corneal stroma swells, the folding of Descemet's membrane can be observed clinically. Descemet's membrane does not regenerate (Klyce et al., 1988a).

1.6.8 Endothelium

The endothelium is the single layer of cells located at the posterior of the cornea which permits the passage of nutrients from the aqueous humor into the cornea (Tuft et al., 1990; Waring et al., 1982). The endothelium is the major cell layer responsible for maintaining the relatively low level of stromal hydration necessary for corneal transparency. Stromal hydration is controlled by the activity of ionic pumps in the plasma membrane of endothelial cells (Joyce et al., 1994). The extracellular ion concentration produced by these pumps draws water from the stroma, thus maintaining the highly organised collagen lamellar structure required for transparency (Klyce and Beuerman 1988). Human corneal endothelial cells are 20 μm wide and 5 μm high and form a hexagonal cell shape (Fig 1.6.7) and numerous lateral, interdigitating cellular processes (Davanger et al., 1985; Sherrard et al., 1990).

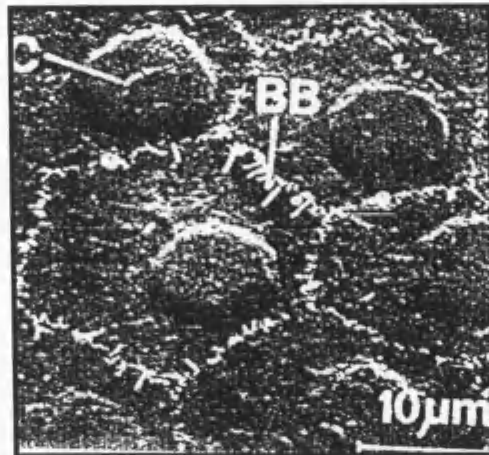


Fig 1.6.7 Scanning electron micrograph of the surface of the corneal endothelium illustrating the hexagonal shape of the cells as well as the other features, including a single cilium (C), and long lateral projections (BB) that bridge from one cell to on to the body of adjacent cells (Svedbergh and Bill 1972).

These processes increase the area of contact between neighbouring cells. The ultrastructural features of the endothelium reveal numerous mitochondria within the cytoplasm indicative of metabolic activity of these cells with an elaborate rough and smooth endoplasmic reticulum and a prominent golgi apparatus reflective of high levels of protein synthesis (Davanger et al., 1985; Sherrard et al., 1990). Tight junctional complexes (ZO-1 and cadherins) of endothelium do not form belts or rings extending around cells, as found in the epithelium (Hirsch et al., 1977). Gap junctions (Cx43) formed between adjacent cells, are located at all levels of the lateral plasma membrane below the tight junctional complexes (Iwamoto et al., 1965; Hirsch et al., 1977; Leuenberger et al., 1973; Hirsch et al., 1976).

The human endothelium is not self renewing and the number of endothelial cells decreases with increasing age (Murphy et al., 1984). The overall rate of cell loss accelerates if the endothelium is injured from trauma, disease or dystrophy (Treffers et al., 1982). The remaining cells become larger and more pleomorphic, and lose the ability to maintain or restore normal barrier and pump function (Oneal et al., 1986; Carlson et al., 1988) and consequently cause opacification. Although the corneal endothelium is considered a non replicating tissue, these cells do possess proliferative capacity. Joyce et

al. (1996) have revealed that corneal endothelial cells, in vivo resemble limbal basal cells in their cell cycle marker profile and are arrested in G1 phase of the cell cycle.

1.7 Epithelial cell homeostasis

Human corneal epithelium is a non-keratinised stratified squamous epithelium composed of 5-6 layers and is subject to a constant process of cell renewal and regeneration. The following review will provide an understanding of the important aspects of human corneal epithelial cell homeostasis.

1.7.1 Epithelial stem Cell

The presence of stem cells is postulated in all self-renewing tissue, where they serve as the reserve for cell renewal and cell proliferation. Epithelial stem cells share the following characteristics which have also been postulated for the stem cells of the corneal epithelium. First, stem cells have a long life span which might be equivalent to the life of the organism which harbours them. Second, they have an almost unlimited potential for cell division (Cotsarelis et al., 1989). Third, stem cells are slow cycling, which indicates a low mitotic activity (Cotsarelis et al., 1989). Fourth, stem cells are the least differentiated cells in the tissue, and lack markers that indicate greater differentiation. Fifth, stem cells can be induced on demand (Sherman et al., 1981) by certain factors to differentiate into transient amplifying cells.

In contrast to stem cells, transient amplifying cells are characterised by a high mitotic rate but a limited proliferative capacity. At a higher level of differentiation than stem cells, transient amplifying cells embark on a pathway leading to further differentiation and ultimately to cell death. After a high but limited number of cell divisions transient amplifying cells further differentiate to post-mitotic cells which have lost the capacity for cell division. These post-mitotic cells then become terminally differentiated and die after a certain time.

1.7.2 Location of stem cells

In contrast to haematopoietic stem cells, which have been identified by antibodies, the existence of stem cells has not been positively proven in any of the remaining self-renewing tissues such as the epithelium of the skin or the ocular surface. In skin it was initially hypothesised that the entire basal layer consisted of stem cells. Then, later the

Langerhans cells were considered to be stem cells, and then radiation dose survival studies suggested that stem cells might comprise 2-7% of basal layer cells (Potten 1979). However the label-retaining cells (LRCs) study using both BrdU and tritiated thymidine revealed that in oral epithelium LRCs are located in discrete regions of tongue and palatal (Bickenbach et al., 1984) and in the murine ear epidermis, in the basal layer, near the periphery of differentiating cell columns (Potten et al., 1974). It was in the 1990s that research using thymidine in murine haired epidermis discovered that the majority of label retaining cells (LRCs) in the skin reside in bulge, region of the hair follicle, with only a small fraction of LRCs in the basal layer of interfollicular epidermis (Cotsarelis et al., 1990). However, a wide body of indirect evidence suggests the presence of stem cells in the limbal corneal region of the corneal epithelium (Davanger and Evensen 1971; Schermer et al., 1986; reviewed by Boulton & Albon 2004).

1.7.3 Location of corneal epithelial stem cells

Evidence for the limbal location of corneal epithelial stem cells is based mainly on the observation that only the limbal basal epithelium contains cells that exhibit two of the aforementioned characteristics of stem cells. First, the limbal basal epithelium contains the least differentiated cells of the corneal epithelium (Schermer et al., 1986). Second, the limbal basal epithelium contains cells, which exhibit the proliferative characteristics of stem cells (Ebato et al., 1987).

1.7.4 Does the limbus contain a stem cell niche?

In the cornea, stem cells are thought to be located in the limbus, the transition zone between corneal and conjunctival epithelium. The concept that epithelial cells in the limbal region are involved in the renewal of corneal epithelium was reported first by Davanger and Evensen in 1971. They observed that pigmented cells in the limbus migrated centripetally. The hypothesis that stem cells are localised in the basal cell layer of the limbus then evolved when Schermer et al. (1986) reported that in situ all corneal epithelial cells except limbal basal cells express keratin 3 (K3). These data suggest that K3 is a differentiation marker and that lack of expression of K3 in the limbal basal cells indicates they are less differentiated than the remainder of cells in the corneal epithelium.

Other lines of evidence that support the local isolation of stem cells in the limbal basal cell layer include: 1) only limbal basal cells retain tritiated thymidine label for long periods, indicating that they have a long cell cycle time (Cotsarelis et al., 1989). 2) Limbal basal cells have a higher proliferative potential in culture than central corneal epithelial cells (Ebato et al., 1987, 1988). 3) Surgical removal of the limbal region results in healing with non-corneal epithelium (Chen et al., 1991; Huang et al., 1991). 4) Limbal transplants result in regeneration of cornea-like epithelium (Kenyon et al., 1989) and 5) limbal basal cells respond to central corneal wounds by undergoing cell division as would be expected of stem cells (Zieska et al., 1992).

1.7.5 The limbo-corneal epithelium

A variety of epithelia subjected to daily wear depend on constant cell renewal for their survival. Proliferative capacity in the stratified epithelia is limited to basal cells in contact with the substratum.

The basal cells covering the outer vascularised limbal rim expressed the K5/K14 cytokeratin pair seen in the basal cells of most stratified epithelia but were devoid of K3 (Schermer et al., 1986). The same observation was later extended to K12, another corneal specific cytokeratin (Kurpakus et al., 1990; Chaloin et al., 1990; Wu et al., 1994). Along the basal cell axis, expression of these proteins starts at the limbo-corneal margin, in concurrence with the stromal transition from vascular to avascular. From this margin to the corneal centre, all basal cells are uniformly positive for K3 and/or K12. Thus, the basal cell layer of the limbus must contain stem and transient amplifying cells, as well as any early precursors that may exist between the two identifiable states (Fig 1.7.1). It has been concluded that bona fide stem cells may represent as much as 10% of the total limbal basal cell population, and as cells move centripetally across the limbo-corneal (LC) demarcation, they concomitantly develop TA cell features and initiate de novo expression of K3 and/or K12.

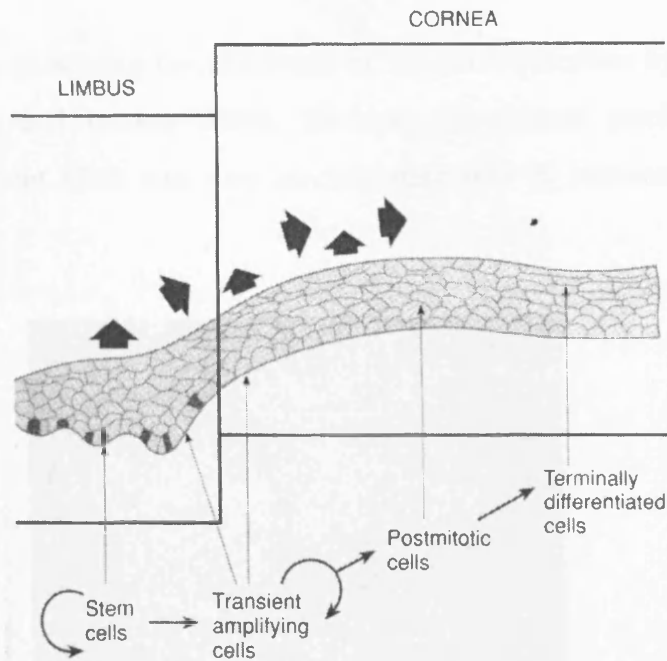


Fig 1.7.1 Limbal and corneal epithelium showing the location of the stem cells and changing characteristics of cells as they move anteriorly and centrally from the limbus (Copied from Kruse et al., 1994).

1.7.6 Proliferation and apoptosis of corneal epithelial cells

1.7.6.1 Epithelial cell proliferation

Epithelial cell proliferation is the fundamental characteristic of corneal epithelium and is confined to its limbal region the transitional zone between the cornea and the conjunctiva, where stem cells are located that could undergo mitotic division (Tseng 1989). The function of stem cells is to replace cells that are continuously lost from the corneal surface. During this process of cell renewal postmitotic cells are displayed into the suprabasal layers, migrate from the periphery to the centre, become mature and finally desquamated (Kruse et al., 1994; Zieske et al., 1992). Lehrer et al. (1998) demonstrated that a large number of normally slow cycling limbal epithelial stem cells could be induced to replicate in response to a single physical or chemical perturbation of the central corneal epithelium (Lehrer et al., 1998). Sun (2004) indicated that corneal epithelium uses three strategies to expand its cell population during wound healing (Fig 1.7.2): (1) recruitment of stem cells to produce more TA cells; (2) increasing the number of times a TA cell can

replicate; and (3) increasing the efficiency of TA cell replication by shortening the cell cycle time (Sun and Lavker 2004). However the control mechanisms behind the proliferation are not clear and play an important role in homeostasis of the corneal epithelium.

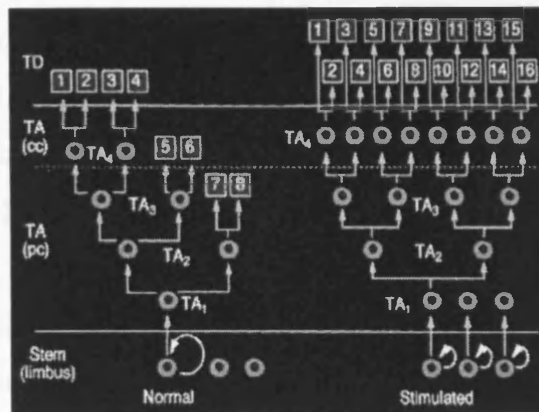


Fig 1.7.2 Three strategies of epithelial proliferation. Under normal conditions, stem cells located in the limbus rarely cycle (large curved arrow). Upon division, stem cells produce regularly cycling TA cells (vertical arrows) located in the peripheral (pc) and central (cc) corneal epithelium. Young TA cells (TA₁, 2, 3) are preferentially located in peripheral cornea, whereas more mature TA cells (TA₄) reside in the central cornea. Under these conditions, not every TA cell will utilise its full potential to divide, since they become terminally differentiated cells (TD; squares, cells 1–4). Upon stimulating wounding, the corneal epithelium expands its cell population. It can recruit more stem cells to divide with a more rapid cell cycle time (small curved arrows) thus producing more TA cells (see TA₁). It can induce the young TA cells to utilise more fully their replicative capacity thus producing more (mature) TA cells (TA₄). Finally, by shortening the cell cycle time (short vertical arrows), the efficiency of TA cell replication is increased (Copied from Lehrer et al., 1998).

1.7.6.2 Apoptosis

Apoptosis, or programmed cell death, is a defined form of controlled or programmed, cell death (Kerr et al., 1972). Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another

form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems (Majno et al., 1995; Arzynkiewicz et al., 1997). Necrosis is a violent form of cell death that is caused by a range of noxious chemicals, biological agents, or physical damage (Wyllie 1997). It is associated with rupturing of cellular membranes, swelling of the cells and random destruction of the cellular structures. The cytosolic proteins of the dying cell are released into the intercellular space, causing an inflammatory reaction (Wyllie 1997). Necrosis typically involves large groups of cells that have become victims of the same pathological assault (Arends and Wyllie 1991).

Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide). Upon receiving specific signals instructing the cells to undergo apoptosis a number of distinctive biochemical and morphological changes occur in the cell (Kerr et al., 1972).

Apoptosis occurs in single cells that separate themselves from neighbouring cells and/or the intracellular matrix (Kerr et al., 1972). The process involves the breakdown of the cytoskeleton, membrane blebbing, and rather than swelling, an apoptotic cell loses volume and shrinks. At the same time the cellular matrix is actively dismantled by caspases. This is an energy-requiring, coordinated process opposite to necrosis, which does not require energy after the initial pathological attack that sets the process in motion (Wyllie 1997). The plasma membrane is never compromised and this important characteristic of membrane integrity allows the cell to retain intact cytoplasmic contents. In the end, neighbouring cells or macrophages will take care of the apoptotic bodies by phagocytosis, averting an inflammatory response.

However, as with most things in life, death also evades classification into two exact categories. Apoptosis and necrosis are often viewed as endpoints on a sliding scale. Some necrotic cell deaths display apoptotic properties such as chromosomal condensation or DNA fragmentation, and sometimes a process that would be defined as apoptosis

possesses some qualities of necrosis (Majno & Joris 1995). The role of apoptosis in cell population control during development has suggested that there are inherent cellular programmes that lead the cell to self-destruct (Sulston et al., 1983).

Also, inhibition of RNA or protein synthesis can, in many cases, abrogate cell death by apoptosis (Wyllie et al., 1984), although it usually accelerates necrosis. Thus, it appears that gene expression is necessary for cell death. Yet, there is another level of complexity, as, in some instances, inhibition of protein or RNA synthesis, or even explosion of nuclei, does not prevent what otherwise appears to be programmed cell death (Eastman et al., 1994). Such cells are thought to be primed for apoptosis.

Caspases

A family of proteins known as caspases are typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes (Fig 1.7.3). The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus. The result of these biochemical changes is the appearance of morphological changes in the cell (Alnemri et al., 1996). So far comprising 14 members (Ahmad et al., 1998), related to interleukin-1 β converting enzyme and termed caspases (Alnemri et al., 1996) have been shown. Caspase-8 is the major caspase that initiates death-receptor-mediated extrinsic apoptotic cascades.

Caspase-9 is a major caspase for cytochrome *c*-mediated apoptotic intrinsic mechanisms thought to be initiated by extracellularly applied stresses (Green et al., 1998). Caspase 3 is known to be an important molecule in the cellular cascade that can be activated as a result of either pathway (Ashkenazia et al., 1998). Caspase activation during apoptosis results in the cleavage of critical cellular substrates, including PARP (poly ADP-ribose polymerase, PARP), which is normally responsible for DNA repair (Tewari et al., 1995).

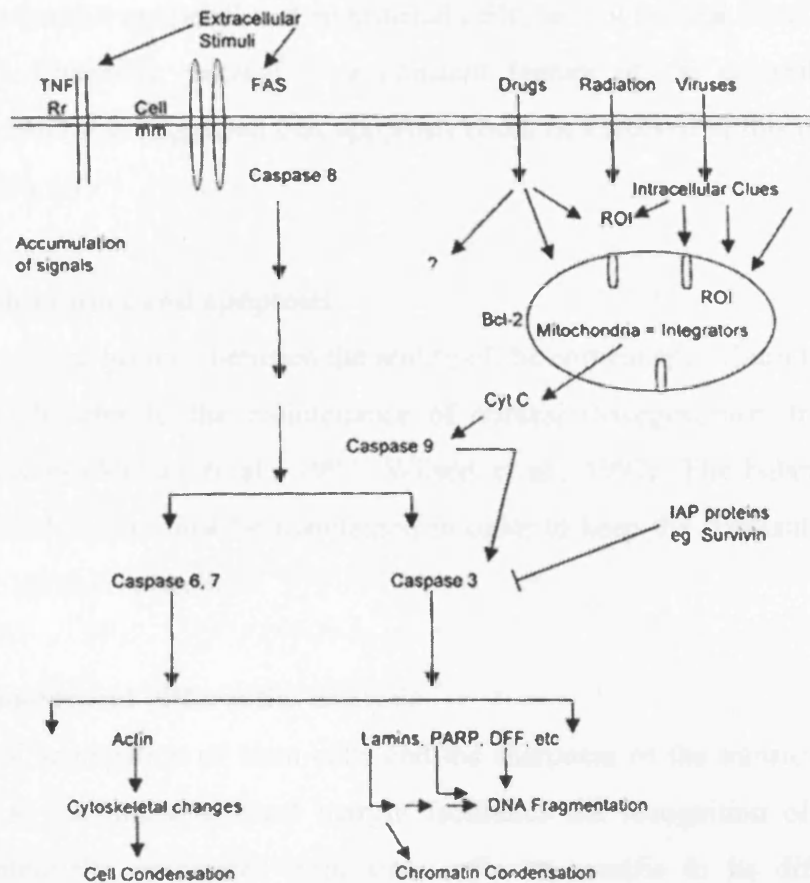


Fig 1.7.3 Outline of apoptosis signaling pathways. The mitochondria act as the principal integrators of signals for apoptosis, and a few examples of cellular targets for executioner caspases are also shown (Copied from Studzinski 1999).

Apoptosis in the cornea

Apoptosis is a programmed cell death, carried out through the activation of an internally encoded suicide programme (Thompson et al., 1995). Wilson et al. (1996a) suggests that apoptosis contributes to the mechanisms of wound healing in the cornea by which anterior keratocytes disappear after epithelial scraping. There exists a variety of extrinsic and intrinsic signals to induce apoptosis. Among these, Fas ligand is a key physiologic

inducer of apoptosis (Nagata and Golstein 1995). In cornea, Fas ligand mRNA and Fas mRNA are expressed in cultures of all three major cell types, whereas Fas ligand protein is present in corneal epithelial and endothelial cells, but not stromal fibroblasts (Wilson et al., 1996a). Epithelial renewal is a constant feature of the corneal epithelium in homeostasis and it is suggested that apoptosis could be involved in this process (Ren and Wilson 1996a, b).

1.7.6.3 Proliferation and apoptosis

The physiological balance between the ability of the corneal epithelium to proliferate and turnover contributes to the maintenance of corneal deturgescence, transparency, and normal division (Mohan et al., 1997; Wilson et al., 1997). The balance between cell growth and cell death must be maintained in order to keep the constant total number of cells in the corneal epithelium.

1.7.7 Limbo-corneal differential analysis

The physical segregation of stem cells and the sharpness of the transition to a different phenotype at the limbal corneal margin facilitates the recognition of components or features potentially associated with stem cells or specific to its differentiation and proliferation. The differentiation of stem cells to transient amplifying cells seems to be stimulated by serum factors, such as retinoic acid. The amplification of transient amplifying cells appears to be supported by polypeptide growth factors such as EGF, aFGF, bFGF or NGF and increasing the concentration of extracellular calcium, and inhibited by TGF and serum factors such as retinoic acid (Kruse et al., 1994). The limbal epithelium is under the influence of the limbal vasculature, while the cornea is avascular (Buskirk et al., 1989). Therefore, factors from serum such as retinoic acid might occur in limbal epithelium in higher concentrations than in the corneal epithelium and the resulting concentration gradients could have regulatory functions. Taken together physiologically occurring regional differences in various regulatory factors might play a role in the regulation of limbal and corneal epithelium. Below is the growing number of

differential features or expression changes in limbal and epithelial cornea (Wolosin et al., 2000).

1.7.7.1 Melanin

Limbal pigmentation (involving melanin) is an intriguing phenomenon, which has the potential to provide significant clues to core features of the limbal corneal epithelium (Wolosin et al., 2000). Unlike corneal cells, basal cells in the limbal region are heavily pigmented and thus, are well protected (Cotsarelis et al., 1989).

1.7.7.2 Enolase

Zieske (1994b) showed that enolase and several other proteins associated with metabolic functions, including cytochrome oxidase and sodium-potassium ATPase, display higher levels in limbal than corneal cells and are likely to represent an intrinsic phenotype feature of these cells. Thus maintaining an ability for metabolic response would be the first strategy for a tissue whose primary role is the preservation of the lineage throughout life.

1.7.7.3 Aldehyde dehydrogenase and transketolase

Two water-soluble proteins, aldehyde dehydrogenase (ALDH) and transketolase (TKT) have been identified as major protein components of the corneal epithelium (Piatigorsky et al., 1998). These proteins have multiple functional roles, absorb UV light, minimising intraocular incidence of these rays and simultaneously reducing UV light-induced radical concentrations through their enzymatic activity. The accumulation of these two proteins has been found to be differentiation and development dependent. In the cornea they are differentially distributed with the highest expression occurring in basal cells and the lowest in apical cells (Kays and Piatigorskiv 1997).

1.7.7.4 Sialylation

At the limbal cornea demarcation, an alpha-2, 3 linked sialyl residues, is added to exposed galactosyl residues present in membrane bound O-linked glycoconjugates, which are most likely found in glycoproteins. The addition of negative charge at the end of O-

linked glycoconjugates with the cell's transition to the corneal domain can be predicted to contribute to decreased compaction of the epithelial layers. Controlling O-linked glycosylation and in particular, sialylation with its net negative charge, may be a universal developmental strategy to tune cell adhesion for optimal function (Sokol, et al., 1990).

1.7.7.5 Gap Junctions and metabolic cooperation

Two gap junction proteins have been identified in the cornea, Cx43 and Cx50. Cx43 is nearly completely circumscribed to basal cells. Cx50 is present in all cell layers (Dong et al., 1994). Matic et al. (1997a) proposed that the fully negative Cx43 cells within the limbus represent stem cells. It was further proposed that absence of gap junctions is an intrinsic, probably essential feature of all epithelial stem cells. According to this view, limbal basal cells weakly positive for Cx43 are likely to represent early progenitors, which in some species start acquiring TA features within the limbal zone. The various changes or differences between the limbal and corneal epithelial domains described up to this point, include proliferative rates and distribution of tissue specific cytokeratins.

1.7.7.6 Basement membrane (BM)

BM composition influences cell behavior and one of the components of the induced behavior is in turn, the development of a secretory pattern aimed at preserving the composition of the inducing BM. For an axially streaming cell system, such as the limbal corneal epithelium, changes in BM composition can thus be taken to represent changes in the cell's secretory activity. Ljubimov et al. (1995) observed that $\alpha 3-5$ chains of type IV collagen in the BM of the central cornea, whereas limbal and conjunctival BM contained $\alpha 1-2$ chains. Laminin $\alpha 2\beta 2$ chains, as well as the $\alpha 5$ chain of type IV collagen, were present exclusively in the limbus. Laminin α chain was detected only in the BM of the limbus. Wessel et al, 1997, identified the long form of type XII collagen in the human cornea and established that this protein is absent from the limbal and conjunctival epithelial BM zones. Carbohydrate composition could also form the basis for basement membrane diversity (Tanzer et al., 1993; Chammas et al., 1993).

1.7.7.7 Retinoic acid, Ca⁺⁺

Retinoic acid is an important modulator of epithelial proliferation and differentiation, which is present in serum in biologically active concentrations (Fuchs et al., 1981). Strickland et al. (1978) showed that in embryonic stem cells retinoic acid induces the differentiation of stem cells. Both limbal and corneal cultures exhibited a biphasic pattern of proliferation in response to increasing concentrations of retinoic acid (Kruse et al., 1990). The proliferation of transient amplifying cells is promoted by increasing concentrations of extracellular calcium (Kruse et al., 1992).

1.7.7.8 Protein kinases

Protein kinases are likely to play central roles in the signal pathways, which control the stabilization of the stem cell phenotype through self-sustaining feedback loops (Bhalla and Iyengar, 1999), as well as the eventual passage into the differentiated state.

Protein kinase C (PKC) is a well-known regulator of cell proliferation. In particular, PKC can mediate G1-phase progression in the cell cycle (Nishi et al., 1998). PKC comprises a family of serine/threonine kinases important in intracellular signalling (Nishizuka 1984; Harrington 1997). In addition to cellular proliferation, PKC has been implicated in the regulation of differentiation, migration, and apoptosis and in tumor promotion (Harrington et al., 1997; Tang et al., 1997). Eleven PKC isoforms have been identified in mammalian cells. These isoforms have distinct cellular location and function and are categorised as classical, novel, and atypical according to their activation sites (Ron et al., 1999). The effect of PKC on the cell cycle has been reported to be both stimulatory and inhibitory, influenced by the PKC isoform and cell type studied as well as by the timing and duration of PKC activation or inhibition (Tang et al., 1997).

Of the multiple families of PKC isoforms, PKC- γ is specific to the limbus (Tseng and Li 1996). All the PKC subtypes are found in the cornea, however, (Joyce et al., 1996) there is a resemblance between epithelial and endothelium (like TGF-beta expression between limbal epithelial and endothelium). Since the human corneal endothelium is highly

quiescent, this pattern may be related to the low proliferative activity of limbal cells. Finally, Sundar et al. (1998) identified a GTPase-associated serine-threonine kinase, which undergoes a marked increase in its expression at the limbal cornea transition in rabbit.

1.7.7.9 TGF- β

The transforming growth factor- β (TGF- β) family of proteins comprises three closely related isoforms in mammals, TGF- β 1, - β 2 and - β 3. Mainly, these are secreted in a biologically inactive latent form in which mature TGF- β s are bound to bimetric latency-associated peptides (LAPs). Their subsequent activation follows the dissociation of the dimetric mature TGF-beta from the latent complex.

TGF- β s are present in small amounts or not at all in corneal epithelium and stroma (Pasqual et al., 1993). In the corneal region, beta 1-LAP antibody did not stain epithelium or stroma, beta 2-LAP antibody stained all epithelial cell layers and the corneal stroma, and beta 3-LAP antibody stained the sub epithelial region alone. The staining pattern in the limbal region was almost the same as in the corneal region, except in the limbal stroma, which was stained with beta 1-LAP antibody in three of eight samples. In the trabecular meshwork, all samples showed clear staining with beta 2-LAP antibody, whereas beta 1-LAP and beta 2-LAP antibody stained faintly in five of eight and four of eight samples, respectively. TGF beta 2-LAP was found in the corneal epithelium and stroma and beta 3-LAP in the subepithelial region, suggesting that TGF-beta 2 and TGF-beta 3 may play essential roles in normal corneal epithelial maintenance in vivo (Nishida et al., 1994).

TGF β s have multifunctional regulatory activities, and are known to be intimately associated with the regulation of wound healing. TGF- β antagonises the actions of EGF, a polypeptide with 53 amino acids and a potent stimulator of proliferation of corneal epithelial cells. It has been found in tear fluid, and various body fluids such as urine, blood plasma and on corneal epithelial cells. Both TGF- β 1 and β 2 inhibit corneal

epithelial cell proliferation promoted by KGF and HGF, and weakly inhibit cell proliferation promoted by EGF (Honma et al., 1997).

1.7.7.10 EGF, KGF and HGF

A number of different growth factors are believed to be involved in the regulation of corneal wound healing. Among them, epidermal growth factor (EGF), keratinocyte growth factor (KGF), and hepatocyte growth factor (HGF) are strong mitogens of corneal epithelial cells.

EGF can stimulate the proliferation of epithelial cells and fibroblasts, as well as various other cell types and exists in various body fluids including urine, milk, saliva, pancreatic juice, cerebrospinal fluid and plasma. Ohashi et al. (1989) and Van Setten et al. (1989) showed that in the eye, EGF is abundant in tears, and could increase corneal epithelial cell proliferation both in vitro and in vivo (Frau et al., 1972; Kitazawa et al., 1990).

KGF and HGF specifically promote proliferation in cells of epithelial origin in a paracrine fashion. KGF mRNA is expressed predominantly in corneal stromal fibroblasts, while KGF receptor mRNA is expressed in corneal epithelial cells but not in stromal fibroblasts (Sotozono et al., 1994). Like EGF, KGF stimulates corneal epithelial cell growth both in vivo and in vitro (Wilson et al., 1993). HGF mRNA is also expressed in cultured human stromal fibroblasts, and its receptor mRNA in corneal epithelial cells (Wilson et al., 1993). Li and Tseng (1997) and Weng et al. (1997) have also indicated that KGF and HGF proteins are secreted by corneal stromal fibroblasts. In conclusion, it is evident that KGF and HGF play important roles in corneal epithelial cell growth as paracrine stimulators from stromal fibroblasts.

1.7.7.11 Cytokines

Cytokines are multipotential glycoproteins which act enzymatically to facilitate intracellular communications. They also play an important role during corneal wound healing. Sotozono et al., 1997, found that the levels of IL-1 α and -6 correlated with the

severity of the injury and that increased IL-1 and 6 in wounded cornea can initiate the events that constitute epithelial wound healing. IL-6 can stimulate epithelial migration in the cornea by up-regulation of collagen synthesis (Nishida et al., 1992), whereas IL-1 accelerates epithelial wound closure in vitro and acts synergistically with EGF (Boisjoly et al., 1993). It is also possible that increased expression of IL-1 in the cornea after an alkali injury may act in conjunction with EGF in tears to stimulate epithelial wound healing, and may also induce KGF and HGF expression in corneal fibroblasts (Li and Tseng 1997; Weng et al., 1997), causing epithelial cell proliferation (Malecaze et al., 1997).

1.7.8 Stromal – epithelial cell interactions in the cornea

Stromal-epithelial interactions are key determinants of corneal function. The communications between these two play an important role in corneal tissues during normal development, homeostasis, and wound healing (Wilson et al., 2000b, 2001). The best characterised stromal to epithelial interactions in the cornea are mediated by the classical paracrine mediators hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF). HGF and KGF are produced by the keratocytes to regulate proliferation, motility, differentiation, and possibly other functions, of epithelial cells (Wilson et al., 1994a, 1996c). Other cytokines produced by keratocytes may also contribute to these interactions. Epithelial to stromal interactions are mediated by cytokines, such as interleukin-1 (IL-1) and soluble Fas ligand that are released by corneal epithelial cells in response to injury. IL-1 appears to be an important regulator of corneal wound healing that modulates functions such as matrix metalloproteinase production, HGF and KGF production, and apoptosis of keratocyte cells following injury (Wilson 1994c). The Fas/Fas ligand system has been shown to contribute to the immune privileged status of the cornea (Wilson et al., 2000b). Cytokine systems may be released from the unwounded corneal epithelium to regulate keratocyte viability and function. There are other, factors that have yet to be identified.

1.7.8.1 Cytokine-mediated interaction of the epithelium, stroma and inflammatory cells

The epithelium, stroma, and nerves participate in homeostasis of the anterior cornea and ocular surface. The lacrimal glands and tear film also contribute to the maintenance of surface smoothness and integrity important to function of the eye. Following injury, these components work together to restore corneal structure and function in most situations. Many cytokines and receptors modulate this process that could also attract immune cells that function to eliminate debris and microbes that may breach the injured surface and gain entry to the corneal stroma (Wilson et al., 2000b).

In conclusion, the corneal epithelium is a complex tissue exhibiting unique patterns of differentiation-linked expression changes subjected to autonomic controls.

1.7.9 Organ culture induced changes in the cornea during storage

The normal anatomy of the cornea is important to its functions. However organ culture induced changes occur in the cornea during storage. The reasons for endothelial deterioration during organ culture storage are unknown, although folds in Descemet's membrane caused by stromal swelling and endothelial cell apoptosis and necrosis are likely to play a major part (Pels and Schuchard 1983; Albon et al., 2000; Armitage and Easty 1997). Epithelial barrier dysfunction could also, at least in part, contribute to stromal swelling and therefore endothelial cell deterioration. In terms of epithelial cell changes and epithelial function during organ culture storage, little information is known.

1.8 Summary

Here the important aspects of the human corneal epithelium have been reviewed. The corneal epithelial stem cells and the physiological regional differences in various regulatory factors and their importance in the regulation of limbal and corneal epithelium have been described. The stromal-epithelial interactions were introduced as a key determinant of corneal function and organ culture as a commonly used procedure in long-term corneal preservation.

Placing a cornea into an organ culture medium is likely to cause endothelial disruption affecting the stroma. In addition stromal-epithelial interactions play an important role in corneal tissue during wound healing (Wilson 2000b). The mechanism of keratocyte loss is also considered to affect the epithelial-stromal interaction (Kruse et al., 1997). Therefore, if disruption of epithelial barrier happens during storage, it may also contribute to stromal disruption and consequently cause stromal swelling.

There is a requirement for a storage technique that will maintain the metabolic function and integrity of donor corneas and especially stem cell viability in organ culture. In order to do this, first we need to understand what changes occur in epithelium during storage.

1.9 Aims

Many corneal disorders are treated most effectively by corneal transplantation, thus there is a need for an efficient storage method to enable the maximum output of high quality corneas for this purpose. In order to achieve this, the structural integrity of the cornea needs to be maintained throughout storage and post transplant. Thus a suitable method of storage is important to maintain the corneal structure and the normal function of cornea.

For successful corneal transplantation, the quality of the donor material is of vital importance. Besides the endothelial cell viability, epithelial cell integrity should also be intact to have a good quality of donor cornea for corneal transplantation and especially if organ cultured corneas become a source of tissue for limbal stem cell transplant. Storage conditions have some effect on corneal endothelial and epithelial cell homeostasis. Therefore development of optimal storage conditions for long term corneal organ culture storage requires knowledge about corneal cell homeostasis. Organ culture attempts to supply the nutrients to maintain cellular metabolism (Armitage et al., 1997). Although the integrity of the endothelium is essential for maintaining corneal transparency, it does not guarantee the viability of the whole cornea (Redbrake et al., 1999).

The other factors of major importance are epithelial cells and their interaction with the stroma to maintain epithelial stromal homeostasis in the cornea. The barrier function of the epithelium and its self-renewing ability play an important role to maintain the normal structure of the cornea. Keratocytes also play a precise role in maintaining the regular organisation of the extra-cellular matrix of the stroma. Most of the overall metabolic activity of the cornea depends on the metabolism of the keratocyte (Muller et al., 1994).

Currently eye bank organ culture stored donor corneas are not used for LSC transplantation, since it is thought that down regulation of limbal stem cells during eye bank storage occurs. The only reliable source of allogeneic limbal tissue is from whole eyes within 24 hours post mortem. This leads to logistical problems in the supply of such

tissue and an extremely limited time for checking the donor's medical history, virological and microbiologic screening and tissue-typing (if necessary).

For organ culture stored cornea to be used in LSC transplantation, the overall viability and functional properties of corneal epithelial cells must be preserved to maintain normal epithelial cell homeostasis together with stem cell viability during organ culture storage. Little is known about the epithelial modifications induced by organ culture preservation. This study aims to characterise the effect of organ culture storage on corneal epithelial cell homeostasis.

In order to determine what these changes are the objectives of this investigation are:

- 1) To determine morphological changes in porcine cornea during organ culture storage
The effect of organ culture on epithelial and stromal thickness and the density of keratocyte will be identified.
- 2) To determine proliferative properties of TACs and stem cells
The cycling status of porcine corneal epithelial and limbal cells over time of culture will be assessed using BrdU immunolabelling.
- 3) To determine if stratification of corneal epithelial cells is maintained during organ culture storage
Stratification in terms of differentiation, will be identified in the corneal epithelium by investigation of specific markers of differentiation (CKs), cell-cell communication (Cx43, Cx50) and cell-cell adherence (ZO-1).
- 4) To determine changes in epithelial cell death
Mechanisms determined in normal homeostasis include, apoptosis will be investigated using the TUNEL assay and immunolocalisation of caspases-3, 8 and 9.

5) Finally to compare porcine corneal changes during organ culture to those of human corneas.

CHAPTER TWO

2. Chapter 2 - General Methods and Procedure Development

2.1 Materials

All chemicals, solutions and media used in these methods, are listed in appendixes I, II and III.

2.2 Methods

2.2.1 Source of tissue

Porcine eyes were obtained from an abattoir within 6hr of death and transported to the laboratory on ice. Human corneas were obtained from the CTS Bristol Eye Bank. All human material had permission for use in research.

2.2.1.1 Organ culture storage

Organ culture was carried out using procedures similar to those used for the UK Corneal Transplant Service (CTS) Eye banking protocols for the storage of human donor corneas. Before removal of the corneoscleral discs, eyes were decontaminated in a class II biological safety cabinet by several washes in sterile phosphate buffer saline (PBS) (pH=4.7), followed by immersion in 3% Betadine for 5 minutes, neutralisation in 0.3% sodium thiosulphate for 1 second, and a final wash in 0.9% NaCl. The corneoscleral disc was then excised from the rest of the globe using a surgical blade. The globe was dissected posterior to the conjunctiva leaving a rim of sclera attached to the cornea. The lens and other sub-corneal tissue were then removed. The corneoscleral disc was then suspended in 80 ml of organ culture medium in a glass DIN bottle using a 5/0-mersilk suture (Ethicon, Surgipath, UK) passed through the scleral rim. The organ culture medium consisted of Eagle's Minimum Essential Medium with Earle's salts and 25mM Hepes buffer, containing 2% fetal calf serum, 2mM glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin, 0.25 µg/ml amphotericin B. 2.5% chondroitin sulphate was also included in the medium (see appendixes I, II, and III).

The corneas were then stored in duplicate in a standard incubator at 34°C for varying periods of time, up to 14 days. Corneas were then bisected and one half was prepared for frozen sectioning and the other half for wax sectioning.

2.2.2 Endothelial assessment of human corneas

To evaluate endothelial cell density and viability of each human cornea, the corneal endothelium was stained for 1 minute in 0.2% trypan blue (Sigma, UK) to identify the number and distribution of dead cells. After a rinse in sterile 0.9% NaCl, one drop of 0.2% Alizarin red (Sigma, UK) in 0.9% NaCl (pH =7.2) was applied to the corneal endothelium for 20 seconds to identify cell borders. The endothelium was then rinsed in 0.9% NaCl and hypotonic 1.8% sucrose was applied to visualise the endothelium under phase-contrast light microscopy to identify number of endothelial cells (few, many or some), size (small, medium), corneal folding, number of dead cells (few, many or some) and if cells are located on the corneal folds, or at the periphery, or both at the end of storage.

2.2.3 Preparation of frozen sections

Cryoembedding medium (Tissue-Tek, Lamb, UK) was placed in the foil mould. Using forceps, the corneal tissue was first snap frozen in a freezing bath (isopentane, cooled in liquid nitrogen), for a few seconds and then immersed in Tissue-Tek medium before returning mould to the freezing bath. Frozen blocks were stored at -80°C.

10µm sections were cut using a cryostat (Lecia Microsystems CM3050s, UK).

The sections were applied directly to clean Super Frost Plus slides (BDH, UK) which electrostatically attract tissue sections, binding them to the slides. Sections were allowed to air-dry at room temperature overnight and then wrapped in aluminium foil and stored at -20°C until time of use.

2.2.4 Preparation of wax sections

Each cornea was fixed in methacarn fixative (60% methanol, 30% chloroform, 10% acid acetic) for no longer than 12 hours. The fixed tissue was then dehydrated in 50% ethanol/chloroform for 30 minutes, followed by two changes of 100% chloroform for 30 minutes each. The tissue was then placed into molten wax for one hour in an oven at 62°C. The hot wax was changed with new molten wax for a further 30 minutes. The cornea was then embedded in a mould containing molten wax by immersion with the cutting surface down. The wax solidified by placing the mould on a cold plate for 30 minutes. The wax blocks were then stored at 4°C until the day of sectioning.

Wax sections of 7 µm were cut using a microtome (Microm HM325 Germany).

Wax sections were floated out in a cold-water bath followed by floating out in a hot water bath containing Albumin (Sigma, UK) bath (40-50°C) to remove wrinkles. They were transferred onto Histobond slides (Lamb UK), and dried in an oven at 56°C overnight to allow adhesion of sections.

Sections were deparaffinised in two changes of xylene for 5 minutes and then rehydrated through one-minute washes of 100%, 90%, 70%, and 50% alcohol before being rinsed in tap water for 10 minutes, followed by rinsing in PBS for 10 minutes.

2.2.5 Histological staining

2.2.5.1. Haematoxylin and Eosin (H&E) Staining

Haematoxylin is the most widely used natural dye in histotechnology. It will stain tissue components such as elastic and collagen fibers, muscle striations, mitochondria, etc, but its common application is as a nuclear dye in standard H&E, the primary staining method for tissue section analysis (Stevens 1990). As a counterstain for haematoxylin, aqueous eosin solutions ranging between 0.5% and 0.2% in strength are most commonly used. Correctly applied, eosin should stain various tissue structures shades of pink-especially collagen, cytoplasm and erythrocytes (Lillie 1977).

Wax sections were de-waxed and rehydrated as above (section 2.2.4). Sections were then immersed in Harris' Haematoxylin stain (BDH, UK) for 5 minutes to stain all nuclei. Sections were then washed in cold running tap water for 10 minutes, counterstained with Eosin (BDH, UK) for 2 minutes and finally washed for 10 minutes in cold running tap water. These sections were then dehydrated by 1-minute immersions through graded alcohols (50%IMS (Industrial methylated spirits), 70%IMS, 90%IMS, 100%IMS, 100%IMS), before two changes in xylene for 1 minute. Sections were then mounted in xylene based DPX mounting medium (BDH, UK). Sections were viewed under a microscope (DMRAZ, Leica, UK) and images captured using Leica Qwin image analysis software (Fig 2.1).

2.2.5.2 Bisbenzimidazole nuclear localisation

A solution of Hoechst 33345 (4 μ g bis-benzimidazole/ml PBS) that labels all cell nuclei was made by diluting a stock solution of 1 mg/ml bisbenzimidazole in PBS. Wax-sections were dewaxed and rehydrated, as described in section 2.2.4. and frozen sections were thawed to room temperature. Sections were stained for 10 minutes with bisbenzimidazole, washed with PBS for 10 minutes and then mounted in Hydromount (BDH, UK). The sections were then stored in the dark until viewed under a microscope (DMRAZ, Leica, UK) equipped with a 100W mercury vapour lamp for illumination, using a filter with excitation 372 nm, and emission 456 nm. Images were captured using Leica QFluoro image analysis software (Fig 2.1.b).

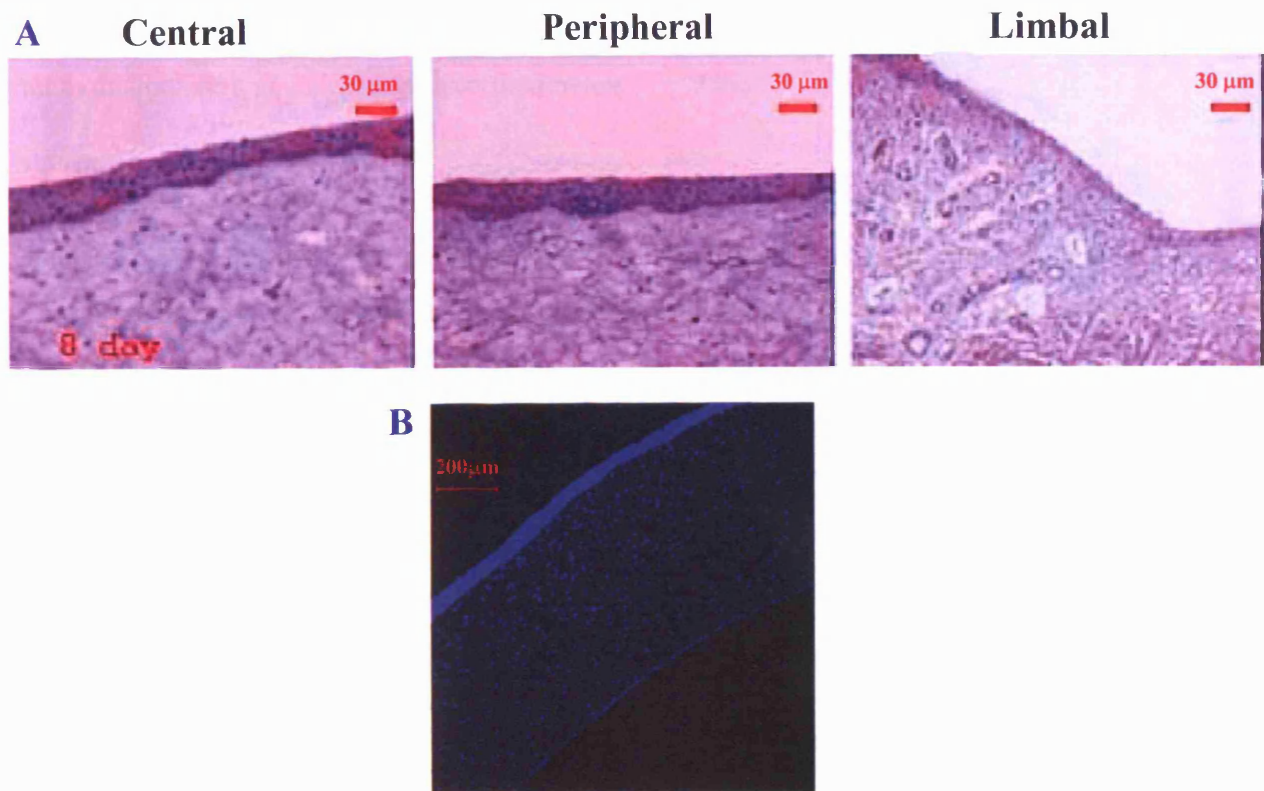


Fig 2.1 A) Haematoxylin and Eosin staining of porcine corneal wax sections in the central, peripheral and limbal regions and after 8 days of organ culture. B) Hoechst staining of wax section of porcine corneal central region at 0 point.

2.2.6 Immunostaining

2.2.6.1. Wax sections

Wax sections were dewaxed and rehydrated as in section 2.2.4. The general protocol for immunohistochemical staining is described below.

Wax sections were washed three times in 10 minute washes in PBS, pretreated with 0.2% Triton X-100 (Sigma, UK) for 10 minutes followed by three further 10 minutes washes in PBS. The sections were then treated with 20 µg/ml proteinase K (Sigma, UK) and washed by three 10-minute washes with PBS. The sections were then incubated in appropriate primary antibody (Table 2.1) overnight at 4°C followed by three, 10-minute washes with PBS. Sections were incubated with secondary antibody (see Table 2.2.) for 2hr in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted in hydromount (BDH, UK) and air dried and wrapped in aluminium-foil until viewing under a DMRAZ microscope (Leica, UK). Images were captured using the QFluoro software program.

2.2.6.2 Frozen sections

Frozen sections were allowed to warm to room temperature, put in acetone for 10 minutes, air dried on the bench for 10 minutes, washed with PBS three 10-minute and then incubated in appropriate primary antibody (Table 2.1.) overnight. These sections were then washed with PBS three times, 10 minutes each. FITC conjugated secondary antibodies (Table 2.2.) were incubated with sections for 2 hr in the dark followed by three, 10 minute washes with PBS. The sections were then mounted in hydromount and air dried and wrapped in aluminium-foil until viewing under DMRAZ microscope (Leica)

Table 2.1 Primary antibodies used for Immunostaining

Primary antibody	Dilution in PBS	Company
Monoclonal mouse anti-Pan Cytokeratin	1/200	Sigma, UK
Monoclonal mouse anti-epithelial keratin – AE5	1/100	ICN, UK
Monoclonal mouse anti human cytokeratin	1/100	Dako, UK
Monoclonal mouse anti epithelial keratin	1/100	MPBiomedicals, UK
Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA)	1/320-1/400	Dako, UK
Anti Ki67 rabbit antibody	1/50	Dako, UK
Anti active Caspase-3 rabbit antibody	1/10	Abcam, UK
Anti human active Caspase-3 antibody	1/250	R & D, UK
FITC conjugate anti rabbit Cleaved caspase-3 antibody	1/100,1/200 1/500	Bio lab, UK
Anti mouse Caspase-9 monoclonal antibody	1/100, 1/200	Abcam, UK
Anti mouse cleaved Caspase-8 monoclonal	1/100, 1/500	Bio lab, UK
Anti rat ZO-1 monoclonal antibody	1/100,1/200 1/400	Chemicon, UK
Anti mouse Connexin- 50 monoclonal antibody	1/100	Zymed, UK
Anti mouse Connexin-43 monoclonal antibody	1/300	Zymed, UK

Table 2.2 Secondary antibodies used for Immunostaining

Secondary antibody	Dilution in PBS	Company
FITC conjugated goat anti mouse IgG	1/320	Sigma, UK
FITC conjugated goat anti rabbit IgG	1/150	Sigma, UK
Alex Fluor 488 goat anti rabbit IgG	1/1000	Molecular probes, UK
Alex Fluor 546 goat anti mouse IgG	1/1000	Molecular probes, UK
Cy3 goat anti rabbit IgG	1/160	Immuno research, UK
Alex Fluor 488 donkey anti mouse IgG	1/1000	Molecular probes, UK
Fluorescein goat anti rat	1/100	Chemicon international, UK

2.2.7 Cell Proliferation in organ cultured corneas

Cellular proliferation is a fundamental biological activity of the cornea that plays an important role in both physiological and pathological processes. In this study the state of cellular proliferation at different time points of porcine organ culture storage were investigated using different proliferative markers such as Ki-67, PCNA and BrdU to determine which was the method to analyse cell proliferation in porcine cornea.

2.2.7.1 Ki-67 immunostaining

The Ki-67 nuclear protein is expressed in all phases of the cell cycle except the resting phase (G₀). Ki-67 is detected in the nucleus of proliferating cells in all active phases of the cell cycle from the late G₁-phase through to the M-phase, but is absent in nonproliferating and early G₁ phase and in cells undergoing DNA repair (Gerdes et al., 1983). Ki67 immunolocalisation has different characteristic patterns in cells depending on their position within the cell cycle. For instance a granular foci in the nucleoplasm, a dot like pattern associated with the nucleolus or a reticulate structure surrounding the condensed chromosomes is reported in late G₁-phase, G₂-phases or mitosis of mouse fibroblasts respectively (Kill 1996 and Starborg et al., 1996).

Immunolocalisation of Ki-67 in human corneas

Frozen sections of human corneas were prepared according to section 2.2.2. Immunostaining of all frozen sections of human corneas using Ki67 antibody (1/50 dilution) as a primary antibody (Dako) and FITC conjugated goat anti rabbit IgG (1/150 dilution) as a secondary antibody (Table 2.1 and 2.2) were performed according to protocol 2.2.6.2. The results demonstrated a dot like pattern of staining associated with the nucleus in central, limbal and peripheral region of epithelial cornea (Fig 2.2).

Immunolocalisation of Ki-67 in porcine cornea

Wax sections were dewaxed and rehydrated as in section 2.2.4. and frozen sections were fixed in acetone and air dried. Immunostaining of all wax and frozen sections of porcine corneas following 1, 2, 4, 6, 8 days in organ culture were performed according to the protocol described in sections 2.2.6.1 and 2.2.6.2. The primary antibody was Ki-67 antibody (1/50, 1/100 dilutions, see table 2.1) and secondary antibody was FITC-conjugated goat anti rabbit IgG (1/150 dilution, table 2.1 and 2.2). No positive specific Ki-67 immunoreactivity was observed in corneal sections. Therefore it seems that anti-human Ki-67 did not cross-react with porcine tissue and therefore could not be used in this study to determine the proliferative status of the porcine corneal epithelium (Fig 2.3).

2.2.7.2 PCNA immunostaining

PCNA is a non-histone nuclear protein that functions as a cofactor for DNA polymerase delta. It is a protein related to the cell cycle and is naturally expressed in proliferating cells. PCNA is a 36 KDa multifunctional protein expressed at high levels in cycling cells during at least 5% of the G-1 phase and the first 35% of the S-phase. It is an essential component of the DNA replication machinery, functioning as the accessory protein for DNA polymerase δ and required for processive chosomal DNA synthesis. The expression of this protein in the cornea has been used as an indicator for corneal cell activation (Gran et al., 1999).

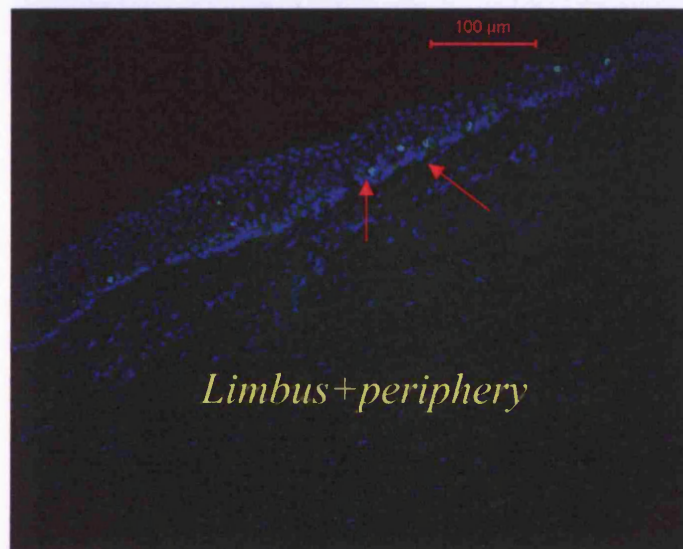
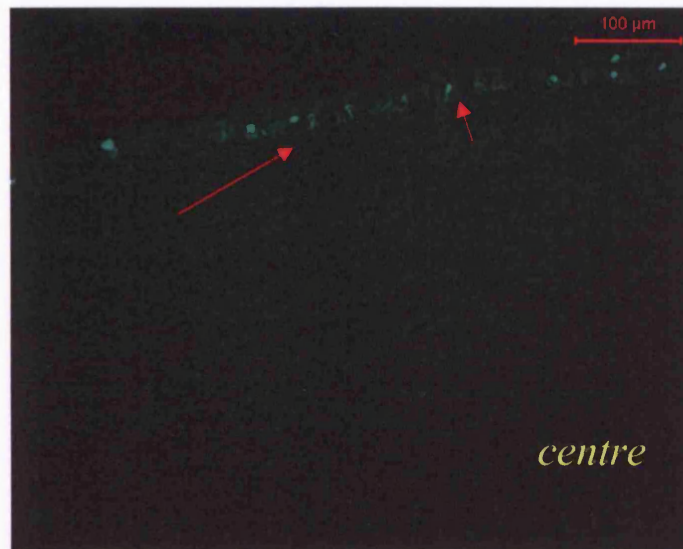


Fig 2.2 Ki67 staining (red arrows) of human frozen corneal sections in the central and limbal regions. Bar represents 100μm.

Fig. 2.2 Ki67 staining of human sections of central and limbal regions of human cornea after 5 days of organ culture. The picture shows the typical staining pattern of a limbus with the corneal epithelium.

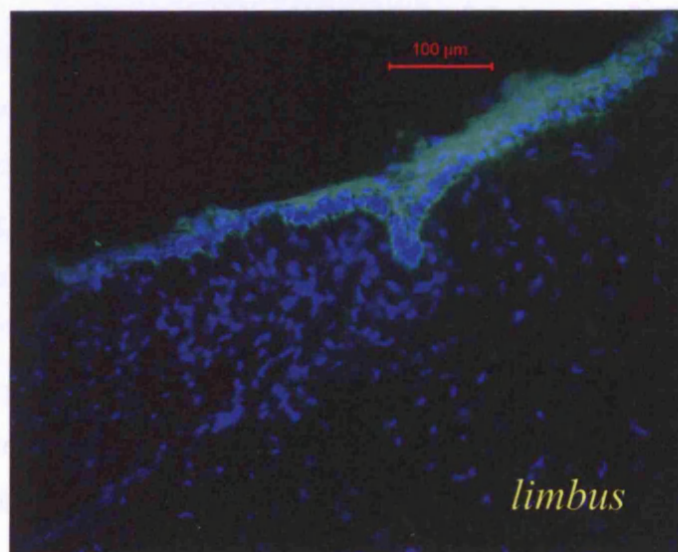
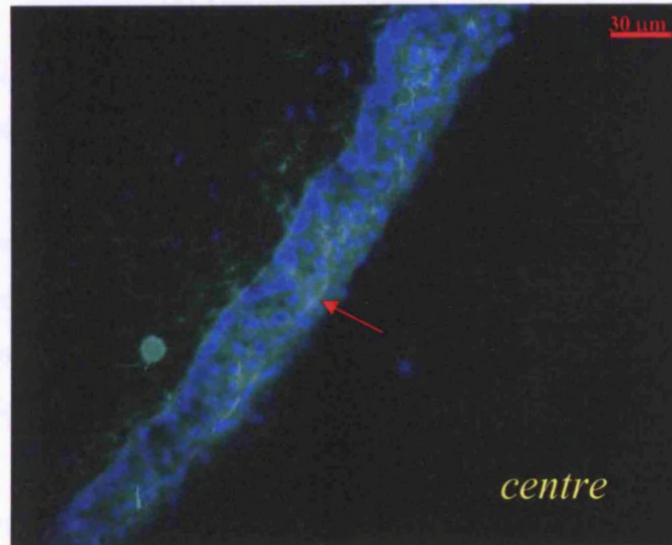


Fig 2.3 Ki67 staining of frozen sections of central and limbal regions of porcine cornea after 8 days of organ culture. The picture shows non specific staining (arrows) of antibody with the corneal epithelium.

Optimisation of immunostaining with anti PCNA

Anti-PCNA antibody was optimised (Table 2.1, 2.2, 2.3, 2.4 and 2.5), including determination of secondary antibody, pre-treatment for antigen retrieval and blocking of non-specific staining.

Antigen visualisation technique was performed using immunofluorescence technique versus immunohistochemistry: Wax sections were dewaxed and rehydrated as in section 2.2.4. and frozen sections were fixed in acetone and air dried. Immunostaining of all frozen and wax sections of porcine corneas following 0, 4 and 8 days in organ culture and human corneas were performed according to protocols 2.2.6.1 and 2.2.6.2 respectively. Anti-PCNA antibody was used as the primary antibody (1/400, 1/320 dilution, table 2.1.) and Alex Fluor 546 goat anti mouse IgG (1/1000 dilution) as the secondary antibody (Table 2.2).

No positive staining was identified in any sections.

Immunostaining of porcine and human corneas were performed using ABC kit: Wax sections were dewaxed and rehydrated as in section 2.2.4. and frozen sections were fixed in acetone and air dried. Immunostaining of all frozen and wax sections of porcine corneas following 0, 4 and 8 days in organ culture and human corneas were performed using ABC complex kit (Dako, UK). Anti-PCNA antibody was used as the primary antibody (1/100, 1/200 dilution, table 2.1.) Goat serum (diluted 1:5 with TBS) were used as blocking reagent, and 1/150 dilution of biotinylated goat anti mouse as a secondary antibody (see table 2.3). The binding was displayed with the biotin-avidin technique and fast red as a substrate. Sections were then air dried and mounted using Loctite and UV light.

A large amount of background staining was seen in both frozen and wax sections. After discussion with Dako, serial dilution and antigen retrieval procedures were recommended.

Table 2.3 Modification of procedure for porcine and human corneas immunostaining using ABC kit

Type of section	Primary antibody	Secondary antibody	Blocking	Result
Wax and frozen	Anti-PCNA, from ABC kit 1/100 or 1/200	biotinylated goat anti mouse 1/150	Goat serum (diluted 1:5 with TBS)	Non-specific staining

Determination of pre-treatment for antigen retrieval and blocking step

The staining was repeated as above, except that this time 0.1% chymotrypsin digestion (prior to Triton X and proteinase K pre-treatment) was used as antigen retrieval treatment to expose epitopes, a combination of 10% goat serum and 20% milk powder were used together as a blocking reagent (because of the high background staining) and the primary antibody dilution reduced to 1/20, 1/50 with 1/150 dilution of biotinylated goat anti mouse secondary. The binding was visualised following the biotin-avidin technique and colour development using fast red. The sections were then counterstained in haematoxylin for two minutes and mounted using loctite and UV light (see table 2.4).

No staining was identified in any of the wax sections and there was little staining in epithelial frozen sections and no specific nuclear staining.

Table 2.4 Modification of procedure for antigen retrieval and blocking step

Type of section	Primary antibody	Secondary antibody	pre-treatment	Blocking	Result
Wax	Anti-PCNA, from ABC kit 1/20 or 1/50	biotinylated goat anti mouse 1/150	0.1% chymotrypsin digestion	10%goat serum (diluted 1:5 with TBS)+ 20% milk powder	Negative
Frozen	Anti-PCNA, from ABC kit 1/20 or 1/50	biotinylated goat anti mouse 1/150	0.1% chymotrypsin digestion	10%goat serum (diluted 1:5 with TBS)+ 20% milk powder	Non specific staining

Further method development for Triton X-100 digestion and removing blocking

The staining was repeated as the procedure described above, with the following modification. For frozen sections, 1/50, 1/100 dilution of PCNA primary antibody with just 0.2% Triton digestion without any blocking. For wax sections, 1/50 and 1/100 dilution of PCNA primary antibody were used once with and without 0.1% chymotrypsin digestion. The blocking step was also removed (see table 2.5.).

No staining was detected in any of the frozen and wax sections, therefore anti- PCNA could not be used as a marker for proliferation.

Table 2.5 Modification procedure for TRITONX-100 digestion and removing blocking

Type of section	Primary antibody	Secondary antibody	pre-treatment	Blocking	Result
Wax	Anti-PCNA, from ABC kit 1/100 or 1/50	biotinylated goat anti mouse 1/150	0.1% chymotrypsin digestion	10%goat serum (diluted 1:5 with TBS)+ 20% milk powder	Negative
Wax	Anti-PCNA, from ABC kit 1/100 or 1/50	biotinylated goat anti mouse 1/150	none	none	Negative
Frozen	Anti-PCNA, from ABC kit 1/20 or 1/50	biotinylated goat anti mouse 1/150	Only 0.2%TritonX-100 for 20 minutes	none	Negative

2.2.7.3 BrdU incorporation assay

Proliferative activity of corneal epithelial cells was carried out using a cell proliferation kit (AMERSHAM). This is a rapid non-radioactive technique in which a thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), is incorporated into replicating DNA and subsequently localised using a specific monoclonal antibody (Gratzner et al., 1982). To increase the sensitivity of this technique, cells to be labelled with BrdU were simultaneously exposed to 5-fluoro-2'-deoxyuridine, an inhibitor of thymidilate synthetase (Ellwart et al., 1985), and thereby increasing BrdU incorporation by lowering competition of endogenous thymidine.

BrdU assay

The cycling status of porcine corneal epithelial and limbal cells, in porcine corneas stored for 0, 3, 6, 9 and 12 days, was assessed following addition of bromodeoxyuridine (1/1000 dilution) into the organ culture media on the day of culture. BrdU was added to triplicates cultures for each time point. The corneas were then bisected and prepared for wax and frozen sectioning as described in sections 2.2.2 and 2.2.3.

Using components of the cell proliferation kit, rehydrated specimens were washed 3 times with PBS. For each section, 100 μ l of the reconstituted nuclease/anti-5-bromo-2'-deoxyuridine was prepared by adding 40 μ l of anti-5-bromo-2'- deoxyuridine monoclonal antibody to 4ml of one vial of freeze-dried nuclease which had been mixed with distilled/deionised water previously. The sections were then incubated for 1 hour in a humidified chamber and washed 3 times with PBS (3 minutes each time). 15 μ l of peroxidase anti-mouse IgG2a was diluted with 1ml of peroxidase anti-mouse IgG2a diluents, and 100 μ l of the solution was added to each specimen. The sections were then incubated for 30 minutes at room temperature. During the incubation period, one aliquot of DAB (25 mg/ml DAB stock) was thawed out and diluted in 50ml PBS. The sections were then washed 3 \times with PBS (3 minutes each time). 5 drops of Substrate/intensifier were added to dilute DAB solution, and stirred vigorously, and then the sections were immersed in the staining solution for 5-10 minutes. The sections were washed 3 \times in distilled water. Finally they were counterstained with Hoechst reagent (section 2.2.5.1.).

After observing the staining, the modifications below were applied to increase the quality of staining for BrdU.

- A. Paraffin sections were used versus frozen sections, as no staining was observed in frozen sections.
- B. 1/500 dilution of BrdU used versus 1/1000, as no staining was observed with 1/1000 dilution.
- C. Different times of visualising with DAB were used for 5-10 mins (several observations under microscope and comparison with negative control) to find the effective time of staining with DAB.
- D. Different counterstains, Eosin, Hoechst, Vector Haematoxylin and Methyl green were used to find the most effective counterstain that could be used to count total cell population nuclei (stained and nonstained BrdU cells) in each specific areas.
- E. Different procedures were used to assess positive proliferative activity of zero time point because of BrdU incorporation difficulties.

Determination of a suitable counterstain

Generally counterstains may consist of single dyes or dye mixtures and depending upon the primary target may colour the cell nucleus, cytoplasm or specific tissue structures (such as collagen). To be effective a counterstain should be of a contrasting, subtle colour which does not intrude on the major stain. Different counterstains, Eosin, Hoechst, Vector haematoxylin and Methyl green were used to find the more effective counterstain that could stain total cell nuclear population of BrdU stained area and therefore could be used for calculating BrdU labelling index.

First Eosin was used as a cytoplasmic stain with bisbenzamide (nuclear staining) to counterstain BrdU stained sections. In order to calculate labelling index in each area of corneal wax sections, two images were captured using QFluoro image analysis. One using bright field filter (Fig 2.4.) to capture BrdU stained cells and the other with a fluorescent filter (Fig 2.5.) to capture the image of total cell nuclei in those areas. Figure 2.5. shows how difficult it was to count cells (Fig 2.5.) counterstained with bisbenzamide even with high magnification (40 x).

In order to overcome this problem Vector haematoxylin and Methyl green counterstains that were non fluorescent and could be seen and captured using Bright field microscopy were tested.

Haematoxylin counterstain vector was used to provide a quick and convenient staining for cell nuclei. Slides were first dewaxed and washed with water and PBS. Haematoxylin was applied directly to the slide, and completely covered the tissue.

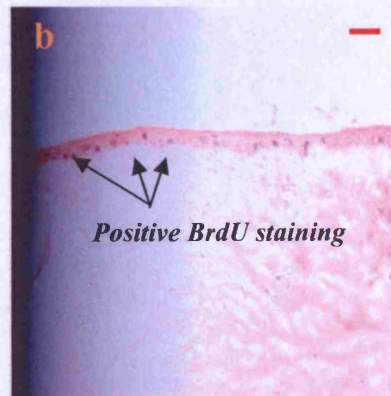
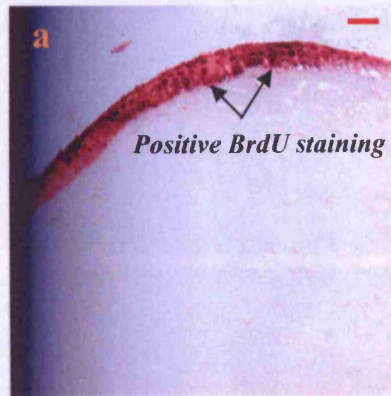


Fig 2.4 Eosin as a counterstain for immunostained porcine corneal wax sections following 4 (a) and 8 days (b) of organ culture. Arrows represent positive BrdU staining. Bar represents 30 μ m.

Fig 2.4 Figure shows images taken from cross-sections stained with hematoxylin using different magnifications, and after 14 days of organ culture. This experiment was carried out alongside the one in which the BrdU was counterstained with eosin immunostaining was with higher magnification as the cells were not clear.

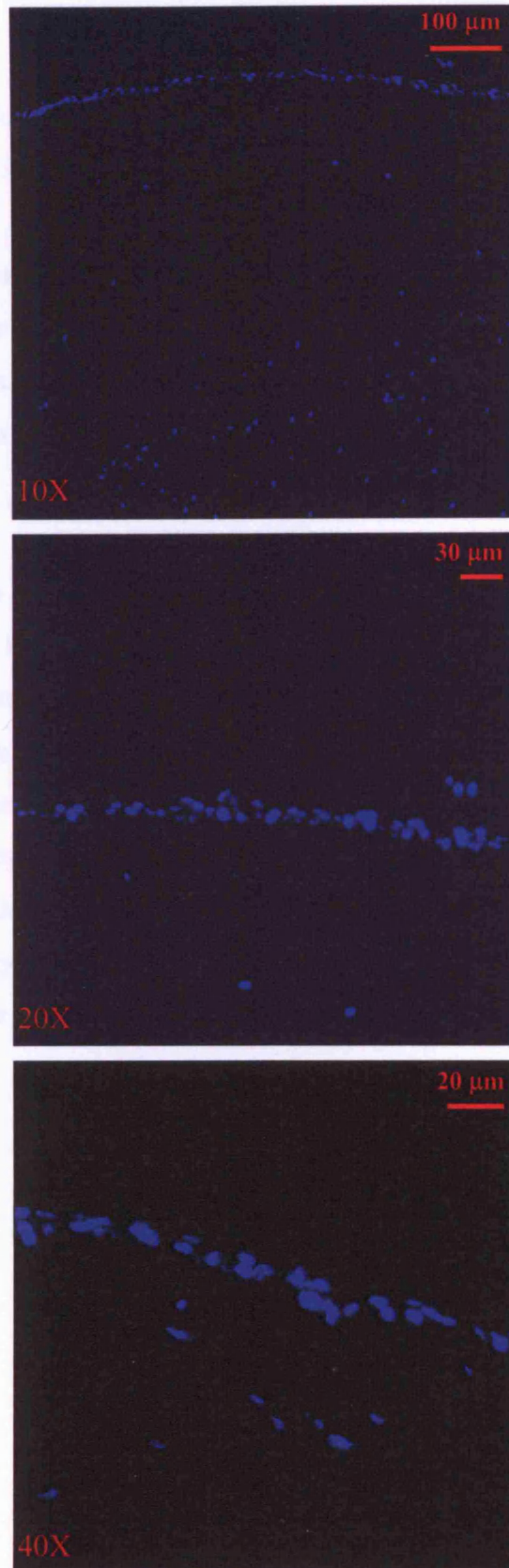


Fig 2.5 Picture shows images taken from corneal wax sections stained with bisbenzamide using different magnifications and after 14 days of organ culture. This counterstain was deemed an unsuitable stain to count the cell nuclei in combination with BrdU immunostaining even with higher magnification as the cells were not clear.

Sections were incubated for 2-45 seconds and rinsed with tap water until the water became colourless and finally the sections were dehydrated and mounted in DPX.

From the results it was obvious that BrdU stained cells were darker than non-BrdU stained cells. However there was not a distinguishable difference between the colour of DAB and haematoxylin stained cells even after 2 seconds.

Therefore methyl green was tried and modified as an alternative method to haematoxylin staining.

Methyl green (Vector) stains cell nuclei light green. It is suitable for use with a wide range of enzymes and is regarded as an ideal alternative to haematoxylin, especially for multiple labelling in immunohistochemistry. The light green colour of this counterstain is of particular advantage to combine with DAB as it also provides an excellent contrast. In order to optimise methyl green as a counterstain, all wax sections were first dewaxed and then stained with preheated Methyl green solution for 1-5 minutes, rinsed with water and dipped in acetone containing 0.05% acetic acid for 5-10 minutes. All sections were then dehydrated by dipping in graded alcohol for a second followed by two times 2 minutes change in xylene; the sections were then mounted with DPX (Table 2.6).

Table 2.6 Modification of procedure for incubation and dehydration of MG

First counterstain	Incubation time with first counterstain	Second counter stain	Fixation procedure	Dehydration procedure	Result
Methyl green solution (Vector)	1-5 mins	none	Acetone for 5-10 minutes	Dip in graded alcohols and 2x 2minutes change in xylene	Best result 2 mins of MG plus 5 mins fixation with acetone
Methyl green solution	1-5 mins	none	None	Dip in graded alcohols and 2x 2 minutes change in xylene	Eosin masked the Methyl green

From the observation it was clear that at least 2 minutes staining was required to ensure good staining, with better result at 3 min (Fig 2.6.a.). Coupling Methyl green staining with eosin was not a good idea because eosin could mask the methyl green staining even after 20 seconds of staining.

In order to use Methyl green as a counterstain with the BrdU assay, Immunolabelling with BrdU cell proliferation kit (Amersham) was coupled with

- a) 2 minutes staining with methyl green, followed by dipping in graded alcohol for 1 second and 5 minutes fixation in acetone, followed by two, 2 minute changes in xylene. The sections were then mounted with DPX (Table 2.7).

DAB staining covered Methyl green staining, as is shown in fig 2.6.b Further modification was needed to balance this effect.

- b) Reducing the dehydration and staining procedure by: 1 minute incubation in methyl green, followed by 1 minute immersion in acetone, plus 30 seconds 100% ethanol dehydration (Table 2.7).

Using this method, DAB did not mask the Methyl green stain or vice versa. However there is still background staining that needed to be eliminated (Table 2.7).

- c) Balance the acetone fixation procedure: by modifying acetone incubation for 60, 30, or 12 seconds.

Best results (background staining was reduced) were obtained following 30 seconds 100% ethanol incubation and 12 seconds acetone fixation (Fig 2.7).

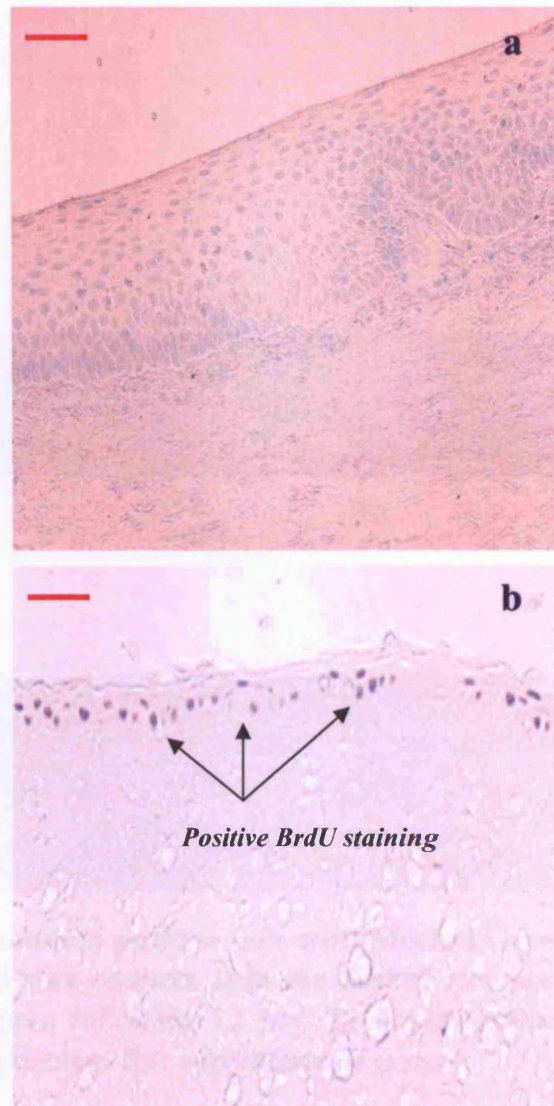


Fig 2.6 Masking of methyl green stain by DAB on corneal wax sections. a) day 0 (without BrdU): stained for 3 minutes in methyl green without DAB processing. Image demonstrates clear staining for methyl green.

b) Day 14 (BrdU added to the medium during organ culture storage). BrdU Immunopositive cells following DAB processing, and counterstaining with methyl green for 3 minutes. DAB masked the methyl green counterstain. Arrows represent positive BrdU staining. Bar represents 20µm.

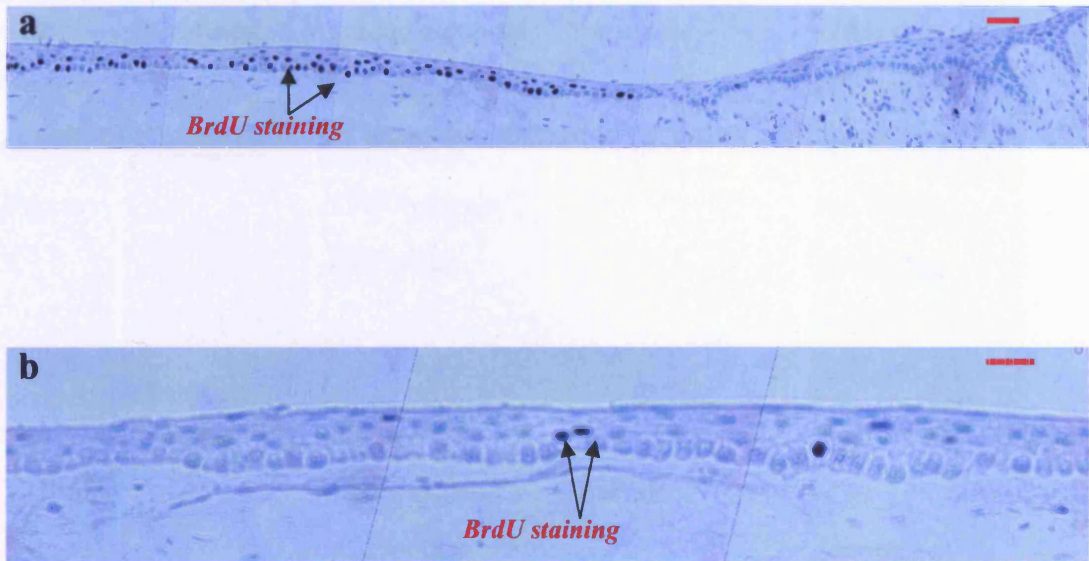


Fig 2.7 BrdU Immuno positive cells with Methyl Green counter staining of porcine corneal wax sections a) in the limbal and peripheral regions b) in the central region, following 12 days in organ culture. Arrows represent positive BrdU staining. Bar represents 20 μ m.

Table 2.7 Modification of procedure for using Methyl green as a counterstain to BrdU immunolocalisation

Counterstain	Incubation time with first counterstain	Dehydration procedure	Acetone fixation	Result
Methyl green solution (Vector)	2 minutes	graded alcohols and xylene	5 minutes	DAB masked Methyl green
Methyl green solution (Vector)	1 minute	30 seconds 100% ethanol and xylene	1 minute	No masking of the Methyl green, but high background
Methyl green solution (Vector)	1 minute	30 seconds 100% ethanol and xylene	30, or 12 seconds	Best result after 12 seconds of acetone fixation

The most appropriate counterstain for BrdU proved to be Methyl green due to its ability to clearly stain cell nuclei. In order to counter stain the BrdU stained sections, they were immersed in preheated (54°C for an hour) methyl green solution for 1 minute and then rinsed through a series washes of distilled water until the water appeared clear. The sections were then dehydrated for 30 seconds in 100% ethanol followed by 12 seconds in acetone and 2 changes of xylene for 2 minutes. Slides were then be mounted in DPX.

Proliferative activity at zero time point

To access proliferative activity for zero time point, moist chamber storage of eyes was carried out using different methods:

The whole eye globe was stored in a moist chamber pot containing Saline soaked gauze at 4°C, 34°C. A 1/500 dilution of BrdU solution was added to the corneal surface (two corneas for each time points 0, 12, 24, 48hrs).

No BrdU staining was observed. One reason could be the lack of sufficient BrdU to be incorporated, or because of the delay in applying BrdU to the porcine cornea.

The globe (fresh eyes obtained directly from the abattoir and processed) was stored in a moist chamber at 4°C. The whole globe eye was suspended upside down in a 60ml pot containing 30 ml of MEM medium with 25 mM HEPES, plus BrdU at a 1/500 dilution and subjected to the BrdU incorporation assay (two corneas for each time points 0, 12, 24 and 48 hours).

No BrdU staining was observed, the reasons for the lack of BrdU incorporation could be either that, the tight junctions of the superficial cells, are preventing, penetration of the BrdU into the corneal epithelium or the proliferation activity of the cells may have shut down.

Fresh porcine eyes were dissected and then placed in dishes containing MEM media with 1/100 dilution of BrdU labelling solution for 30 minutes at 37°C.

No staining was observed at this stage.

1%, 5%, and 20% of N-Acetyl Cysteine (Sigma, UK) was added to porcine cornea to make the cornea permeable to BrdU for about 10 minutes and then drops of BrdU solution added to the cornea of porcine eyes. All corneas were dissected, half were frozen and half waxed and used for BrdU labelling.

Staining seemed to appear in the limbal region only after 1% treatment with N-Acetyl Cystein.

Optimised protocol for BrdU incorporation assay

Once the BrdU technique had been optimised, the technique was repeated using the optimised procedures below:

- A. 5 minute staining with DAB was used versus 5-10 minutes.
- B. Methyl green staining was used versus eosin and hoechst staining according to the optimized procedure for methyl green staining.

Calculation of mitotic index

The BrdU staining was performed and the mitotic index was calculated according to formula: $\frac{\text{Number of labelled cells}}{\text{Total number of cells in that region}} \times 100$

Total number of cells in that region.

2.2.8. Analysis of cell death in porcine cornea

To investigate changes in epithelial cell death, apoptosis was analysed using two different techniques. Firstly, the TUNEL assay (terminal deoxynucleotide transferase mediated dUTP-digoxigenin nick end labelling) was used to label DNA fragments of 180 bp nucleosomal units produced by the apoptotic process. Secondly to confirm apoptosis immunodetection of caspases (3, 8 and 9) which are the main effectors of apoptosis. Their activation leads to the characteristic morphological changes of the apoptotic cell such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing (Nicholson et al., 1995; Medema et al., 1997; Green 1996). Activation of the caspase signalling pathway creates a cascade of caspase enzymes that will cleave proteins essential for cell survival and lead to apoptosis.

2.2.8.1 TUNEL staining

Standard TUNEL method

The TUNEL detection of porcine corneal epithelial and limbal cells in porcine corneas stored for 0, 3, 6, 9 and 12 days was assessed using an Apoptag Apoptosis Detection kit (Chemicon International USA) that labels apoptotic cells by modifying DNA fragments utilising terminal deoxynucleotidyl transferase (TdT) mediated digoxigenin end labeling. The corneas were first bisected and prepared for wax sectioning as described in sections 2.2.2. Wax sections were cut, dewaxed and rehydrated as in section 2.2.4. The sections were pretreated with Triton X-100. The sections were then washed twice with PBS (2 minutes each time). They were then pretreated with 20 µg/ml of proteinase K. The sections were then washed twice with PBS (2 minutes each time). After that 3 % hydrogen peroxidase in PBS was applied to sections for 5 minutes.

The sections were then washed twice with PBS (2 minutes each time). 50µL of equilibration buffer were applied directly on each section for 20 min. The excess was gently taped off and immediately afterwards 33µl of TdT enzyme was diluted with 77 µl of reaction buffer to make 110 µl of working strength TdT enzyme, and then 50µl of the solution was added to each specimen. The slides were then incubated in a humidified chamber using plastic cover slips (to assure that a constant volume of solution is applied per unit of specimen area) at 37°C for 1 hour. 1 ml of stop wash buffer was diluted with 34 ml of distilled water to make 35 ml of working strength wash buffer, and then 40µl of the solution was added to each specimen. The slides were incubated with working strength for 20 min.

The slides were then immersed in 70% ethanol in a coplin jar and stored at -20°C overnight. The samples were then washed with 2x 2 minutes of PBS. The 50µl of anti-dioxigenin conjugate was applied to each section for 1 hour. The sections were then washed 4x with PBS (2 minutes each time). Enough peroxidase substrate was applied to each specimen for 5 minutes. The specimens were then washed in 3 changes of distilled

water in a coplin jar for 1 min and then incubated in distilled water in a coplin jar for 5 minutes at room temperature.

The TUNEL stained sections were then immersed in preheated (54°C for an hour) methyl green solution and rinsed through a series of washes of distilled water until the water appeared clear. The sections were then dehydrated for 12 seconds in butanol following 2 changes of xylene for 2 min. They were then mounted in DPX.

No specific staining was observed at this stage so the method was optimised according to modifications described below:

- Different pre-treatment procedures using pre-treatment with Triton X-100 for 0, 60 minutes at 0.2% and 10, 15, and 20 minutes at 0.5% concentration of respectively.
- Different time of incubation with proteinase K at 0, 5, 10 and 25 minutes.
- Different time of developing colour in peroxidase substrate (5-10 minutes).
- Different thickness of corneal wax sections using 7, 16 and 10 μm sections.

Refinement of TUNEL method

The treatment procedure and colour development was carried out as follows:

The staining was repeated using the procedure described above. Modifications of this procedure are detailed below in table 2.8.

- 1) Pre treatment procedure using 0.2 % of Triton X-100 for 20 minutes.
- 2) 25 minutes incubation with proteinase K.
- 2) Between (5-10) mins colour developing time in peroxidase substrate.

At this stage only some staining was recognised and appeared to be non specific. Further modifications was required.

Table 2.8 Modification to develop pre-treatment procedure and colour development

Type of section and thickness	Triton X-100	Proteinase K	DAB processing	Result
7 μ m, wax	0.2% for 20 mins	25 minutes	5-10	Non-specific staining

In order to develop pre-treatment procedure the staining was repeated using the standard TUNEL procedure, and modifications below were also applied to corneal wax sections (see table 2.9):

- 1) Pre-treatment procedure using extra 0.2 % of Triton X-100 for 1 hour.
- 2) Immersion in 70% ethanol in coplin jar, sealed with Parafilm overnight after washing the sections with stop wash buffer solution.

Although faint staining was observed for 0 day, but there no staining for 3 and 12 days was appeared. Sections stored in organ culture for longer time need a different pre-treatment procedure (Fig 2.8.a and b).

Table 2.9 Modification of procedure for pre-treatment procedure and immersion in 70 % ethanol

Type of section and thickness	Triton X-100	Proteinase-K	Result
7 μ m, wax	0.2% for one hour	—	Positive staining for 0 day (+). Negative staining for 3 and 12 days (-).

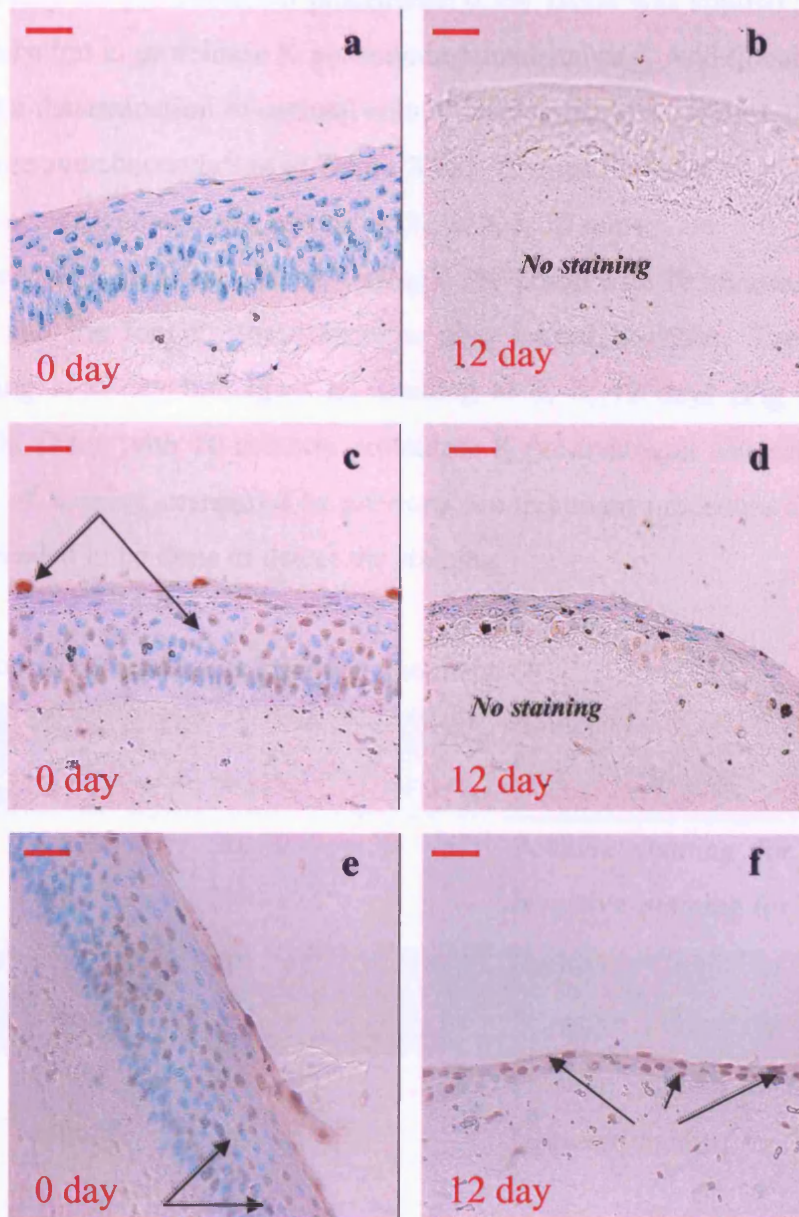


Fig 2.8 Optimisation of TUNEL assay. TUNEL (arrows) modification for finding suitable pre-treatment procedure in the central region of porcine corneal wax sections and after 0 and 12 days of organ culture using:

a, b) 7µm section pretreated with 0.2% TritonX-100 for one hour, no staining was detected at this time point.

c, d) 7 µm sections treated with 0.5% TritonX-100 for 20 min +10 minutes proteinase K. More effective staining (compared to procedure a) was observed with this pre-treatment at day 0, but there is still no staining at day 12.

e, f) 10 µm sections treated with 0.5% TritonX-100 + 10 minutes proteinase K. The staining seems to be even more effective (especially at 12 days) compared to other modifications. Bar represents 20µm. Arrows represents positive staining.

To further develop the pre-treatment procedures, 0.5% Triton was applied followed by a 10 minute incubation in proteinase K generated optimal staining. Additional modification was required for determination of optimal colour development (see table 2.10):

- 1) Different time and concentration of Triton X100, 20 mins for 0.5%.
- 2) Different time of incubation for proteinase K, at 0, 5, 10 mins.

The staining seemed to be more effective using 0.5% Triton with 10 minutes proteinase K pre-treatment and the longer stored sections also looked healthier. There was some positive staining at 0 day but again no staining at 3, 6, 12 days (Fig 2.8.c and d). Therefore 0.5% Triton with 10 minutes proteinase K pre-treatment was established as a better method of staining compared to previous pre-treatment procedure although extra modification needed to be done to detect the staining.

Table 2.10 Modification procedure for pre-treatment

Type of section	Triton X-100	Proteinase K	Result
7 μ m, wax	0.5% for 20 minutes	0	Positive staining for 0 day (+). Negative staining for 3 and 12 (-)
7 μ m, wax	0.5% for 20 minutes	5	Positive staining for 0 day (++) Negative staining for 3 and 12 (-).
7 μ m, wax	0.5% for 20 minutes	10	Positive staining for 0 day (+++). Negative staining for 3 and 12 (-).

To allow better visualisation of apoptotic cells, the thickness of corneal sections were also developed: Apoptotic cells are smaller than neighbouring viable cells, they also fragment into smaller apoptotic bodies, therefore cutting very thin section, can underestimate apoptotic frequency. Therefore the staining was repeated with the procedure described in section 2.2.8.1., and 0.5% Triton for 20 mins followed by 10 minutes proteinase K pre-treatment using both 7 and 16 μ m sectioned corneas (see table 2.11). Corneas were also left for 3 days in the 60°C incubator overnight.

The staining appeared to work for 16 μm sections (day 3) however, 16 μm sections of 0-day corneas seemed to show less staining compare to thinner (7 μm) sections. Therefore thicker corneal sections seem to work better especially for corneas stored longer in medium. However thinner corneal sections showed better staining at 0 points. Therefore 10 μm sections seems to be a good candidate for both long and short time points to be examined.

Table 2.11 Modification procedure for thickness development

Type of section	Triton X-100	Proteinase K	Result
7 μm , wax	0.5% for 20 minutes	10	Positive staining for 0 day (+++). Negative staining for 3 and 12 (-).
16 μm , wax	0.5% for 20 minutes	10	Positive staining for 0 (+). Positive staining for 3 days (+).

To optimise the section thickness, staining was repeated using the procedure described in section 2.2.8.1., and 10 μm sections on 0, 6, 12 days corneal sections (see table 2.12.). The staining seemed to work for 10 μm sectioned corneas at 0, 6 and 12 days, with especially large amount of staining at 12 days (Fig 2.8.e and f). The amount of staining at 0 day was also less for 10 μm compared to 7 μm sections. Taken together from all these results 10 μm sections were chosen as the best choice. However to avoid over pre-treatment with Triton X-100, different times of incubation with Triton X were applied and the results were compared.

Table 2.12 Modification of procedure to find the most suitable thickness for corneal sections

Type of section	Triton X-100	Proteinase K	Result
7 μm , wax	0.5% for 20 minutes	10	Positive staining for 0 day (+++). Negative staining for 3 and 12 (-).
10 μm , wax	0.5% for 20 minutes	10	Positive staining for 0 (+++). Positive staining for 6 and 12 days (+).

To further optimise the 10 μm sections, they were incubated in Triton X-100 for 10, 15, 20 minutes to determine the optimal incubation time (see table 2.13.).

The results showed that reducing staining to 10 minutes does not work for 6 day sections and staining seemed to be more effective using 15 minutes pre-treatment with 0.5% Triton X-100 plus 10 minutes proteinase K (Fig 2.9).

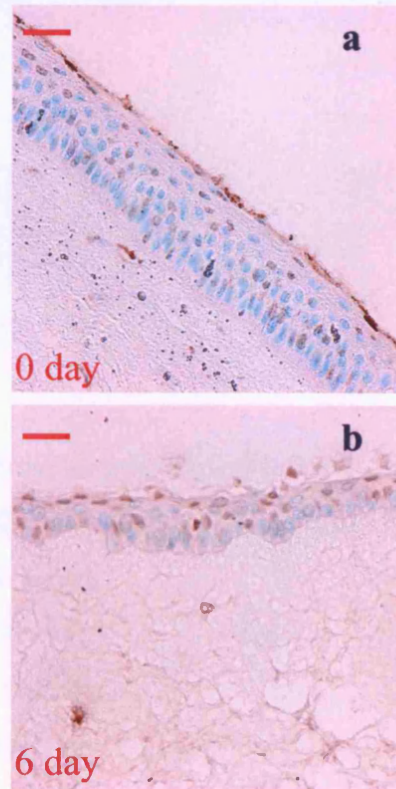


Fig 2.9 The effect of the application of various incubation time of Tritonx-100 on porcine corneal wax sections (central region) using TUNEL assay .

a) 10 μm section with 0.5% Triton for 20 mins +10 mins proteinaseK

b) 10 μm section 0.5% Triton for 15 mins +10 mins proteinase K

As shown by these pictures, the level of background staining appears less after 15 minutes incubation (b). Therefore the most suitable incubation time is 15 minutes (b). Bar represents 20 μm .

Table 2.13 Modification procedure for optimisation of 10 µm sections

Type of section	Triton X-100	Proteinase K	Result
10 µm, wax	0.5% for 10 minutes	10	Positive staining for 0 day (+++). Negative staining for 6 day (-).
10 µm, wax	0.5% for 15 minutes	10	Positive staining for 0 day (++++). Positive staining for 6 day (+++).
10 µm, wax	0.5% for 20 minutes	10	Positive staining for 0 day (+++). Positive staining for 6 days (+++).

Optimization of methyl green staining for TUNEL assay

Vector Methyl green (h-3402) was used to stain nuclei as described in sections and modified as below for TUNEL assay:

Several different incubation times (1, 1.5, 2, 3 minutes) with Methyl green were applied plus replacement of ethanol with butanol (2, 3, 5, 6, 7, 8, 9, 12, and 15 seconds) for dehydration procedure and finally the best suitable procedure was: Methyl green staining for 1.5 minutes followed by series washes of distilled water until the water appeared clear, followed by 12 seconds dehydration in butanol plus two changes of xylene for two changes of xylene for 2 minutes. Sections were then mounted in DPX.

Accepted optimised TUNEL technique:

Once the TUNEL technique had been optimised, the technique was repeated according to section 2.2.8.1. and with the following optimised procedures:

10µm corneal sections

15 minutes pre-treatment with 0.5%Triton X -100

10 minutes proteinase K

Methyl green was used as a counterstain according to the procedure described above.

Calculation of mitotic index for TUNEL assay

TUNEL staining was performed according to section 2.2.8.1.3. and the mitotic index was calculated according to formula: the number of TUNEL labeled cells x 100/total number of cells in that region.

2.2.8.2 Immunodetection of caspase-3

Caspase-3 is one of the key executioners of apoptosis as it is either partially or totally responsible for proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP- ribose) polymerase (PARP) (Alnemri 1994). Activation of caspase-3 requires proteolytic processing of its inactive zymogen into the activated forms p17 and p12 (Nicholson 1995).

Methods development

Several antibodies from different companies against active caspase-3 were used (Table 2.1 and 2.14.) to confirm the apoptotic activity of corneal epithelial cells in wax section at different times of organ culture storage. All these antibodies were raised against caspase-3 and detect the cleaved active form of caspase-3. In order to generate a positive control for apoptosis, wound organ cultured bovine corneas (previously shown to demonstrate caspase-3 active immunostaining and a gift from Louise Carrington), were used. In brief bovine eyes were obtained less than four hours post mortem and three wounded corneas organ culture were set up, serum-free Towells T8 medium 1 μ m staurosporine (Sigma, UK) was added to just above the limbal region, and 100 μ l of this medium added drop wise onto the wound. Corneas were kept in culture for a further 24hour and then fixed and processed as detailed in sections 2.2.3 and 2.2.4.

Immunohistochemical staining of waxed and frozen sections of porcine corneas were performed as described in section 2.2.6.1 and 2.2.6.2. For abcam antibody, both wax and frozen sections were used plus 1/10 dilution of rabbit polyclonal to active caspase-3 antibody (against the p17 fragment of the active caspase-3) and 0.5% Triton X-100 for 10 minutes. In the case of the R&D antibody, frozen sections plus 0.5% Triton X-100 were

used for 10 minutes at 1/250 dilution of rabbit anti caspase-3 active antibody were used. The staining was then followed by 1/1000 dilution of Alex Flour 488 goat anti rabbit IgG (Molecular probes) for 2 hours (see table 2.14).

For Biolab antibody both wax and frozen sections were used plus 0.5% Triton X-100 for 10 minutes plus 1/100, 1/200 and 1/500 dilution of FITC conjugate rabbit anti caspase-3 active antibody (against the cleavage site of caspase-3 at ASP175) overnight.

Staining for activated caspase-3 with Abcam antibody showed cytoplasmic staining for activated caspase-3. The staining was observed throughout the suprabasal layer of porcine wax corneal sections in limbal region at 0, 3, 12, days of organ culture storage (Fig 2.10). At 0 day higher levels of positive staining appeared in the suprabasal layers of the peripheral and central regions, while at 3 and 12 days, it appeared to be localised to basal layer especially in the peripheral region. No specific immunoreactivity was detected with this antibody in frozen sections.

There was no staining observed with the R&D antibody for frozen sections and only one cornea (0 point) showed some staining through all suprabasal layers of epithelium.

It's likely that this antibody does cross react with porcine tissue, thus producing non specific staining.

As with Biolab antibody, non specific staining was observed at all dilutions of Biolab caspase antibody (Fig 2.11). Therefore, in order to find if the high background is reduced by blocking, the staining was repeated using wax sections with 0.5% Triton X-100 for 15 minutes, at 1/100 dilution of antibody and 10% BSA.

Only limited non-specific staining was observed at 0 and 12 days with this antibody (Fig 2.12.a).

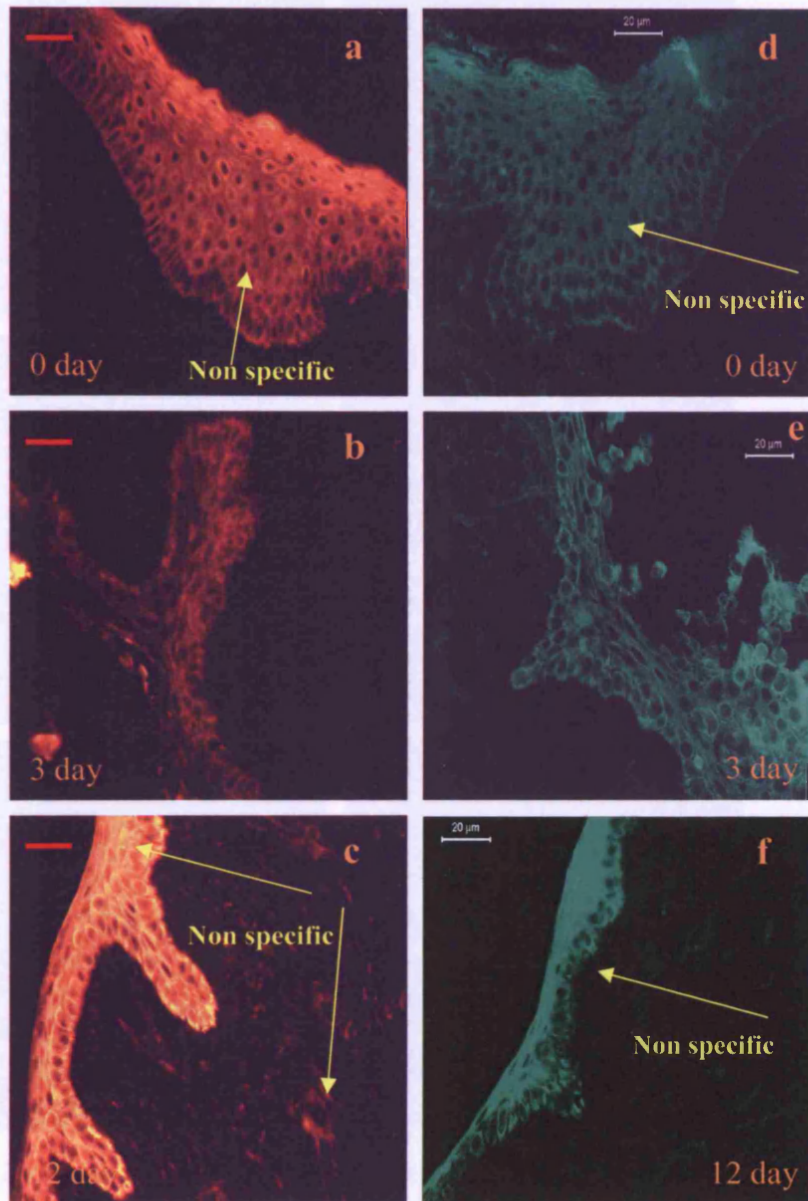


Fig 2.10 Immunoreactivity of caspase-3 (Abcam) with wax sections of porcine cornea in the limbal region and after 0, 3 and 12 days of organ culture, using 2 different secondary antibodies. a, b, and c have been stained with Cy3 and d, e and f with Alex fluor 488 conjugates.

All pictures show cytoplasmic non-specific caspase-3 staining (arrows) in suprabasal layer of the epithelium. Bar represents 20μm.

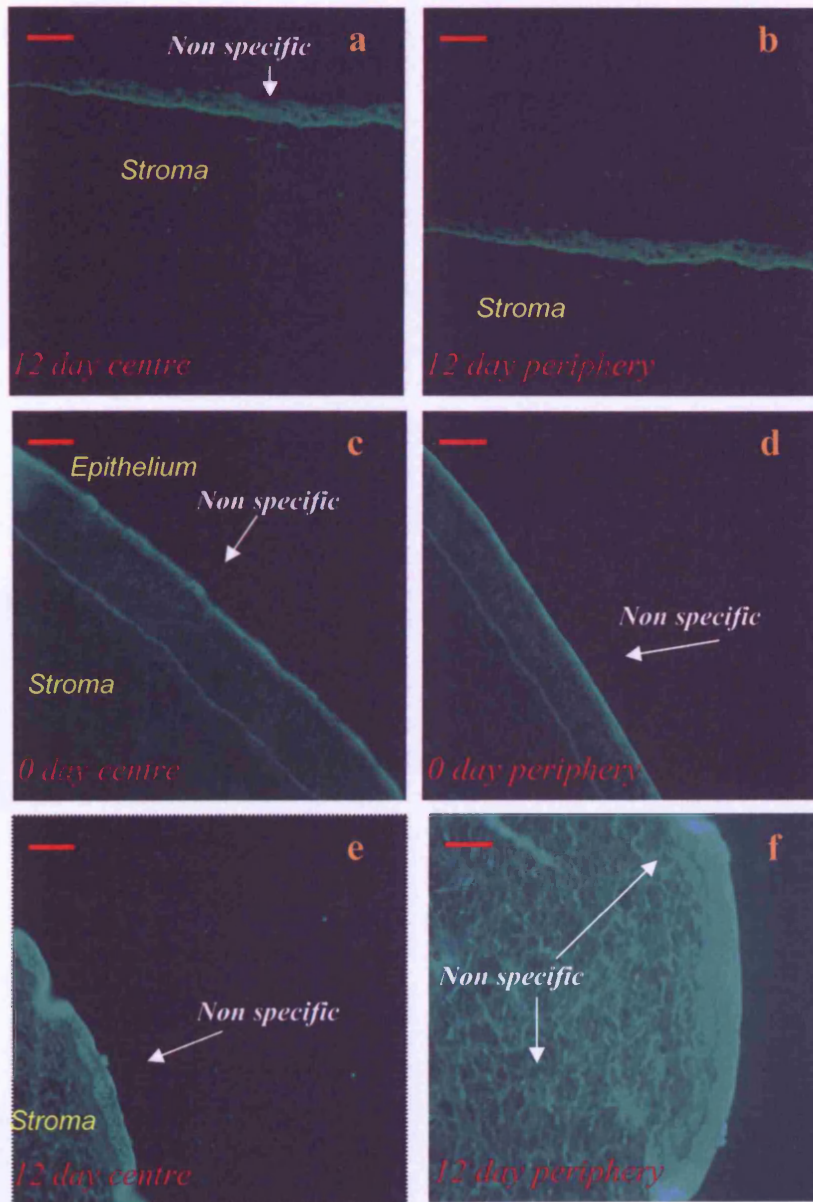


Fig 2.11 Caspase-3 (Biolab) immunostaining in the frozen (a and b) and wax sections of porcine cornea (c, d, e and f) in the central and peripheral region and after 0 and 12 days of organ culture. All pictures shows non-specific immunoreactivity (arrows) of caspase 3 with corneal sections. Bar represents 30µm.

Type of section	Primary antibody	Secondary antibody	Triton X-100	Result
Frozen	Rabbit polyclonal anti caspase-3 antibody 1/10 (abcam, UK)	Alex Fluor 488 goat anti rabbit 1/1000	0.1% for 10 mins	Neg
Wax	Rabbit polyclonal anti caspase-3 antibody 1/10 (abcam, UK)	Alex Fluor 488 goat anti rabbit 1/1000 or Cy3 conjugate	0.1% for 10 mins	Cytoplasmic non-specific staining
Frozen	Rabbit polyclonal anti caspase-3 antibody 1/250 (R&D, UK)	Alex Fluor 488 goat anti rabbit 1/1000	0.2% for 10 mins	Negative & non-specific
Frozen	FITC conjugate rabbit caspase-3 antibody 1/500, 1/200, 1/100 (Bio lab, UK)	N/A	0.5% for 10 mins	Non-specific staining
Wax	FITC conjugate rabbit caspase-3 antibody 1/500, 1/200, 1/100 (Bio lab, UK)	N/A	0.5% for 10 mins	Non-specific staining
Wax	FITC conjugate rabbit caspase-3 antibody 1/100 (Bio lab, UK)	N/A	0.5% for 15 minutes+10% BSA	Non-specific staining

Table 2.14 Antibodies and methods used for caspase-3 staining

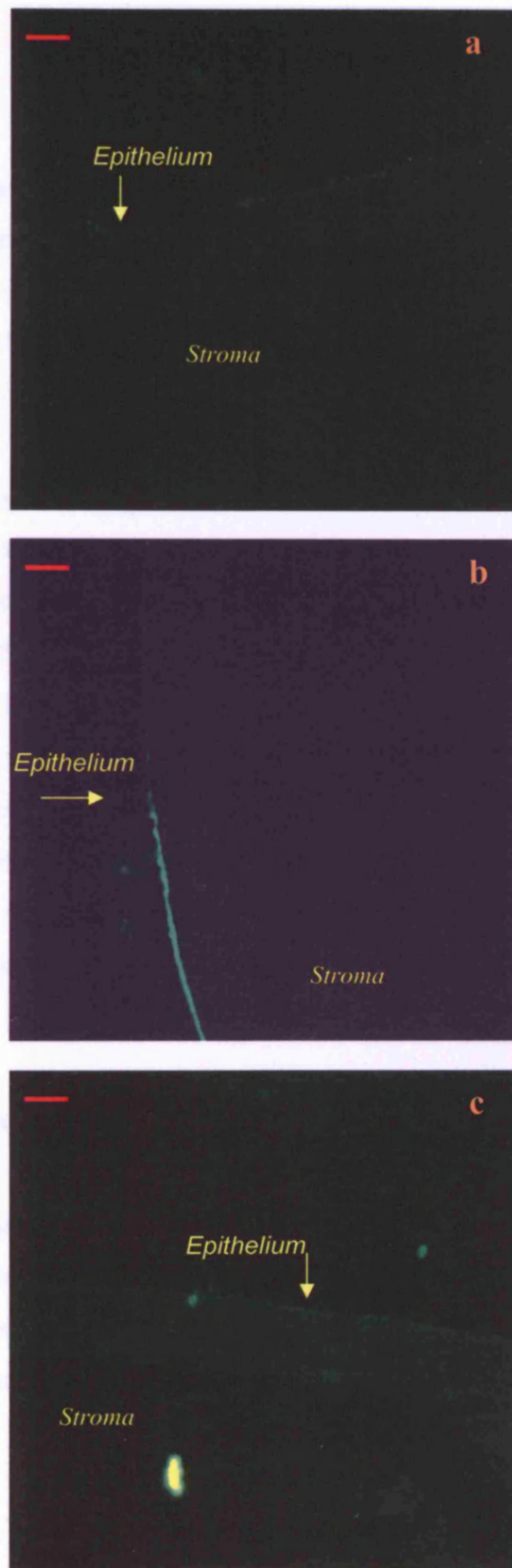


Fig 2.12 Caspase-3, 8 and 9 immunostaining of wax section of porcine cornea in the central region and at day 0. Positive immunoreactivity of caspase 8 is shown in corneal epithelial flat cells (b). No specific immunoreactivity of porcine section was detected with any other caspases, 8 and 9 (a, c). Bar represents 30 μ m.

2.2.8.3 Immunolocalisation of caspase-8

Apoptosis induced by death ligands such as CD95 (Fas/APO-1) and tumor necrosis factor receptor (TNFR1) activates caspase-8, leading to the release of the active subunits p18 and p10 of caspase 8 (Muzio et al., 1996; Fernandes et al., 1996). Activated caspase-8 is able to cleave and activate downstream caspases such as caspases-1, 3, 6 and 7.

Immunodetection of caspase-8 in frozen corneal section

Frozen sections were allowed to come to room temperature, put in acetone for 10 minutes, and dried on bench for 10 minutes. All the samples were then washed with three, 10 minutes with PBS. The sections were then pretreated with 0.5% Triton X-100 for 10 minutes, followed by three 10 minutes washes with PBS. The sections were then incubated with 1/500 and 1/100 of anti caspase 8 antibody (raised in mouse and against small subunit of caspase-8 resulting from cleavage at Asp 384) overnight (see table 2.15.) followed by 2hr incubation with 1/1000 dilution of Alex Fluor 488 donkey anti mouse secondary antibody in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted and viewed as described in above section.

Result:

No staining was detected with any dilution of antibody.

Immunodetection of caspase-8 in wax corneal section

The staining was repeated with wax sections as described in section 2.2.8.3.1. with 0.5% Triton X-100 for 15 minutes, 1/100 dilution of antibody and 10% donkey serum was also used as blocking reagent (see table 2.15).

Result:

Positive immunoreactivity of caspase-8 was identified as shown in fig 2.12b. Thereafter all caspase 8 immunodetection was carried out on wax corneal sections using this protocol.

Table 2.15 Antibodies and methods used for caspase-8 immunostaining

Type of section	Primary antibody	Secondary antibody	Treatment	Result
Frozen	Mouse monoclonal anti caspase-8 antibody 1/500 or 1/100 (Biolab, UK)	Alex flour 488 donkey anti mouse 1/1000 (Molecular probes)	0.5%Triton X-100 for 10 mins	Neg
Wax	Mouse monoclonal anti caspase-8 antibody 1/100 (Biolab, UK)	Alex Flour 488 donkey anti mouse 1/1000 (Molecular probes)	0.5%Triton X-100 for 10 mins	positive

2.2.8.4 Immunodetection of caspase-9

Caspase-9 is an important member of the cysteine aspartic acid protease (caspase) family (Duan 1996). Upon apoptotic stimulation, cytochrome c released from mitochondria associates with procaspase-9 (47 kDa)/Apaf 1. This complex processes procaspase-9 into large active subunit (35 kDa or 17 kDa) and a small subunit (10 kDa) by self-cleavage at Asp315 (Liu 1996, Zou 1999). Cleaved caspase-9 further processes other caspase members including caspase-3 and caspase-7, to initiate a caspase cascade leading to programmed cell death (Deveraux 1998; Macfarlane 1997). In addition to self cleavage, procaspase-9 also can be cleaved in vivo by caspase 3 at Asp330. This process serves as positive feedback to amplify the apoptotic signal in the caspase activation pathway (Zou 1999).

Immunodetection of caspase-9 in frozen corneal section

Frozen sections were allowed to come to room temperature, put in acetone for 10 minutes, and dried on the bench for 10 minutes. All the samples were then, washed three times, 10 minutes with PBS. The sections were then pretreated with 0.5% Triton X-100 for 10 minutes, followed by three 10 minutes washes with PBS. The sections were then incubated with 1/200 and 1/100 of anti caspase-9 antibody (raised in mouse and against caspase 9 prodomin, see table 2.16.) followed by 2hr incubation with 1/1000 dilution of Alex Flour 488 donkey anti mouse secondary antibody in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted and viewed as described in above section.

Result:

No staining was observed with any dilution of antibody.

Wax section staining of caspase-9

The staining was repeated with wax sections as described above with 0.5% Triton x-100 for 15 minutes, 1/100 dilution of antibody and 10% donkey serum was also used as blocking reagent (see table 2.16).

Result:

Some staining was observed only at 0 day with this antibody (Fig 2.12.c).

Table 2.16 Antibodies and methods used for caspase-9 staining

Type of section	Primary antibody	Secondary antibody	Treatment	Result
Frozen	Mouse monoclonal anti caspase-9 antibody 1/200 or 1/100 (abcam, UK)	Alex Fluor 488 Donkey anti mouse 1/1000 (Molecular probe)	0.5% Triton X-100 for 15 mins	Neg
Wax	Mouse monoclonal anti caspase-9 antibody 1/200 (abcam, UK)	Alex Fluor 488 Donkey anti mouse 1/1000 (Molecular probe)	0.5% Triton X-100 for 15 mins+10%donkey serum	Neg (only at day 0)

2.2.9 Immunodetection of AE5

This antibody is specific for basic 64 K keratin which, together with the acidic 55 K keratin, has been designated as a marker for corneal type differentiation (Sun et al., 1984, Cooper et al., 1985). Studies on the expression of the 64 K keratin in vivo and in cultured rabbit corneal epithelial revealed that stem cells are located in the limbus, the transitional zone between the cornea and the conjunctiva. This antibody also reacts with lip and snout epithelia (cow and rabbit) (Schemer et al., 1986). For AE5 antibody, unfixed, frozen section worked much better than formaldehyde-fixed sections. This product was presented as the purified IgG fraction ascites production in phosphate buffered saline, pH 7.2.

2.2.9.1 Immunodetection of AE5 in frozen corneal section

Frozen sections were allowed coming to room temperature, put in acetone for 10 minutes, and dried on bench for 10 minutes. All the samples were then, washed three times, 10 minutes with PBS. The sections were then pretreated with 0.1% Triton X-100 for 10 minutes, followed by three 10 minutes washes with PBS. The sections were then incubated with 1/100 dilution of monoclonal anti AE5 antibody overnight (see table 2.17) followed by 2hour incubation with 1/1000 dilution of Alex Fluor 488 donkey anti mouse



IgG in the dark, followed by three, 10 minute wash with PBS. The sections were then mounted in hydromount and wrapped in aluminium-foil to visualize under DMRAZ microscope (Leica, UK).

Results:

Positive immunoreactivity of AE5 was identified as shown in fig 2.13. Thereafter all AE5 immunodetection was carried out on wax corneal sections using this protocol.

Table 2.17 Antibodies and methods used for AE5 staining

Type of section	Primary antibody	Secondary antibody	Treatment	Result
Frozen	Monoclonal mouse anti – AE5 cytokeratin 1/100 (MP Bipmedicals, UK)	Alex flour488 donkey anti mouse 1/1000 (molecular probe)	0.1% Triton X-100 for 10 mins	positive

2.2.10 Pan Cytokeratin

Monoclonal mouse anti human cytokeratin, clone AE1/AE3 is intended for laboratory use to identify qualitatively by light microscopy two epitopes present on a majority of epithelial cytokeratins in acetone fixed frozen and formalin fixed, paraffin embedded tissue Immunohistochemical test methods.

AE1/AE3 is a cocktail of two monoclonal antibodies that were obtained by immunizing mice with human callus keratin. AE1/AE3 has been shown to identify the majority of human cytokeratins and thus may be used as a tool for the positive identification of cells of simple and stratified epithelial origin (Tseng et al., 1982; Woodcock et al., 1982). Antibody AE1 immunoreacts with an antigenic determinant present on most of the

subfamily A cytokeratins, including cytokeratins 10, 13, 14, 15, 16 and 19 (MWs of 56.5, 54, 50, 50, 48, and 40 KDa respectively) but not numbers 12, 17 and 18 (55, 47 and 45KDa) (Sun 1984). Antibody AE3 reacts with an antigenic determinant shared by the subfamily B cytokeratins including numbers 1 and 2, 3, 4, 5, 6, 7 and 8 (MWs of 65, 67,64,59 58, 56, 54, and 52 KD, respectively) (Eichner 1984).

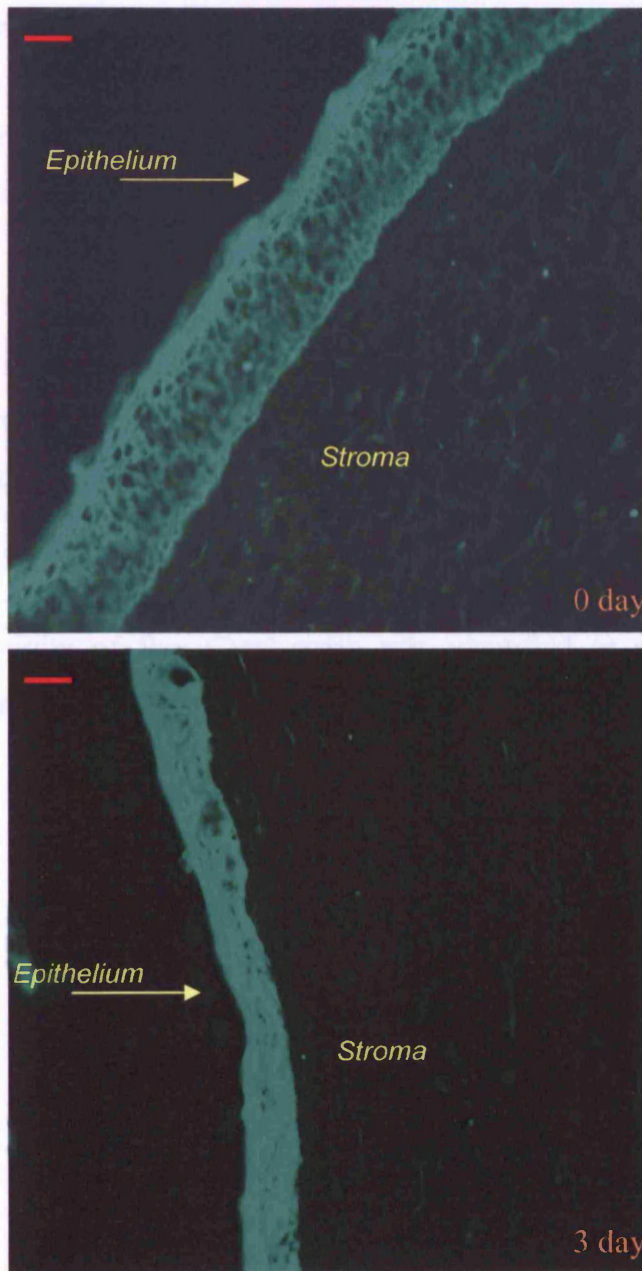


Fig 2.13 Positive immunostaining of AE5 with the peripheral epithelium in frozen section of porcine cornea and after a) 0, and b) 3 days of organ culture. Bar represents 30 μ m.

2.2.10.1 Immunodetection of AE1/AE3 in frozen corneal section

Frozen sections were allowed to come to room temperature, put in acetone for 10 minutes, and dried on the bench for 10 minutes. All the samples were then, washed three times, 10 minutes with PBS. The sections were then pretreated with 0.1% Triton X-100 for 10 minutes, followed by three 10 minutes washes with PBS. The sections were then incubated with 1/100 of monoclonal mouse anti human cytokeratin AE1/AE3 from Dako over night (see table 2.18) followed by 2hour incubation with 1/1000 dilution of Alex Fluor 488 donkey anti mouse IgG in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted in hydromount and dried and wrapped in aluminium-foil to visualize under DMRAZ microscope (Leica, UK).

Results:

Positive immunoreactivity of Pan Cytokeratin was identified as shown in Fig 2.14 Thereafter all pan immunodetection was carried out on wax corneal sections using this protocol.

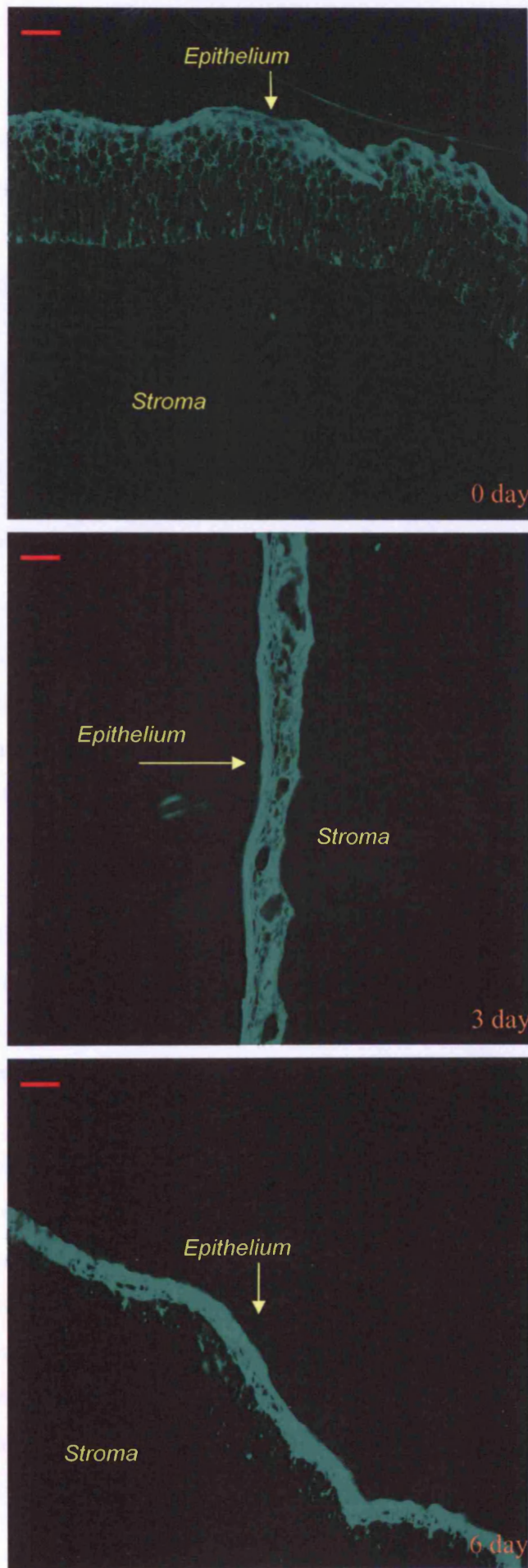


Fig 2.14 Positive immunostaining of AE3 with frozen sections of porcine cornea in the epithelial peripheral region and after a) 0, b) 3 and c) 6 days of organ culture. Bar represents 30 μ m.

Table 2.18 Antibodies and methods used for AE1/AE3 staining

Type of section	Primary antibody	Secondary antibody	Treatment	Result
frozen	Monoclonal mouse anti human Pan cytokeratin AE1/AE3 1/100 (Dako, UK)	Alex flour 488 donkey anti mouse 1/1000 (molecular	0.1% Triton X-100 for 10 mins	positive

2.2.11 Immunodetection of connexin 43

Connexin 43 is a member of the large family of gap junction proteins. Connexins assemble as a hexamer and are transported to the plasma membrane to create a hemichannel that can associate with hemichannels on nearby cells to create cell to cell channels. Clusters of these channels assemble to make gap junctions. Gap junction communication is important in development and regulation of cell growth. Phosphorylation of Cx43 has been shown to be important in regulating assembly and function of gap junctions (Musil et al., 1990, 1991). Ser 368 of Cx43 is phosphorylated by protein kinase C (PKC), which decreases cell to cell communication (Lampe et al., 2000). Src can also interact with and phosphorylate Cx43 to alter gap junction communication (Giepmans et al., 2001).

2.2.11.1 Immunodetection of Connexin 43 in frozen corneal section

Frozen sections were allowed to come to room temperature, put in acetone for 10 minutes, and dried on the bench for 10 minutes. All the samples were then washed three times, 10 minutes with PBS. The sections were then pretreated with 0.1% Triton X-100 for 10 minutes, followed by three 10 minutes washes with PBS. The sections were then incubated with 1/500 of mouse anti connexin 43 antibody (Zymed, UK) recognise connexin 43 when the serine at residue 368 is unphosphorylated and phosphorelated at sites besides Ser 368) and 1/300 of mouse anti connexin43 from Sigma (recognize 130-

143 un phosphorelated sites of connexin 43) over night (see table 2.19) followed by 2 hr incubation with 1/1000 dilution of Alex Fluor 488 donkey anti mouse IgG in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted in hydromount and wrapped in aluminium-foil to visualise under DMRAZ microscope (Leica).

Results:

No staining was detected at 0 point although some staining was observed with both antibodies at day 12, with some background staining at day 3 (Fig 2.15.a).

2.2.11.2 Blocking procedure for connexin 43 staining

A combination of 20% donkey serum with 10% of BSA was applied in order to eliminate this huge amount of background. Fresh corneas were also used instead of previous 0 point cornea, in order to see if the time of leaving cornea before fixation could affect connexin 43 staining (see table 2.19).

Result:

Positive immunoreactivity of connexin 43 was identified as shown in fig 2.15.b. Thereafter all connexin 43 immunodetection was carried out on frozen corneal sections using this protocol.

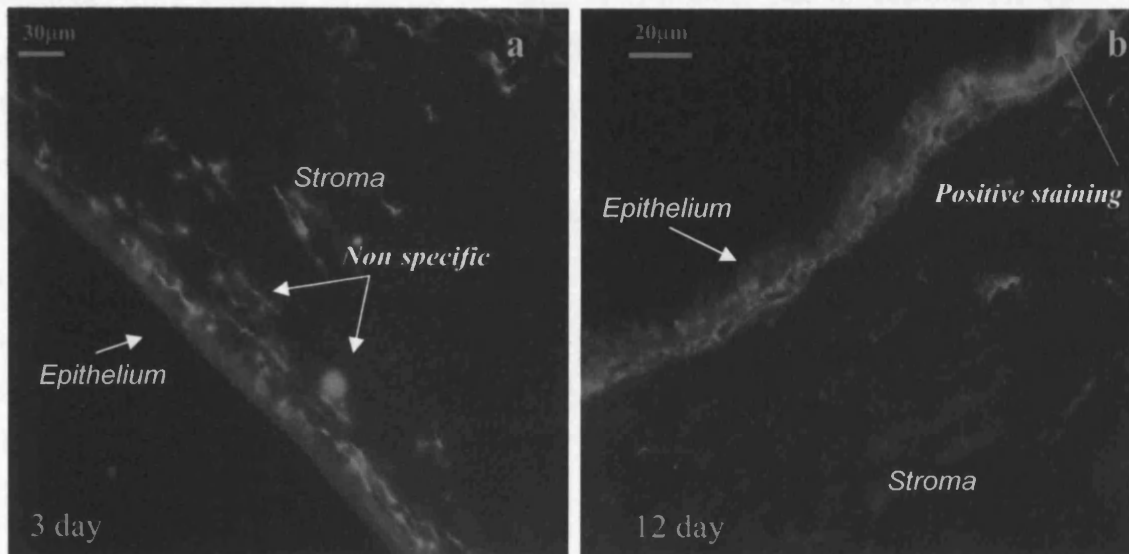


Fig 2.15 Connexin 43 (Sigma) immunolocalisation in the porcine cornea (frozen sections) without blocking and after 3 days (a) and with blocking and after 12 days of organ culture (b). Huge background staining (white arrows) has shown with corneal section without adding blocking (a) and positive immunoreactivity of Cx43 in basal cells of epithelium with less background staining (blue arrow) after using 20% donkey serum plus 10%BSA as a blocking (b).

2.2.12 Immunodetection of connexin 50

Mouse anti-Connexin 50 antibody (Zymed) does not cross react with other connexin proteins and is specific for connexin 50. Anti connexin 50 recognises an epitope in the cytoplasmic tail located between residues 290-440.

2.2.12.1 Immunodetection of connexin 50 in frozen corneal section

Frozen sections were allowed to come to room temperature, put in acetone for 10 minutes, and dried on the bench for 10 minutes. All the samples were then, washed three times, 10 minutes with PBS. The sections were then pretreated with 0.1% Triton X-100 for 10 minutes, followed by three 10 minutes washes with PBS. The sections were then incubated with 1/25 dilution of mouse anti connexin 50 antibody (Zymed, UK) followed by 2 hour incubation with 1/1000 dilution of Alex Fluor 488 donkey anti mouse IgG in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted in hydromount and wrapped in aluminium-foil to visualise under DMRAZ microscope (Leica).

Result:

Positive immunoreactivity of connexin 50 was identified. Thereafter all Connexin 50 immunodetection was carried out on frozen corneal sections using this protocol.

Table 2.19 Antibodies and methods used for connexin 43 staining

Type of section	Primary antibody	Secondary antibody	Treatment	Result
Frozen	Mouse anti connexin 43 1/500 (Zymed, UK)	Alex Fluor 488 donkey anti mouse (1/1000)	0.1% Triton X-100 for 10 mins	Positive+background
Frozen	Mouse anti connexin 43 1/300 (Sigma, UK)	Alex Fluor 488 donkey anti mouse (1/1000)	0.1% Triton X-100 for 10 mins	Positive+background
Frozen	Mouse anti connexin 43 1/300 (Sigma, UK)	Alex Fluor 488 donkey anti mouse (1/000)	0.1%Triton X-100 mins+20% donkey serum+10% BSA	Positive

2.2.13 ZO-1 staining

The tight junction is a unique intercellular structure, formed by several intracellular zonula occludens (ZO) proteins and the transmembrane occludins and claudins. ZO-1 ZO-2 and ZO-3 are members of the membrane associated guanylated kinase homologue family (Tsukita et al., 1999). They are peripheral membrane proteins located at the points of the TJ-membrane contact in epithelial and endothelial cells (Anderson et al., 1988; Haskins et al., 1998).

2.2.13.1 Immunodetection of ZO-1 in frozen corneal section

Frozen sections were allowed to come to room temperature, put in acetone for 10 minutes, and dried on bench for 10 minutes. All the samples were then washed, three times, 10 minutes with PBS. The sections were then pretreated with 0.5%, 0.1% Triton X-100 for 10 minutes, followed by three, and 10 minutes washes with PBS. The sections were then incubated with 1/100, 1/200 and 1/400 of rat anti ZO-1 antibody (detects a 225 KD polypeptide that is localised exclusively to the cytoplasmic surface of the tight junction in a variety of epithelia) over night (see table 2.20.) followed by 2hour incubation with 1/1000 dilution of FITC conjugate goat anti rat IgG in the dark, followed by three, 10 minute washes with PBS. The sections were then mounted in hydromount and dried and wrapped in aluminium-foil to visualise under DMRAZ microscope (Leica, UK).

Result:

Considerable background staining was detected (Fig 2.16.a) and the 1/400 dilution of antibody was chosen versus 1/100 and 1/200 dilutions and 0.1% Triton X-100 was used versus 0.5%.

2.2.13.2 Blocking procedure for ZO-1 staining

First 10% goat serum was tried as a blocking reagent and because it was not efficient (Fig 2.16b). A combination of 20% goat serum with 10% of BSA was applied to eliminate the huge amount of background (see table 2.20., fig 2.16c).

Result:

Positive immunoreactivity of ZO-1 was identified as shown in fig 2.16c. Thereafter all ZO-1 immunodetection was carried out on wax corneal sections using this protocol.

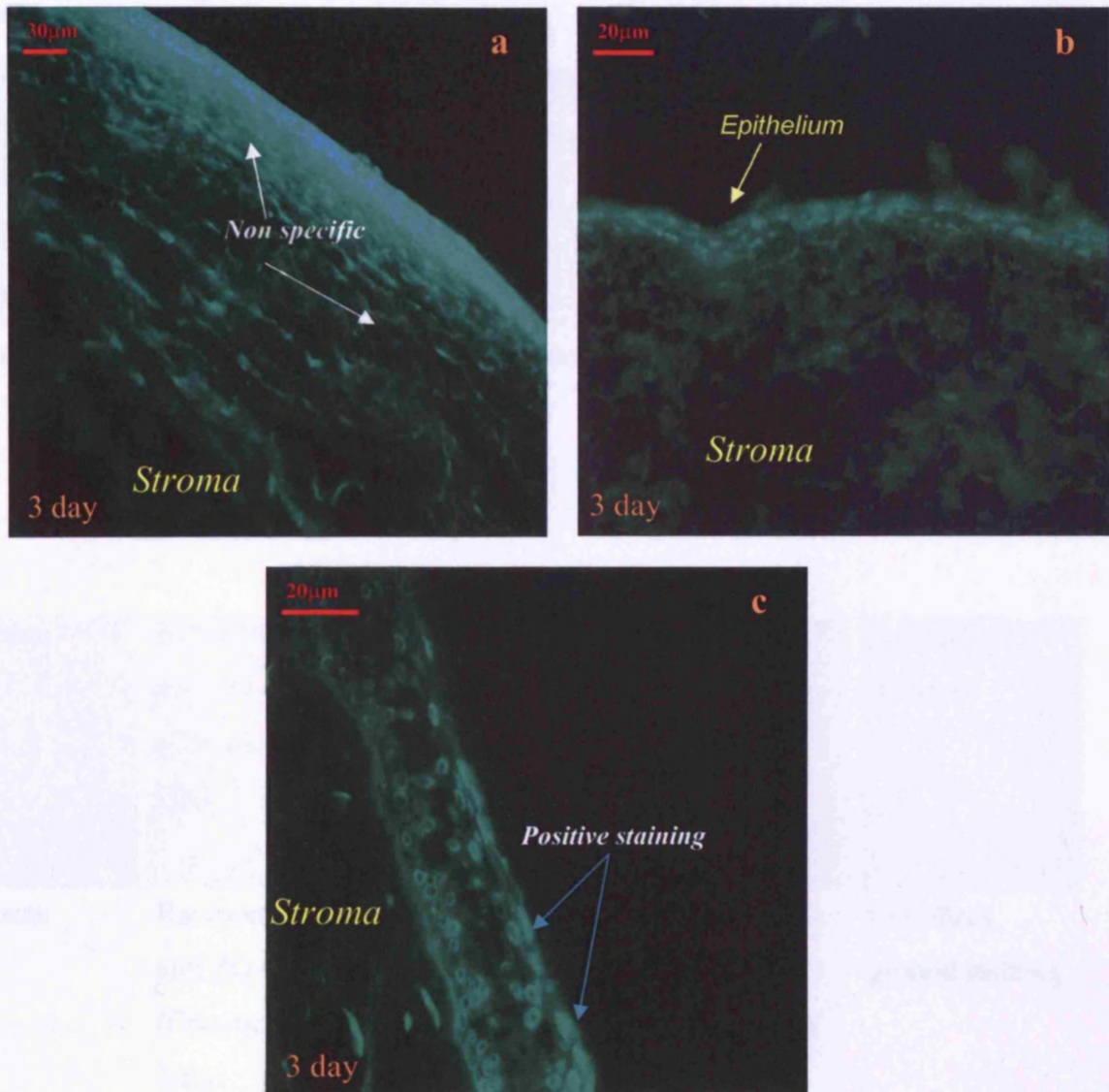


Fig 2.16 ZO-1 immunostaining of porcine corneal frozen sections (central region) without blocking and after 3 days of organ culture shows non specific (white arrows) staining (a), using 10% goat serum as a blocking (b) and double blocking with 10% goat serum plus 10% BSA (c). Figure c shows how double blocking with goat serum plus 10% BSA causes a great amount of reduction in background staining and greater staining (blue arrows).

Table 2.20 Antibodies and methods used for ZO-1 staining

Type of section	Primary antibody	Secondary antibody	Treatment	Result
Frozen	Rat monoclonal anti ZO-1 1/100 (Chemicon, UK)	FITC conjugate goat Anti rat 1/1000 (molecular probe)	0.5% Triton X-100 for 10 mins	Back ground staining
Frozen	Rat monoclonal anti ZO-1 1/200 (Chemicon, UK)	FITC conjugate goat Anti rat 1/1000 (molecular probe)	0.5% Triton X-100 for 10 mins	Back ground staining
Frozen	Rat monoclonal anti ZO-1 1/400 (Chemicon, UK)	FITC conjugate goat Anti rat 1/1000 (molecular probe)	0.5% Triton X-100 for 10 mins	Back ground staining
Frozen	Rat monoclonal anti ZO-1 1/400 (Chemicon, UK)	FITC conjugate goat Anti rat 1/1000	0.1% Triton X-100 for 10 mins+10%goat serum	Less Back ground staining
Frozen	Rat monoclonal anti ZO-1 1/400 (Chemicon, UK)	FITC conjugate goat Anti rat (1/1000)	0.1% Triton X-100 for 10 mins+20%goat serum+10%BSA	positive

2.2.14 Statistical analysis

Statistical analysis performed using Graph Pad Prism version 3.00 for Windows (Graph Pad Software, San Diego, USA). Column statistics analysis were performed to compute descriptive statistics, to test for normality, and to compare the mean (or median) of the group against a theoretical value of 100 using a one-sample t test or Wilcoxon rank sum test. One way ANOVA, unpaired t-test and Pearson's correlation test were used for parametric data. Kruskal-Wallis test and Mann-Whitney test and Spearman correlation calculation test, were performed when the data was not parametric. $P < 0.05$ was considered statistically significant.

CHAPTER THREE

3. Chapter 3 - Identification of Changes in Porcine Corneas During Organ Culture Storage

3.1 Introduction

This investigation was prompted by the need to understand the effect of storage time on epithelial cell homeostasis in porcine cornea during organ culture storage.

Organ culture storage of corneas has been established as a routine method to preserve corneas for transplantation. Storage for 28 days is routinely accepted in the UK (Armitage et al., 1997). Successful grafts have also been achieved with corneas stored in organ culture for even longer periods (Ehlers et al., 1999).

In preclinical studies, the porcine cornea is used routinely as an animal model due to its relative similarity to the human cornea and its availability in great numbers from abattoirs. The porcine corneal epithelium is highly stratified, like human cornea and has seven to nine cell layers which are classified as basal, wing and superficial cells (Reichl et al., 2003). In addition the porcine cornea has a Bowmans layer similar to that in the humans cornea (Sweatt et al., 1999). To date the porcine cornea has been successfully used in several investigations into organ culture storage (Rieck et al., 2003; Reichl et al., 2003; Reim 2001).

Several morphological properties of porcine cornea will be investigated in this chapter to determine the effect of organ culture preservation method on corneal cell homeostasis. The effect on epithelial and stromal thickness and the density of keratocytes will be identified. This will allow us to understand the overall changes that occur in corneal homeostasis during organ culture and provide a basis for subsequent investigations described in later chapters.

3.2 Experimental design

Using organ culture preservation methods, porcine corneas were preserved up to 14 days in modified organ culture medium at 34°C. The epithelial morphology, numbers of cell

layers, epithelial and stromal thickness, and keratocyte number and density were assessed following haematoxylin and eosin or hoechst staining. Images were analysed by light and fluorescence microscopy respectively using Leica QFluoro image analysis software. Corel Draw image analysis was then used to measure epithelial and stromal thickness. Keratocyte number and density was then quantified in anterior, middle and posterior regions using Corel Draw (to divide stroma to anterior, middle and posterior), Image Pro Plus (to count number of keratocytes) and Scion image (to measure the area) software.

3.2.1 Organ culture storage and wax sectioning

Porcine corneas were stored maintained to the protocol described in section 2.2.1.1, up to 14 days in organ culture storage. The corneas were then processed for tissue freezing (section 2.2.3.) and paraffin embedding (section 2.2.4.). Three, 7 μ m wax sections from each cornea were cut and put on to the slides and left overnight at 56°C to measure epithelial and stromal thickness and keratocyte number and density in the central corneal region.

3.2.2 Quantification of epithelial cell layers and thickness in porcine stored cornea

In order to measure the epithelial thickness in porcine stored cornea, 77 porcine corneal wax sections (see section 3.2.1) were stained with haematoxylin and eosin as described in section 2.2.5.

Then images were captured from 77 corneas (3 sections for each cornea), using Leica QFluoro image analysis software (77 \times 3=231 images) and analysed using Corel Draw 8 and 9. Three measurements for each image and 693 measurements in total (231 \times 3=693) were obtained to quantify epithelial thickness in the central region of porcine stored cornea.

The numbers of cell layers was also counted from haematoxylin and eosin porcine corneal wax sections under light microscopic visual field (40X). Three corneas were used for each time point of culture (up to 14 days) and three sections for each cornea (9 \times 14= 126 observations).

3.2.3 Quantification of corneal stromal thickness and keratocyte number & density in porcine stored cornea

In order to measure stromal thickness and keratocyte number and density in porcine stored cornea, wax corneal sections were first stained with bisbenzimidazole: 70 porcine corneal wax sections (refer to section 3.2.1) were stained with Hoechst 33345 according to the procedure described in section 2.2.5.2.

In order to measure the stromal thickness in the central cornea, stained sections of porcine central cornea were first imaged using Leica QFluoro image analysis software. Images were captured from 70 corneas, and 3 sections were used for each cornea. Between 2 and 3 pictures were taken from each section. The pictures were then grouped (using Corel Draw) to form the complete images of the central cornea (976µm). These images were then analysed using Corel Draw 8 and 9 to measure stromal thickness. Three measurements for each image and 630 (210×3=630) measurements in total were used to quantify stromal thickness in the stromal central region of porcine stored cornea (Fig 3.1).

3.2.4 Image analysis of stromal section for keratocyte number and density measurement

In order to measure keratocyte number and density, images of 62 stromal sections were obtained (section 3.2.3). Using Corel Draw, each stroma was then divided into 3 parts anterior, middle and posterior regions (Fig 3.1). Then the Scion image program was used to measure the areas in each part of the anterior, middle and posterior stromal regions. Finally the Image ProPlus software (image solutions, UK) was used to count the number of keratocytes in three different parts of the anterior, middle and posterior regions. Keratocyte density was then calculated using the formula:

$$\text{Keratocyte density} = \frac{\text{No of ks}}{\text{area}} \times \frac{\text{STo}}{\text{STn}}$$

No of ks = Number of keratocyte in appropriate time and region

STo=Stromal thickness in 0 day, STn=stromal thickness in appropriate time

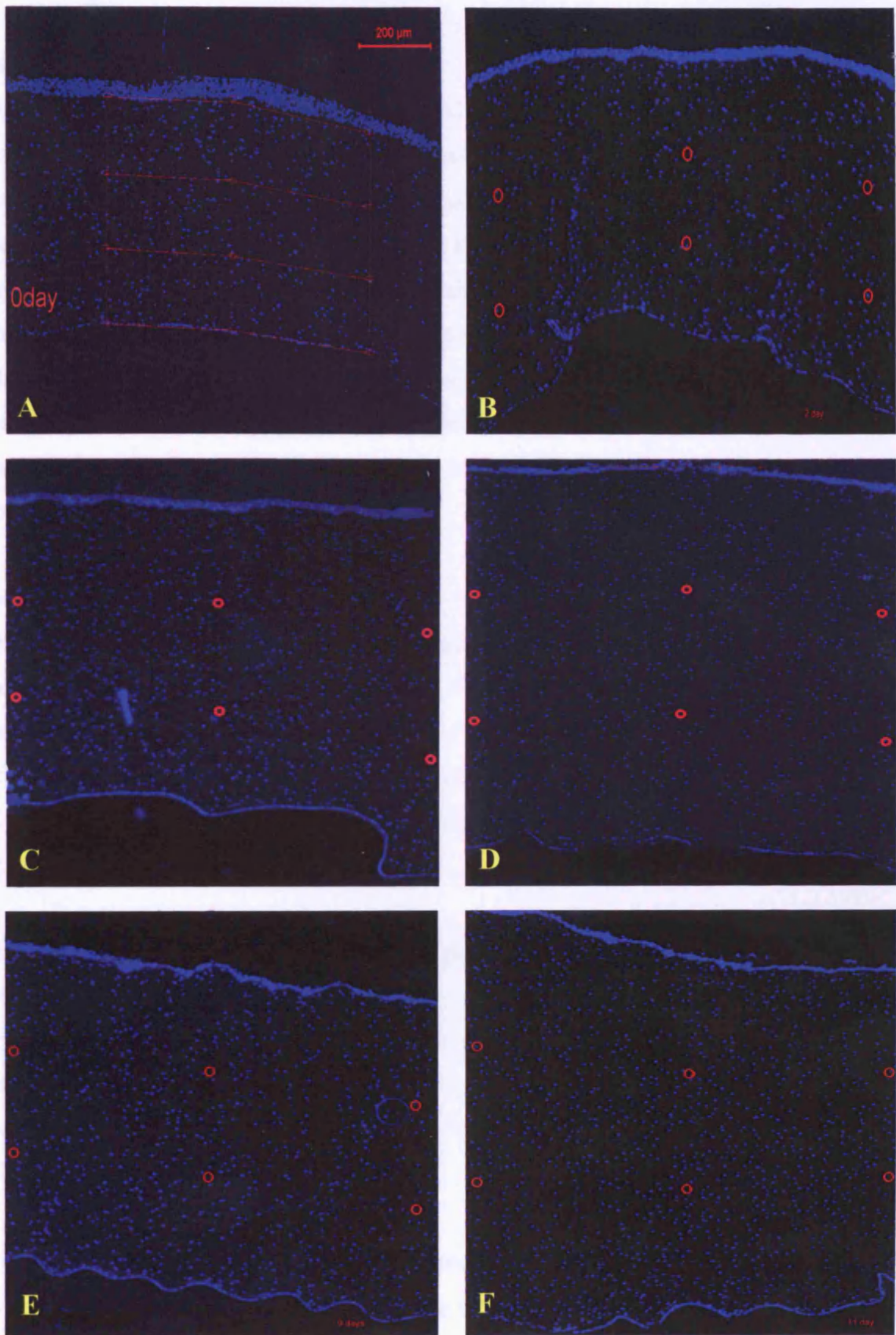


Fig 3.1 Hoechst staining of wax sections of porcine cornea central regions following 0 (A), 2 (B), 6 (C), 8(D), 9(E), and 11(F) days of organ culture. The red points and connecting lines demonstrate the division of regions. Bar represents 200μm.

3.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Prism normality test was also performed for deviations of data from Gaussian distribution using Kolmogorov-Smirnov (KS) test and $p > 0.1$ was considered as significant. One way ANOVA and unpaired t test was performed for parametric data, Kruskal-Wallis test and Mann-Whitney test for non parametric data and $p < 0.05$ was considered to be statistically significant. The statistical analyses of regression were also performed to analyse the relationships between epithelial or stromal data with time. Regression analysis was also performed to analyse the relationships between keratocyte number or density data with time. Column and row analyses were also performed to discover mean value and sample variation in each time point of organ culture storage.

3.3 Results:

3.3.1 Epithelial morphology and thickness in porcine stored cornea

In order to identify the epithelial morphological changes occurring during organ culture storage, haematoxylin and eosin staining of corneal wax sections were investigated over 0 to 14 days of organ culture. The results for epithelial morphology, thickness and number of cell layers were combined together to understand the possible changes that can occur during storage at each time point of culture.

Figure 3.2 and Figure 3.3 show some of the epithelial differences (central region) that occurred during organ storage of porcine cornea. A decrease in the number of epithelial cell layers was observed with increasing organ storage time. The statistical analysis of regression has also demonstrated a significant negative correlation (Fig 3.4) between organ culture storage time and epithelial thickness ($P < 0.0001$ and Spearman correlation $r_s = -0.9714$).

Porcine epithelial thickness (central region) decreased linearly from a maximum mean value of $45.2 \pm 3.6 \mu\text{m}$ ($n=6$ corneas) at 0 day to a minimum value of $7.7 \pm 0.6 \mu\text{m}$ ($n=6$) by day 14 of organ culture storage (Table 3.1). The number of cell layers has also

decreased from 7.1 ± 0.3 (n=9), at day 0 to 1.4 ± 0.2 number (n=3) at 14 days of organ culture (Fig 3.3.).

At day 0 it can be seen that the external corneal epithelial surface was smooth, and the central epithelium is $45.2 \pm 3.6 \mu\text{m}$ (n=6 corneas) thickness (Table 3.1) with 7-8 layers (7.1 ± 0.3 n=9 corneas) in its center. The number of epithelial cell layers increased towards the periphery with a maximum of 12 cell layers in the limbus (Fig 3.2 A, B, C).

After 2 days of organ culture, the central cornea lost its uniformity with a mean thickness of $31.5 \pm 5 \mu\text{m}$ (n=6), in its centre (Table 3.1). The number of cell layers at day 2 changed from 3 to 5 layer in the central (4 ± 0.2 , n=3) and peripheral, and 8 layers in the limbus (Figure 3.3).

Figure 3.2. shows that the epithelial thickness decreased to 4 layers in the centre (3.7 ± 0.2 , n=3) and 3-2 layers in the periphery and in the limbus after 4 days of organ culture (Figure 3. 2 D, E and F). Epithelial analysis of thickness also revealed a $23.7 \pm 0.6 \mu\text{m}$ (n=4) thickness in the central region at this time point (Table 3.1). this Decrease in epithelial thickness was even more obvious after 5 days (26.5 ± 1.9 , n=6) of organ culture.

There was also an obvious drop of epithelial thickness at day 7 ($18.7 \pm 2.1 \mu\text{m}$, n=6) with 1 to 3 layers (2.2 ± 0.2 n=3) in its centre. The number of cell layers continued to decrease after 7 days of organ culture. At day 8, it was only 1 or 2 (2 ± 0.2 , n=3) layers (Figure 3.3) at the points of minimum thickness ($17.5 \pm 3.4 \mu\text{m}$, n=6) in the center of the epithelium, 2-3 layers in the periphery and 1-2 layers in the limbus.

Further thinning of the epithelium was observed with increasing storage time. At 11 days of organ culture, the epithelium reached 1-2 (1.7 ± 0.2 , n=3) layers in its centre and approximately 1-2 layers in the limbus. Similar patterns are also obvious at 12 (1.3 ± 0.1 , n=3), 13 (1.3 ± 0.2 , n=3), and 14 (1.4 ± 0.2 , n=3) days of storage (Figure 3.2 J, K and L).

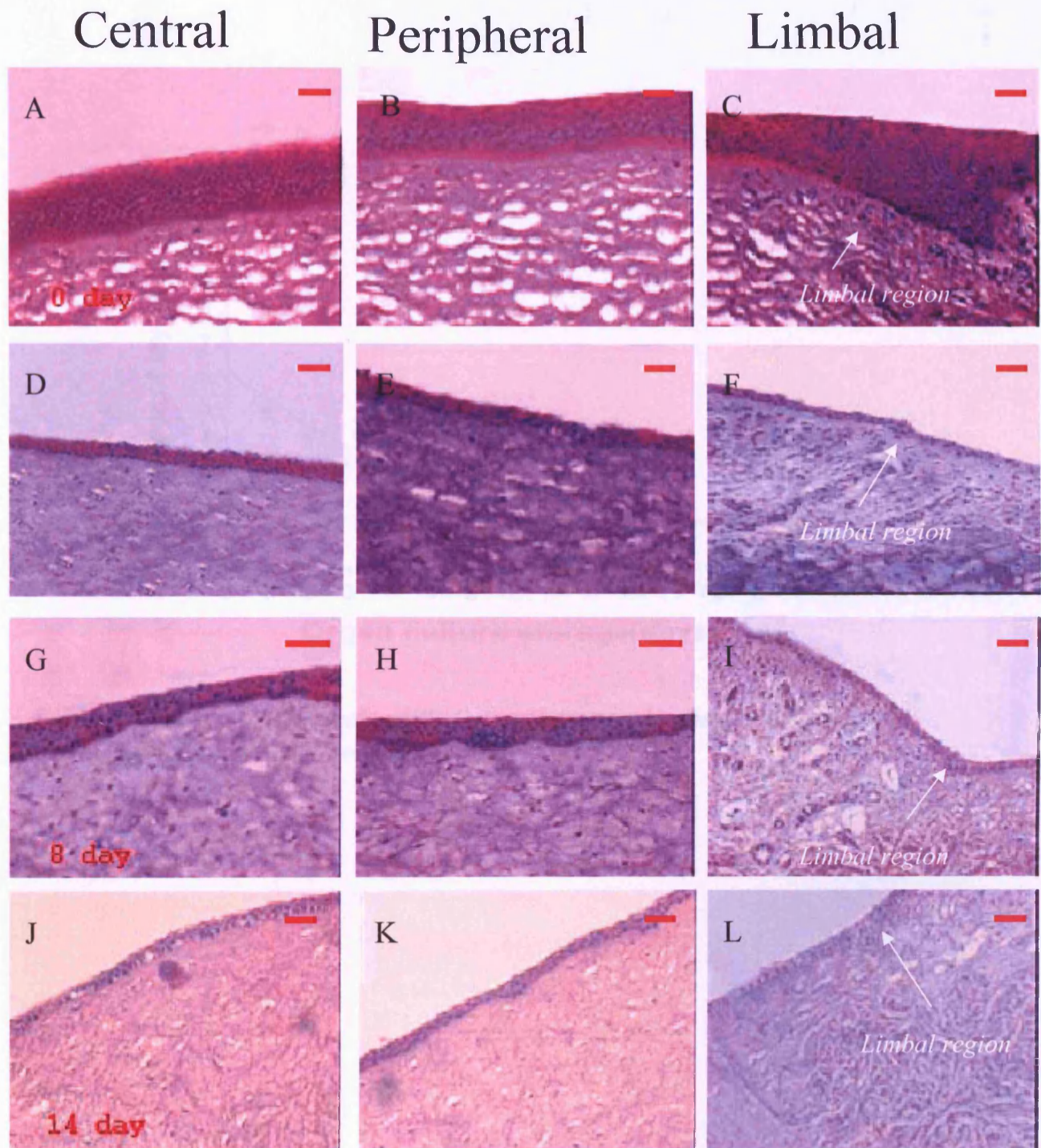


Fig 3.2 Haematoxylin and Eosin staining of wax sections of the central, limbal and peripheral regions of porcine corneas after 0 (A-C) day, 4 (D-F) day, 8 (G-I) day, 14 (J-L) day. A decrease in corneal epithelial thickness was observed with increasing time in organ culture. Bar represents 30 μ m.

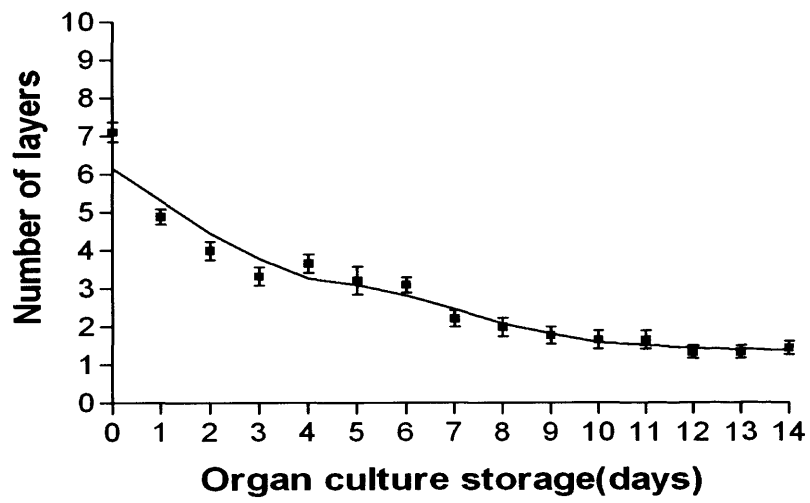


Figure 3.3 Corneal epithelial cell layers (central region) as a function of organ storage time.

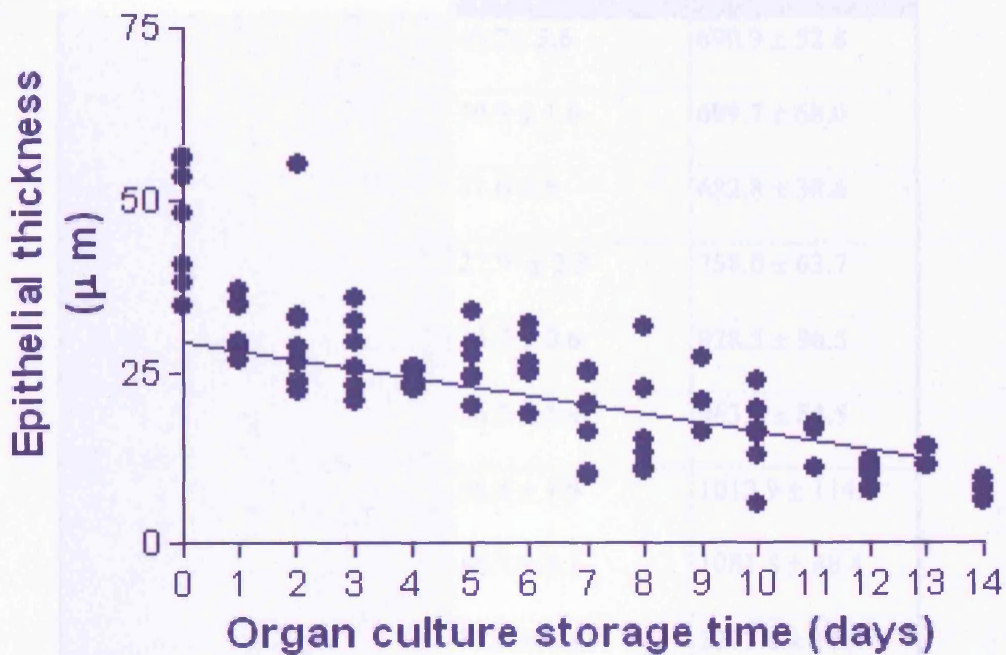


Fig 3.4 Epithelial thickness of porcine cornea with organ storage time (Spearman's rank correlation coefficient, $r_s = -0.9714$, $p < 0.0001$, $n = 77$ corneas).

Table 3.1 Epithelial and vascular (Mean \pm SD, n) thickness in control series of porcine corneas during organ culture storage.

<i>Organ culture storage time (days)</i>	Epithelial thickness	Stromal thickness
	Mean \pm SEM	Mean \pm SEM
0	45.2 \pm 3.6	690.9 \pm 52.8
1	30.9 \pm 1.6	699.7 \pm 68.0
2	31.6 \pm 5	682.8 \pm 38.6
3	27.9 \pm 2.3	758.0 \pm 63.7
4	23.7 \pm 0.6	928.5 \pm 96.5
5	26.5 \pm 1.9	963.9 \pm 64.5
6	26.4 \pm 1.9	1012.9 \pm 114.7
7	18.7 \pm 2.1	1081.8 \pm 48.4
8	17.5 \pm 3.4	1273.6 \pm 125.1
9	21.5 \pm 3.1	1080.5 \pm 122.3
10	15.8 \pm 2.5	1202.1 \pm 80.5
11	15.2 \pm 2.0	1058.8 \pm 61.4
12	10.2 \pm 0.7	1193.2 \pm 163.6
13	12.9 \pm 1.3	1111.7 \pm 6.1
14	7.7 \pm 0.6	1569.1 \pm 126.4

Table 3.1 Epithelial and stromal (Mean \pm SEM) thickness in central region of porcine cornea during organ culture storage.

3.3.2 Quantification of corneal stromal thickness in porcine stored cornea

Fig 3.5. showed a positive significant correlation between stromal thickness of porcine corneas and organ culture storage time ($p < 0.0001$ and Spearman rank correlation $r_s = 0.8821$). The stromal thickness increased from a mean value of $691 \pm 53 \mu\text{m}$ at day 0 to a maximum of $1569 \pm 126 \mu\text{m}$ at 14 days of organ culture storage (Fig 3.1, 3.5).

3.3.3 Keratocyte density in porcine stored cornea

Keratocyte density in the central stromal region was quantified, at different times of porcine organ culture storage. A significant decrease in keratocyte density (anterior, posterior and middle) occurred in the corneal stroma with increasing time of organ culture ($p < 0.0001$). Linear regression analysis showed an decrease of keratocyte density in the anterior ($r=0.77$ $n=62$) middle ($r=0.78$, $n=62$) posterior ($r=0.79$, $n=62$) and total ($r=0.58$, $n=62$) central stromal region with increasing time of organ culture (Fig 3.6).

In the anterior region of the stroma there was a decrease in keratocyte density (Table 3.2) from 1485.8 ± 325.8 cells/ mm^2 at 0 day to 153.2 ± 49 cells/ mm^2 at day 14 ($p < 0.0001$) (Fig 3.6, Table 3.2). In the mid stromal region there was a decrease from 1250 ± 297.8 mm^2 at day 0 to 148.9 ± 46.3 cells/ mm^2 ($p < 0.0001$) at day 14 (Fig 3.6, Table 3.2). The posterior stroma showed a decrease in keratocyte density from 1191.917 ± 268.342 cells/ mm^2 at day 0 to 163.3 ± 50.6 ($p < 0.0001$) at day 14 (Fig 3.6, Table 3.2). The total central stroma also showed a decrease in the density of keratocytes with time in organ culture from 2370.5 ± 1343.947 cells/ mm^2 at day 0 to 381.6 ± 159.5 cells/ mm^2 ($p < 0.0001$) at day 14 (Fig 3.7, Table 3.2).

One way ANOVA has also shown that there was not any significant difference in keratocyte density between various levels within the stroma ($p > 0.05$). This study has therefore demonstrated that there was a homogenous distribution of keratocyte density in all parts of the central stromal region (Table 3.2).

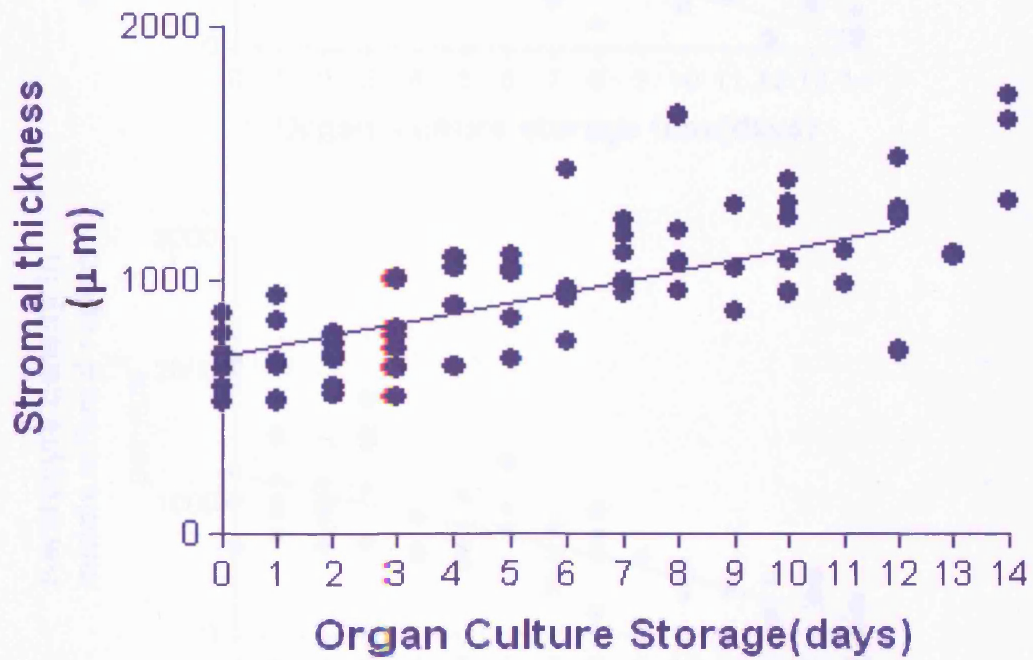


Fig 3.5 Stromal thickness of porcine cornea with organ storage time (Spearman's rank correlation coefficient, $r_s=0.8821$, $p<0.0001$, $n=70$ corneas).

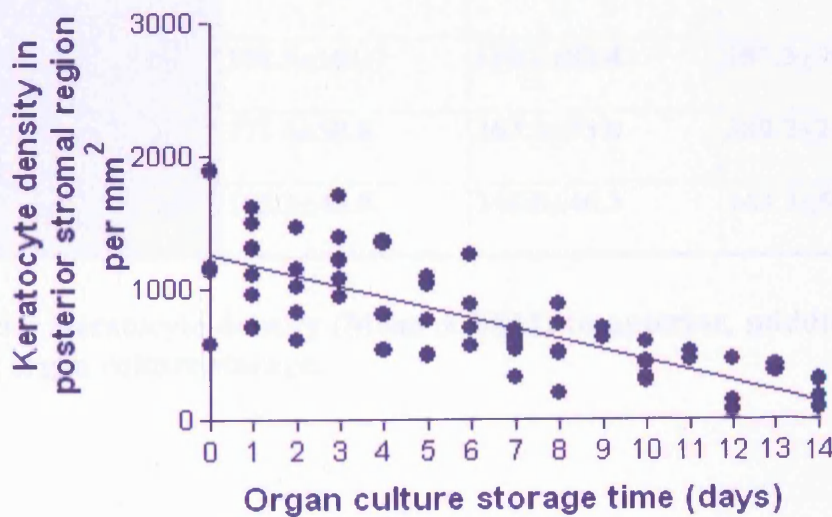
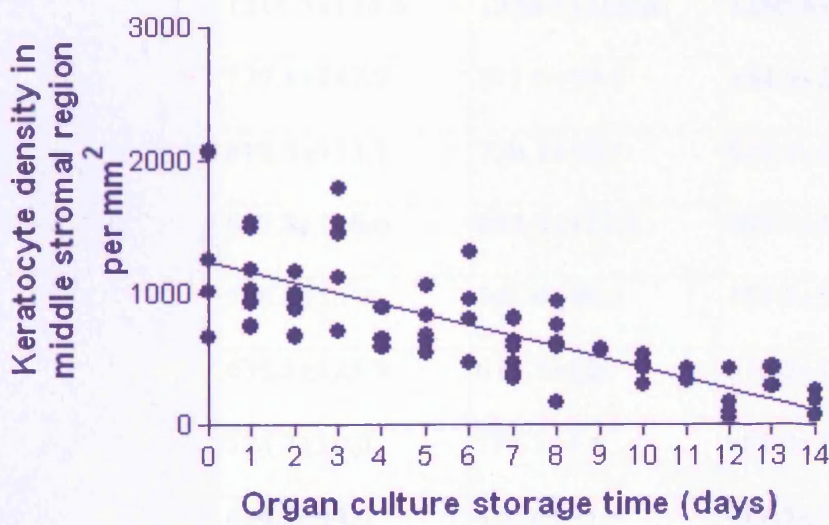
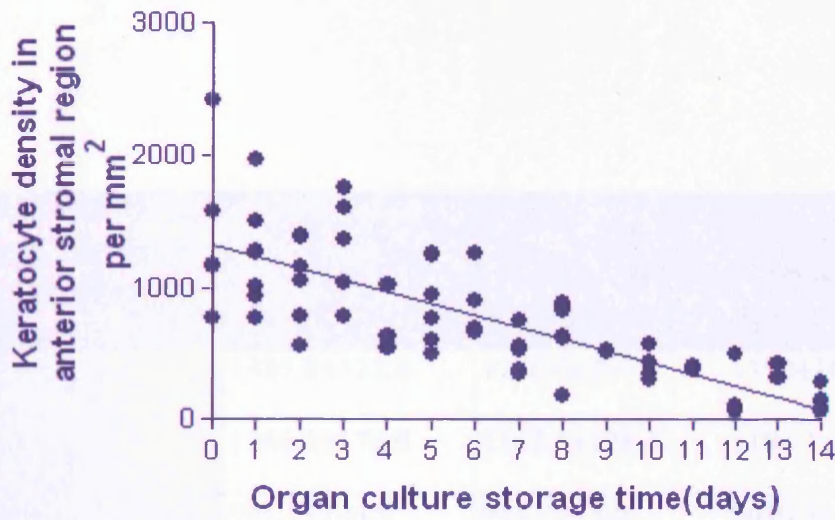


Fig 3.6 Keratocyte density in anterior ($r=0.77$, $n=62$), middle ($r=0.78$, $n=62$) and posterior ($r=0.79$, $n=62$) corneal regions ($p<0.0001$).

Organ culture storage time (days)	Anterior	Middle	Posterior	Total
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
0	1485.8±352.8	1250.0±297.8	1192±268.3	2370.5±1344
1	1246.2±179.9	1152.6±128.5	1196.1±138.9	2523.8±355.7
2	991.5±144.9	931.7±78.0	1010.3±146.1	2466.9±455.7
3	1316.3±178.6	1320.1±186.6	1260.4±131.8	2888.6±716.6
4	739.8±147.9	711.0±90.4	894.9±238.8	1152.7±284.2
5	819.9±133.1	750.1±88.3	825.9±107	2012.3±396.2
6	887.8±138.6	833.3±173.1	845.7±149.4	2332.2±676.3
7	528.5±58.7	543.0±70.2	559.8±51.6	1477.5±265.6
8	633.1±123.9	618.7±126	570.3±113.5	1739.3±429.2
9	524.7±10.0	577.2±5.5	663.0±34.3	611.0±29.3
10	424.9±44.6	410.6±43.9	417.5±53.1	1252.9±140.2
11	400.8±14.2	383.7±42.7	491.3±30.7	433.7±23.3
12	198.5±101.7	188.2±82.4	191.3±90.4	577.8±273.1
13	371.8±50.8	363.3±73.0	389.2±25.8	371.5±45.2
14	153.2±48.9	148.8±46.3	163.3±50.6	381.6±159.5

Table 3.2 Keratocyte density (Mean ± SEM) in anterior, middle, posterior stroma during organ culture storage.

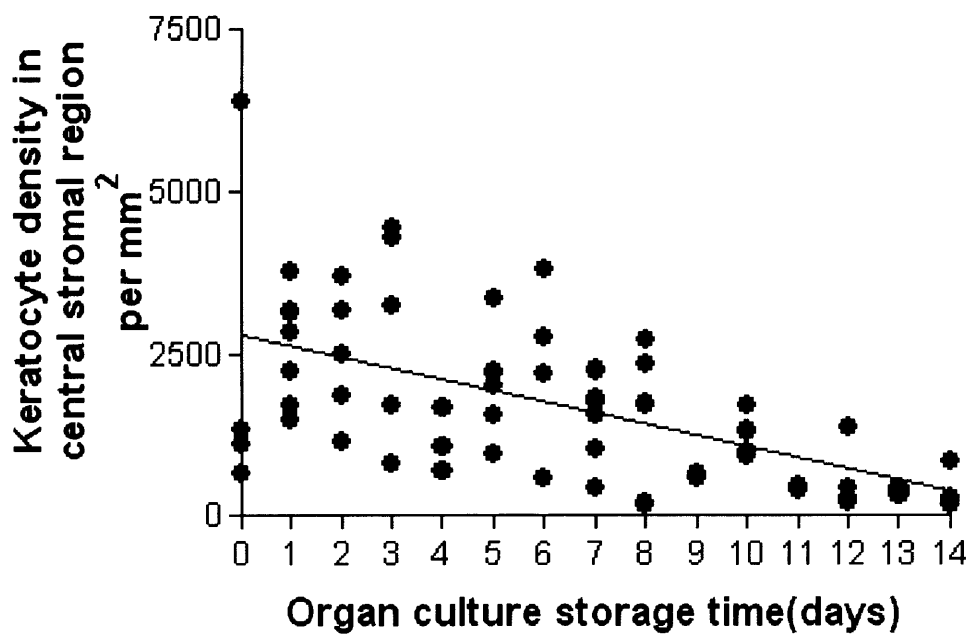


Fig 3.7 Keratocyte density in central stromal region ($r=0.58$, $n=62$ and $p<0.0001$).

3.4 Discussion:

There have been a variety of studies (Thuret et al., 2004, 2003; Crewe and Armitage 2001; Pedersen et al., 2001; Albon et al., 2000; Armitage et al., 1997; Foreman et al., 1996, Redbrake et al., 1998; Frueh et al., 1995; Ehlers et al., 1999) undertaken to investigate the changes in corneal morphology and cellularity of human corneas during organ culture storage. The results of this chapter have demonstrated a positive relationship between the degree of porcine corneal disruption and the amount of stromal swelling with storage time, in agreement with Crewe and Armitage (2001). Keratocyte density was also affected during porcine corneal storage time. A detailed comparison of these data with human cornea will be discussed in chapter 7.

A reduction in the number of cell layers was observed in the central corneal epithelium from 7-8 layers in day 0 to 1-2 layers after 14 days of organ culture. Epithelial thickness decreased from $45.2 \pm 3.6 \mu\text{m}$ at day 0 prior to organ culture to $7.7 \pm 6 \mu\text{m}$ thick after 14 days in organ culture storage. This is in agreement with the finding of Crewe and Armitage (2001) who demonstrated a reduction in the depth of the epithelium during human corneal storage.

The tendency of stroma to absorb water and to swell is usually counteracted by a fluid control mechanism located in the endothelium (Fatt et al., 1965). If this mechanism is disrupted water will enter the stroma and an increase in the stromal thickness, corneal oedema will result. The endothelium, via the apically located cell membrane bound NaK-ATPase mediated pump system, ensures that the leak rate of water into the stroma is exactly matched by the rate of the metabolic pump removing stromal water (Kim et al., 1994). If the barrier function is lost through the disruption of this fragile endothelial monolayer the stroma will become oedematous and the cornea will lose transparency.

During organ culture storage, the entire cornea was submerged in culture medium. A loss of epithelial cells was clearly observed. Degradation of the corneal epithelial barrier may be a contributory factor to stromal swelling resulting in an additional and substantial

influx of water across the epithelial surface leading to the stromal swelling that happens during organ culture storage.

Kampmeier et al. (2000) have shown that the porcine cornea is about 780 μm thick and when enucleated, swells in physiologic saline solution at a rate of about 26 μm per hour (Tungler et al., 1997). Wilson et al., (1984) have also shown that when a hypotonic solution is applied to the corneal surface the stroma swells. This well known osmotic response causes a surprising hydration change in the posterior stroma and permits swelling in the anterior-posterior direction. These observations are consistent with the increase in stromal swelling that happened during porcine cornea culture storage. From this study it was also shown that there was a significant correlation between the thicknesses of the porcine stroma with increased time in organ culture. The stromal thickness of our organ cultured cornea increased from a mean value of $691 \pm 53 \mu\text{m}$ at day 0 to a maximum value of $1569 \pm 126 \mu\text{m}$ after 14 days of organ culture storage, in agreement with Redbrake et al., (1997) findings in human corneal storage (from 652.5 at day 0 to 1509.70 μm after 28 days of organ culture storage).

There are other factors, including increase in the permeability of the epithelium and the endothelium due to a loss of cell-cell adhesion (McLaughlin et al., 1985; Wang et al., 1993) and epithelial hypoxia that could also be associated with corneal swelling (Klyce 1981).

During organ culture, epithelial hypoxia may occur as a result of changing conditions of aerobic to anaerobic respiration metabolism, as occurs in the organ culture storage of porcine corneas. Klyce (1981) showed that epithelial hypoxia enhances epithelial lactate production and release to the stroma. This process causes an increase in stromal lactate concentration and a decrease in stromal NaCl concentration (primarily through dilution). If stromal lactate accumulation increase in osmotic load and dilutional effect on [NaCl], stromal oedema is produced (effect on endothelial permeability transport). Therefore the tendency to swell in the porcine organ culture model could be due to both loss of

epithelial cell layer or increase in permeability as well as endothelial disruption (e.g. endothelial cell deterioration and changes in endothelial transport system).

The keratocytes of the cornea were first described by Toynebee in 1841, but histological analysis of keratocyte was not performed until much later (Sverdlick et al., 1954) using silver impregnation studies. Sverdlick showed cells in the corneal stroma with large, flattened and eccentric nuclei, and a highly vacuolated cytoplasm. These cells lay between the collagen lamellae and seemed to contact each other by a syncytium of long cytoplasmic processes. They also connect by a variety of specialized membrane junctions.

Our observation revealed decrease in keratocyte density with time of storage. This could be due to stromal swelling and the keratocyte might spread out over a larger area.

However observation of the limbal cornea during organ culture also revealed the existence of a higher population of keratocytes in the anterior stroma, which might be due to migrating activated keratocytes to this area to initiate a healing process. Some increase in keratocyte numbers was also observed at days 5 to 8 in anterior, middle and posterior regions of the stroma that could be due to keratocyte proliferation. Further investigation needs to be done to understand the mechanism of keratocyte proliferation, apoptosis and their interaction with epithelial cells to maintain corneal homeostasis during organ culture storage.

Corneal epithelium is subject to a constant process of cell renewal and regeneration. Cells in the uppermost layer of the corneal epithelium are continuously desquamated from the surface and must be replaced by cell proliferation (Klyce et al., 1985; Zieske et al., 1992; Kruse et al., 1994). The corneal epithelium exists in a state of dynamic equilibrium, with the superficial cells being constantly shed into the tear film, with a turnover period of 4–6 days. The special localisation of dividing cells in the basal layer of the corneal epithelium showed that proliferation is limited to basal cells (Hanna et al., 1961). Only cells which are in contact with basement membrane have the ability for mitotic cell division. Cells

that are displaced into the suprabasal layers become post-mitotic and lose their capability for cell division (Laver et al., 1991). To accomplish its self renewal process, the corneal epithelium and the epithelia of other self renewing tissues rely on the presence of stem and transient amplifying cells, which are the only cells with proliferative potential (Lavker et al., 1993, Morrison et al., 1997).

3.5 Conclusions

In the porcine cornea epithelial cells can be seen visibly to slough off during storage. The histological study showed corneal stromal swelling and epithelial sloughing in preserved cornea. The demand for tissue regeneration requires proliferation of stem cells and their differentiation into transient amplifying cells which subsequently proliferate and migrate centripetally and anteriorly to replace cell loss from the surface. Any disturbance in this process could result in unsuccessful epithelial renewal.

Lack or down-regulation of proliferation, changes in epithelial cell death and/or stratification of cornea are important factors that play a role in the disruption of epithelial cell renewal and need further investigation.

CHAPTER FOUR

4. Chapter 4 - Stratification of Porcine Corneal Epithelial Cells During Organ Culture Storage

4.1 Introduction

The functional properties of the corneal epithelium are to provide a cellular layer that is transparent, impermeable to penetration by physiological solutes and refractory to pathogen attachment. In order to accomplish these physiological functions, the epithelium must develop a distinctive organisation of the cytoskeleton and have an asymmetric distribution of the membrane proteins between the apical and basolateral membranes (Chifflet et al., 2004; Wolosin et al., 2000).

Stratification of the corneal epithelium is maintained by efficient epithelial cell-cell adherence and cell matrix adhesion in order to provide a competent barrier against fluid loss and pathogen entrance (Surge and Zieskie 1997). The resistance barrier of the corneal epithelium is to a great extent generated by zonula occludens (ZO, tight junctions), which are formed between the apical parts of the cell in most stratified epithelia (Mclaughlin et al., 1985; Wang et al., 1993). Tight junctions function to provide a paracellular permeability barrier (Mclaughlin et al., 1985; Wang et al., 1993). The presence of tight junctions between squamous cells in the superficial layer and the transepithelial resistance of corneal epithelium are highly correlated (Wang et al., 1993). Therefore alteration of ZO-1 which is one of the membrane-associated proteins of tight junctions (Furse et al., 1993; Stevenson et al., 1999) may be a factor in corneal storage worth investigation.

The cytokeratin filament system within the corneal epithelium provides a structural framework for the epithelial cytoplasm. It provides cytoskeletal components that anchor cells tightly to one another and to their substrate through the desmosomes and hemidesmosomes. Cytokeratins form a family of at least 20 intermediate filaments found in epithelial cells and their expression depends on the type and state of differentiation of the epithelium (Steinert et al., 1988; Franke et al., 1981b; Moll et al., 1990). They can be divided into acidic and basic-neutral subfamilies according to their relative charges. In

vivo, a basic keratin is usually paired with an acidic keratin to form a heterodimer. In corneal epithelium keratin 3 (64 kDa, basic) and keratin 12 (55 kDa, acidic) pairs are specifically expressed (Liu et al., 1993; Liu et al., 1994). K3/K12 is a marker for terminal differentiation in the corneal epithelium, it stains all differentiated cells of the corneal and limbal epithelium but not conjunctival epithelium (Schermer et al., 1986). AE3 antibody reacts with a number of basic (65-67 KDa) epithelial keratins (1, 2, 3, 4, 5, 6 and 7), which are found in all epithelial cells, and AE1 recognises a group of acidic (56.5 KDa) keratins (10, 13, 14, 15, 16, 19) (Moll et al., 1982; Cooper et al., 1984; Eichner et al., 1984).

In organ culture the organisation of cytokeratins, which are one of the three cytoplasmic filament types in corneal epithelial cells, may also be altered.

Gap junctions mediate cell-to-cell communication and play an important role in re-epithelialisation in the cornea (Wolosin et al., 2000). Gap junctions are specialised cell membrane structures forming intercellular channels (Loewenstein et al., 1981; Beyer et al., 1990) that are composed of a variety of transmembrane proteins (polypeptides) called connexins (Cx) (Loewenstein et al., 1981; Simon et al., 1988; Beyer et al., 1990; Goodenough et al., 1996). The gap junctions allow the direct passage of ions from the cytoplasm of one cell to the next which allows the conduction of electrical impulses to travel from one cell to another and to maintain organ size and properties rates of proliferation and/or differentiation in self renewing tissues they need to constantly adjust to the changes in cell loss rates. Gap junctions are suited to contribute to this control, especially in a tissue such as corneal epithelium (Wolosin et al., 2000). Their role in direct cell-cell communication may affect cell proliferation, differentiation, and apoptosis (Simon et al., 1998; Beyer et al., 1990; Gabbiani et al., 1978).

Cx 43 and Cx50 are the two connexins identified in the human ocular surface epithelium so far (Dong et al., 1994). Under normal conditions, expression of Cx43 is noted in the basal cell layer of the human corneal but not limbal epithelium, suggesting that the

expression of Cx43 denotes the differentiation of SCs into corneal TACs (Matic et al., 1997a; Wolosin et al., 2000).

The aim of this chapter was to determine changes in stratification of corneal epithelium during organ culture storage. This is important for determination of barrier properties of epithelium and maintenance of efficient epithelial cell homeostasis. Therefore, firstly, changes in cytokeratins were studied to determine the differentiation process of corneal epithelial cells during organ culture (using AE5 and AE1/AE3 markers). Second cell-cell communication (Cx43 and 50 immunolocalisation) and cell-cell adherence (tight junctions, ZO-1) were investigated to determine the properties of intercellular junctions in corneal epithelial cells in culture storage.

4.2 Experimental design

In this study cellular differentiation was determined by immunolocalisation with antibodies raised against cytokeratin 3 (AE5) and cytokeratins 10, 13, 14, 15, 16, 19 (AE1) and cytokeratins 1, 2, 3, 4, 5, 6 and 7 (AE3). The distribution and presence of gap junction proteins and tight junctions were determined by immunolocalisation with antibodies raised against connexins 43 and 50; and ZO-1 respectively. For each marker, corneal sections were used in duplicate for 0, 3, 6, 9 and 12 days of organ culture.

4.2.1. Immunolocalisation for cytokeratin

Cytokeratin 3 and Pan cytokeratin immunolocalisation were studied in porcine corneal frozen section using antibodies raised against epidermal cytokeratin 3 (AE5), cytokeratins 10, 13, 14, 15, 16, 19 (AE1) and cytokeratins 1, 2, 3, 4, 5, 6 and 7 (AE3) as described in sections 2.2.9 and 2.2.10.

4.2.2 Immunolocalisation for Cx43 and Cx50

Cx43 and 50 immunolocalisation were investigated in porcine corneal frozen section using antibodies raised against Cx43 and Cx50 as described in sections 2.2.11 and 2.2.12.

4.2.3 Immunolocalisation for ZO-1

ZO-1 immunolocalisation was studied in porcine corneal frozen section using antibodies raised against ZO-1 as described in section 2.2.13.

4.3 Results:

4.3.1 Cytokeratin 3 immunolocalisation in porcine stored cornea

The immunolocalisation of cytokeratin 3 in the porcine stored cornea showed (Fig 4.1) a homogenous strong cytoplasmic staining for CK3 in the basal and suprabasal cells in the central and peripheral epithelium, in contrast to the more limited suprabasal cells staining in the limbal region. The limbal region demonstrated only some superficial cells staining at day 3 (f) compared to the more uniform suprabasal layer staining in the limbus at day 0 (c). This pattern was unchanged in the central, peripheral and limbal regions from day 6 to 12 (j and k) and in the greatly thinned limbal epithelium at day 12 (l).

4.3.2 Pan cytokeratin immunolocalisation in organ culture stored cornea

Immunostaining with AE1/AE3 for 0, 3, 6, 9 and 12 days of porcine corneal culture showed a homogeneous uniform intracytoplasmic distribution pattern throughout the central, peripheral, and limbal regions of epithelium (Fig 4.2). Staining intensity remained constant from day 3 (d, e and f) to 12 (m, n and o) days of organ culture.

4.3.3 Connexin 43 immunolocalisation in porcine stored cornea

Immunofluorescent staining of organ culture porcine corneas stored (Fig 4.3.1) for connexin 43 antibody demonstrated the presence of very faint staining in the basal and suprabasal cells in the central and peripheral regions at day 0. Punctate staining was observed in keratocytes, with more staining in the anterior compared to posterior and middle region (Fig 4.3.2). After 3 days of organ culture the diffuse staining was more evident especially in the basal and suprabasal layer in the peripheral region (e) compared to the central area (d). The staining seemed to be absent in the peripheral region (h) after 6 days of organ culture, although some staining could be observed in the epithelial wing cells in the central (g) region. The diffuse staining was apparent again at day 9 and some

positive staining was observed in the basal layer of the peripheral region (n) with the patchy staining in the central (m) at day 12. No staining was evident in the limbal regions at any time point of organ culture (c, f, I, l and o).

Fig 4.1 Fluorescent localisation of AE5 in cryosection of porcine corneas maintained in organ culture for various time points. The pictures show strong cytoplasmic staining of AE5 in basal and suprabasal layers of epithelium in the central (a, d, g and j) and peripheral (b, e, h, and k), regions in contrast to the more limited suprabasal cells staining (arrow) in the limbal area (c, f, I, and l). More superficial staining was observed in epithelium at day 3 (f, arrow) compared to the uniform suprabasal pattern at day 0 (c). This pattern was unchanged in the central, peripheral and limbal regions from day 9 (g, h and I) to 12 (j, k and l). Bar represents 30 μ m.

Central

Peripheral

Limbal

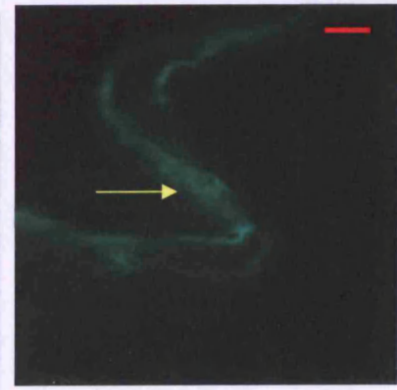
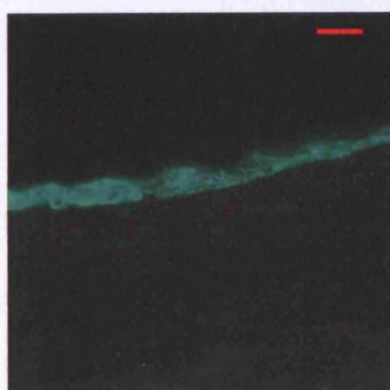
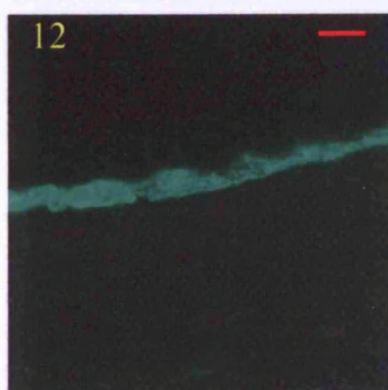
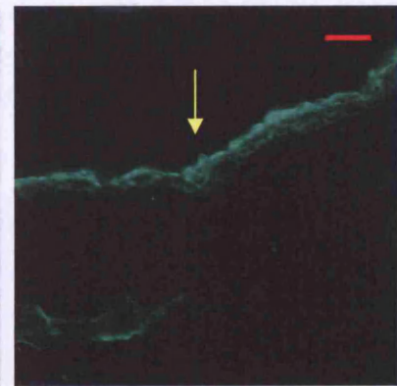
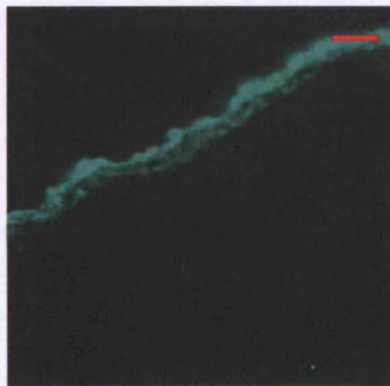
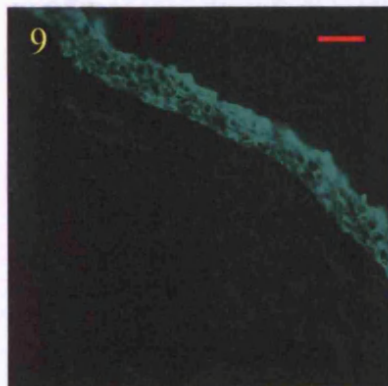
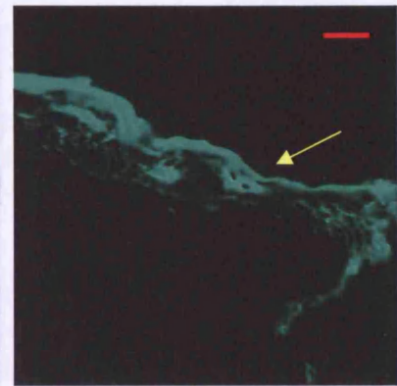
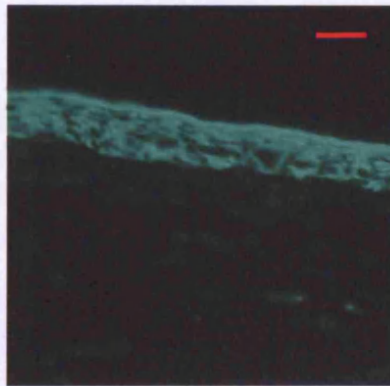
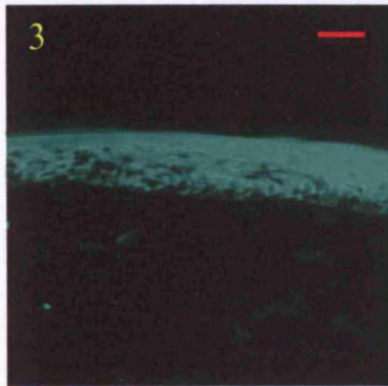
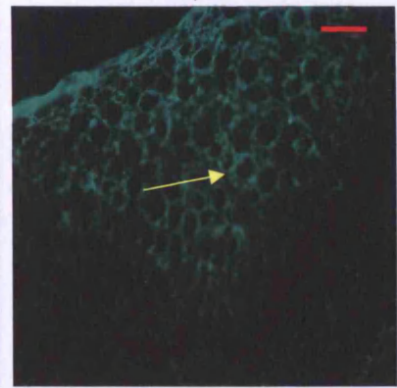
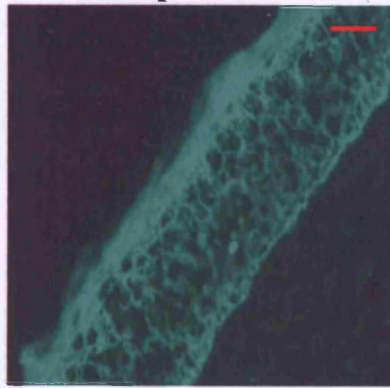
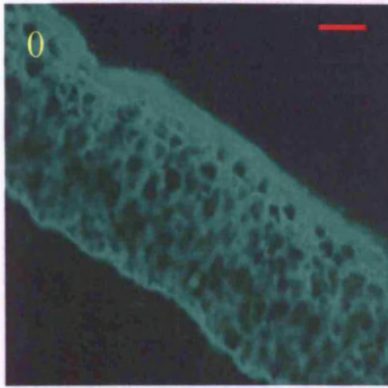


Fig 4.2 Fluorescent localisation of AE1/AE3 in cryosections of porcine corneas in organ culture. Various time points of porcine corneal organ cultured were compared; a, d, g, J and m are central b, e , h, k and n are peripheral and c, f, i, l and o shows limbal regions.

The picture shows the cytoplasmic immunolocalisation of AE1 and AE3 in all basal and suprabasal layers of epithelium in the central (a, d, g, j and m), peripheral (b, e, h, k and n) and limbal (c, f, I, l and o) regions. The intensity of staining remained constant from day 3 (d, e and f) to 12 (m and o) of organ culture. Bar represents 30 μ m.

Central

Peripheral

Limbal

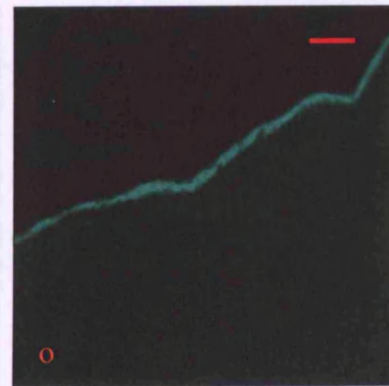
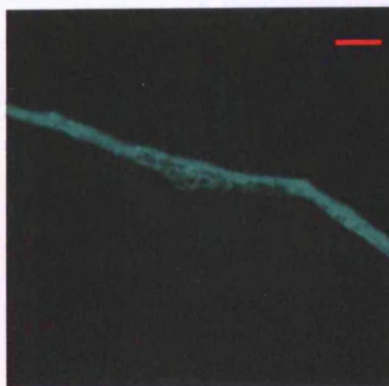
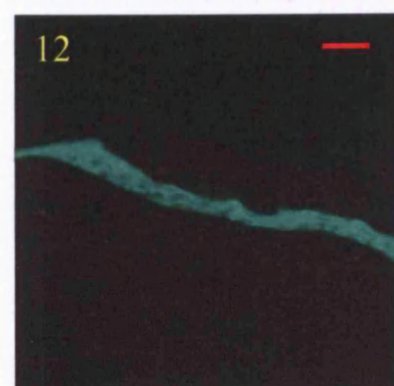
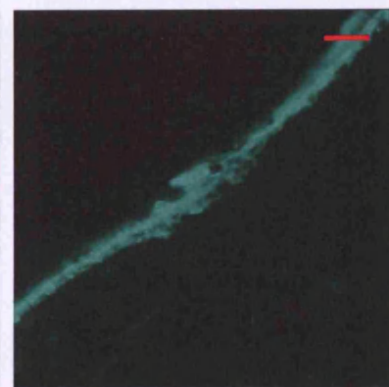
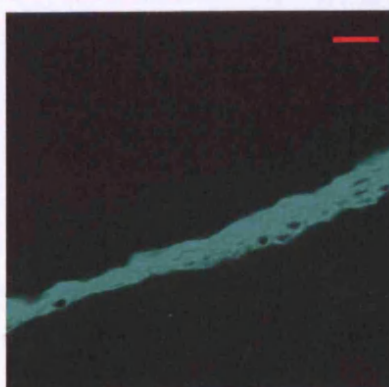
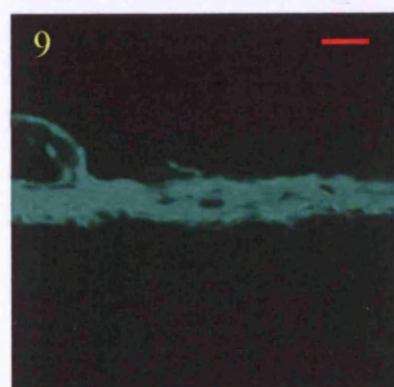
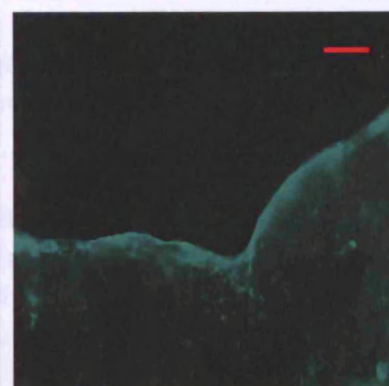
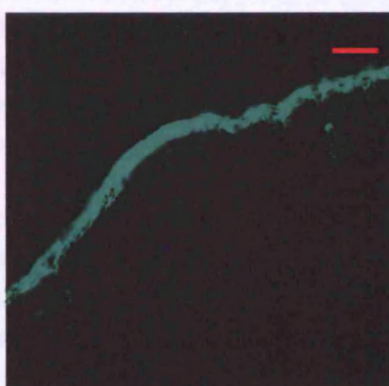
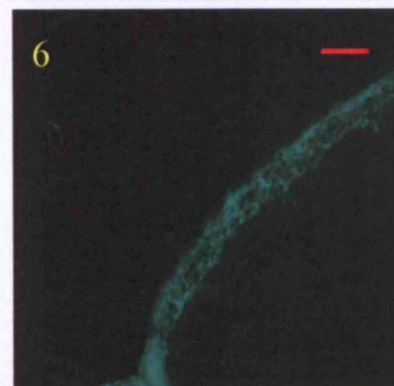
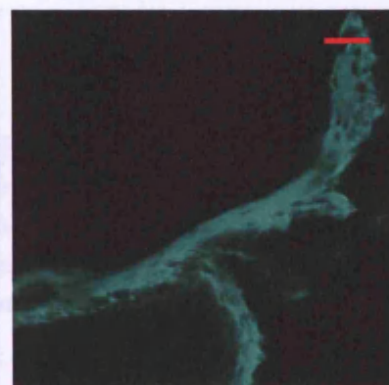
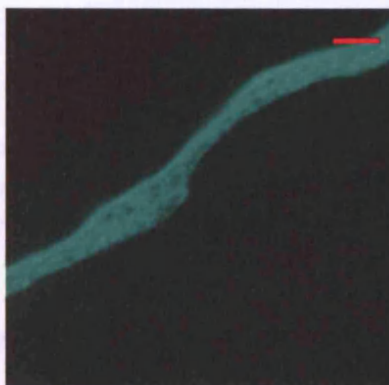
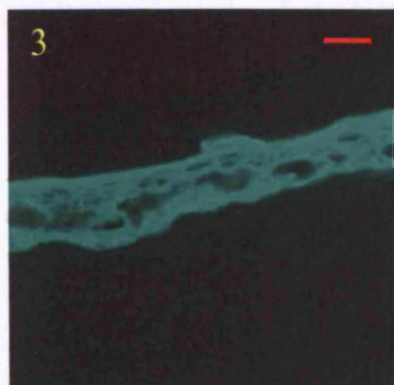
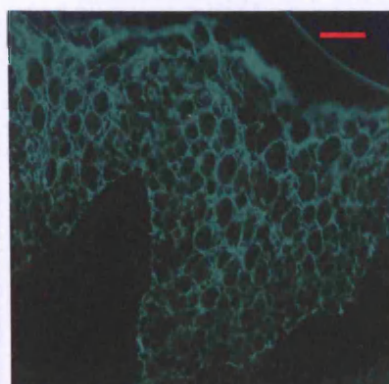
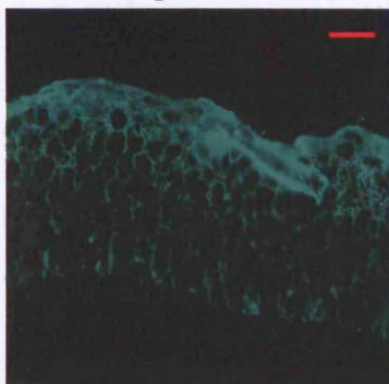
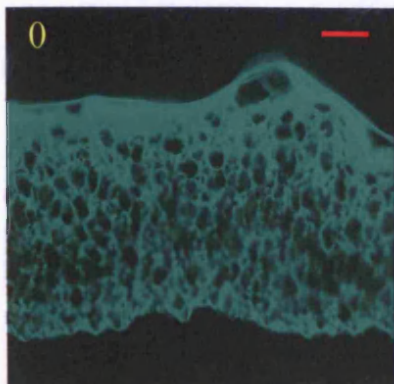


Fig 4.3.1 Immunofluorescent staining of connexin 43 in cryosections of porcine corneas maintained in organ culture for various time points. (Bisbenzimidazole nuclear staining (blue) of these images were shown in the following page). Figs a, b and c show the presence of very faint Cx43 staining in the basal and suprabasal layer of corneal epithelium in the central (a) and peripheral (b) regions (day 0).

Figures d, e and f, shows corneal epithelium after 3 days of organ culture, the diffuse (arrows) staining was more in the basal and suprabasal layer of the periphery (e) compare to the centre (d). Arrow shows (g) only central epithelial staining at day 6 (wing cells).

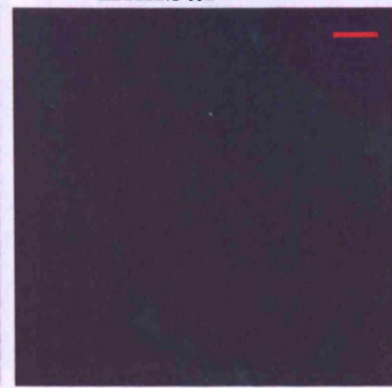
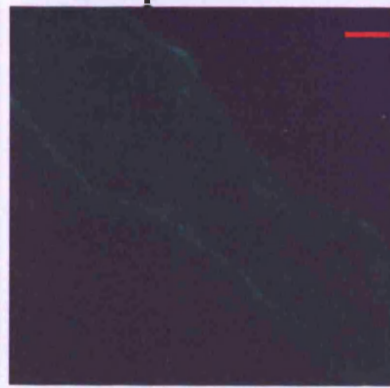
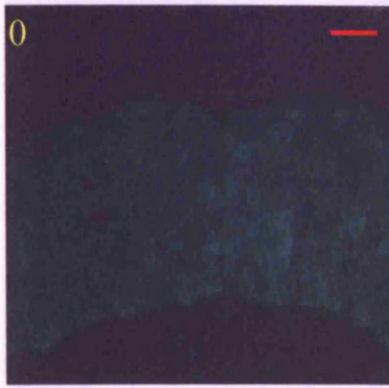
Fig (j) shows diffuse staining (arrow) at day 9. Positive staining in the basal layer (arrow) of epithelium in the periphery (n) with patchy (arrow) staining in the central (m) at day 12. No staining was evident in limbal regions at any time points of organ culture (c, f, i, l and o). Bar represents 30 μ m.

Central

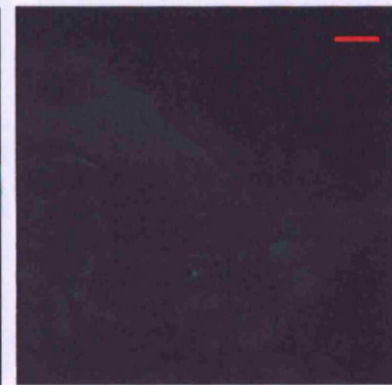
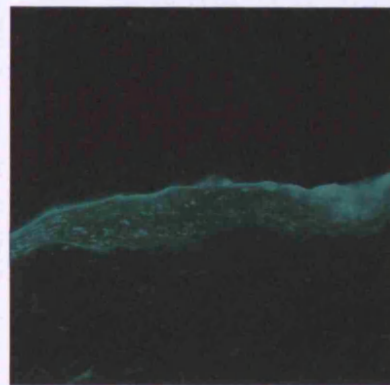
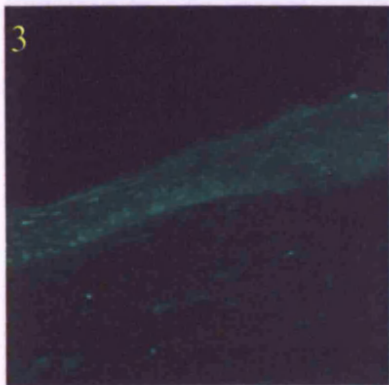
Peripheral

Limbal

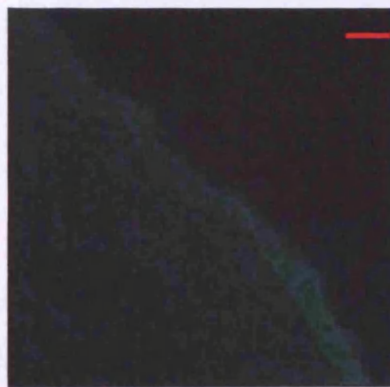
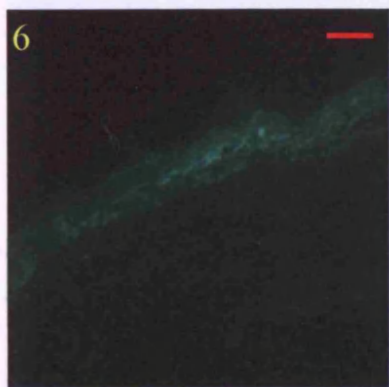
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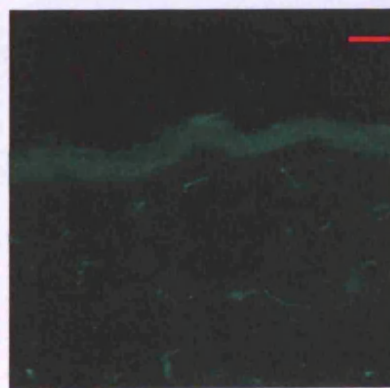
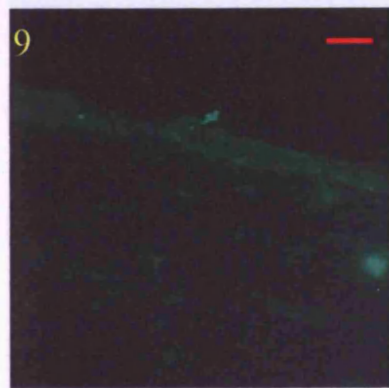
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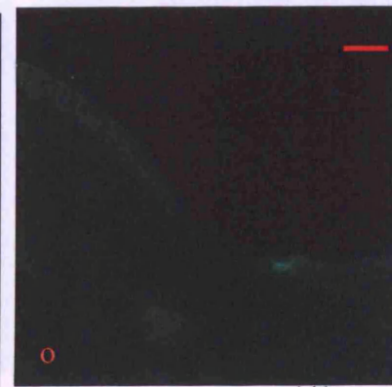
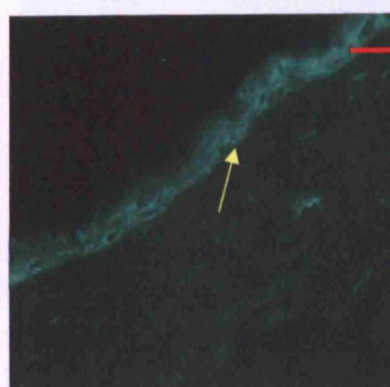
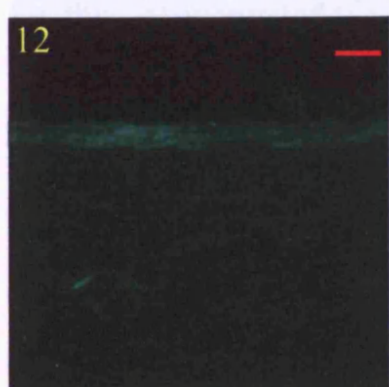
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9



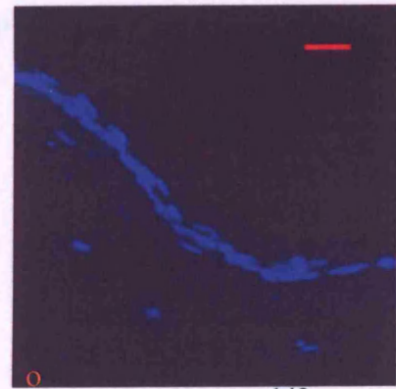
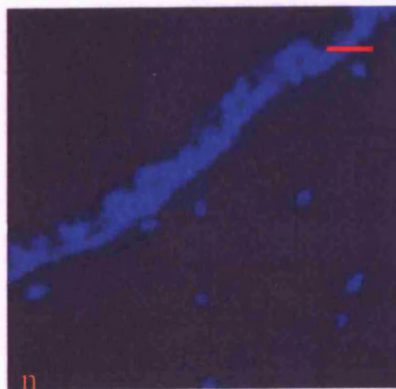
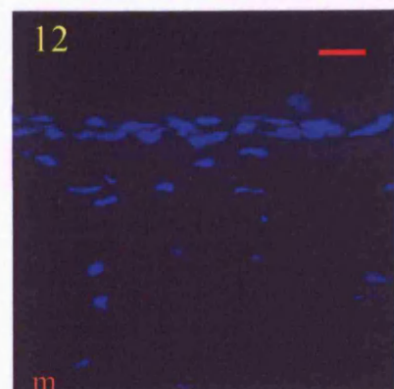
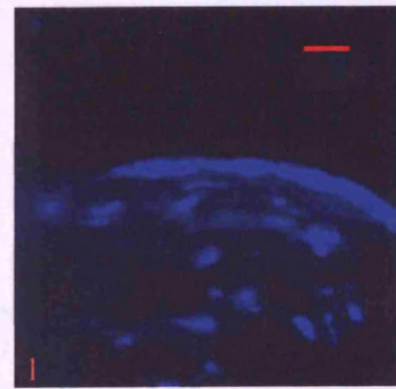
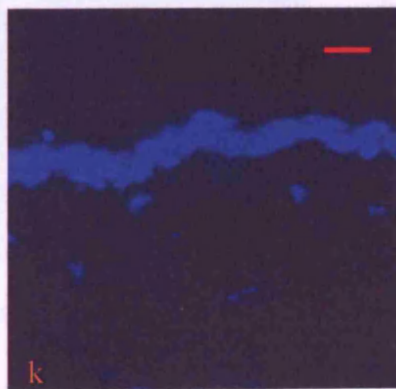
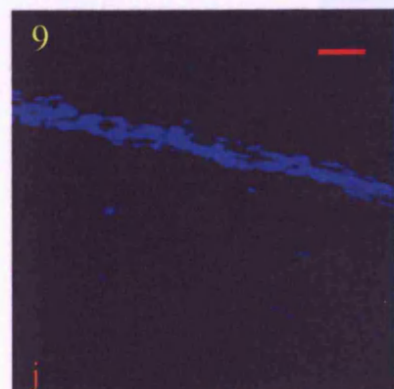
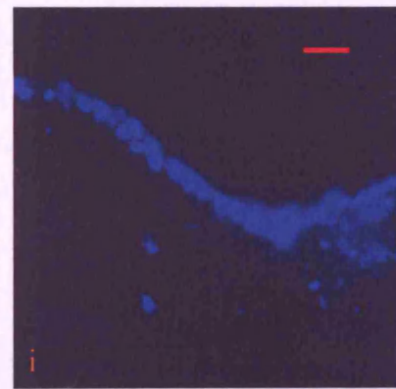
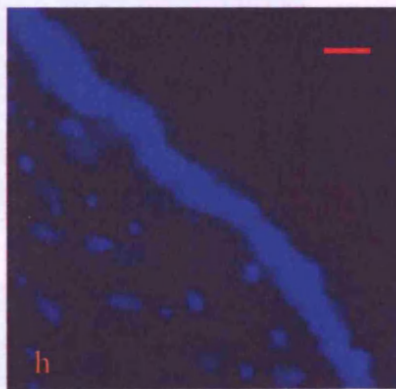
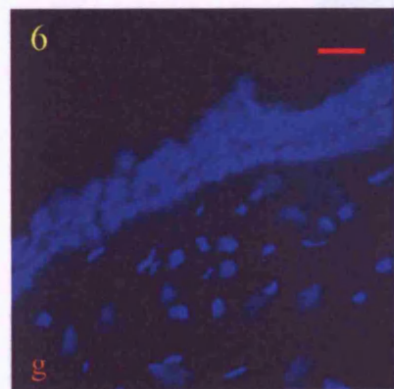
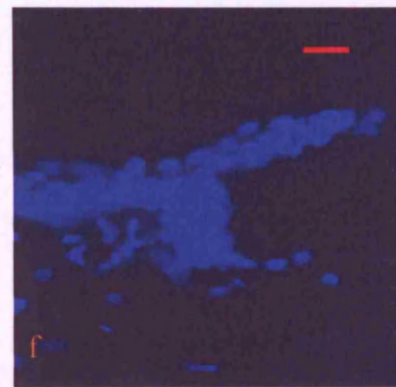
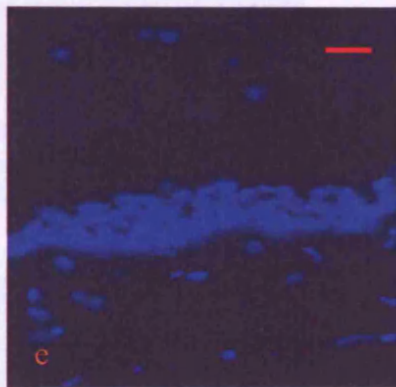
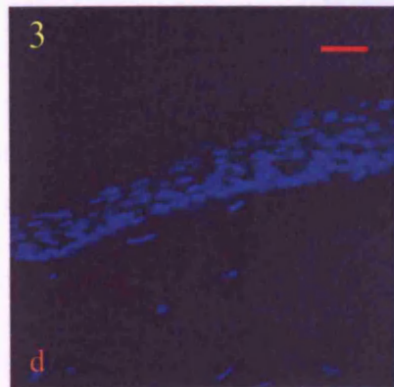
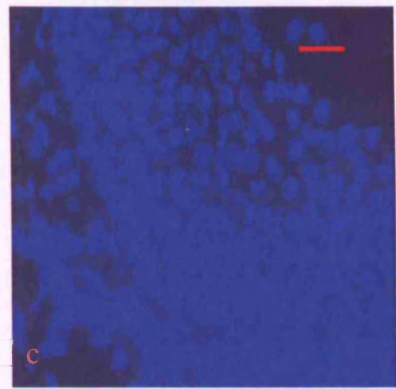
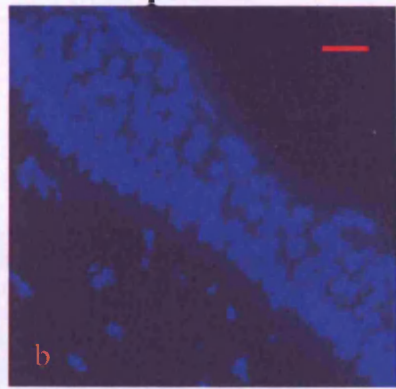
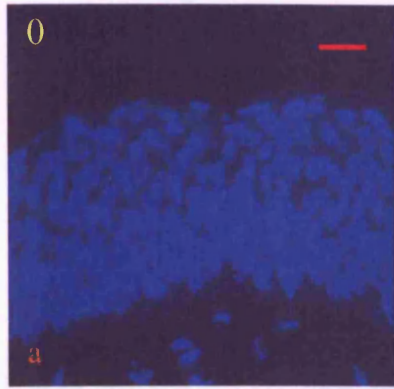
12



Central

Peripheral

Limbal



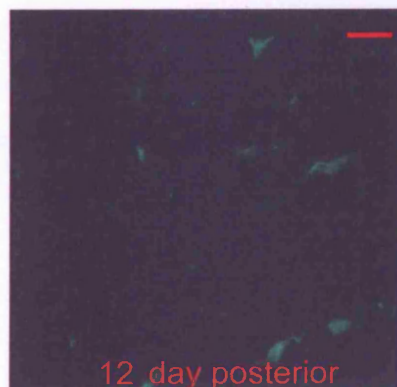
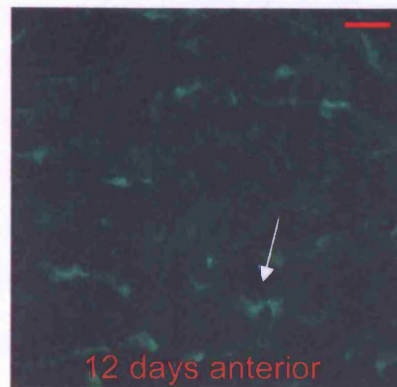


Fig 4.3.2 Cx43 immuno labelling of keratocytes in anterior, middle and posterior regions of stroma after 12 days of organ culture. Greater amount of staining was present in the anterior region compared to that in the middle and posterior. Bar represents 20 μ m.

4.3.4 Connexin 50 immunolocalisation in porcine corneas maintained in organ culture

Cx50 staining of the porcine corneal sections showed the presence of strong suprabasal staining, with fainter staining in the wing cells in the peripheral and central regions at day 0 (Fig 4.4). No immunostaining was detected in the stroma. Diffuse basal and suprabasal staining was displayed in both the peripheral (e) and central (d) regions of epithelium after 3 days of organ culture storage. Cx50 staining was absent in porcine cornea at day 6 (g and h). However diffuse, punctate staining was appeared again after 9 (j and k) days of organ culture. No staining was evident in the limbal regions at any time points of organ culture (c, f, i and l).

4.3.5 ZO-1 immunolocalisation in porcine corneas maintained in organ culture

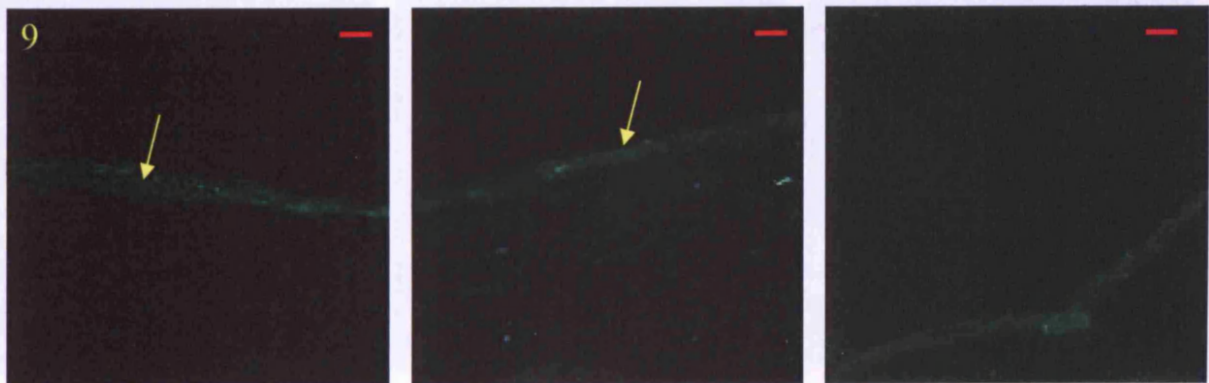
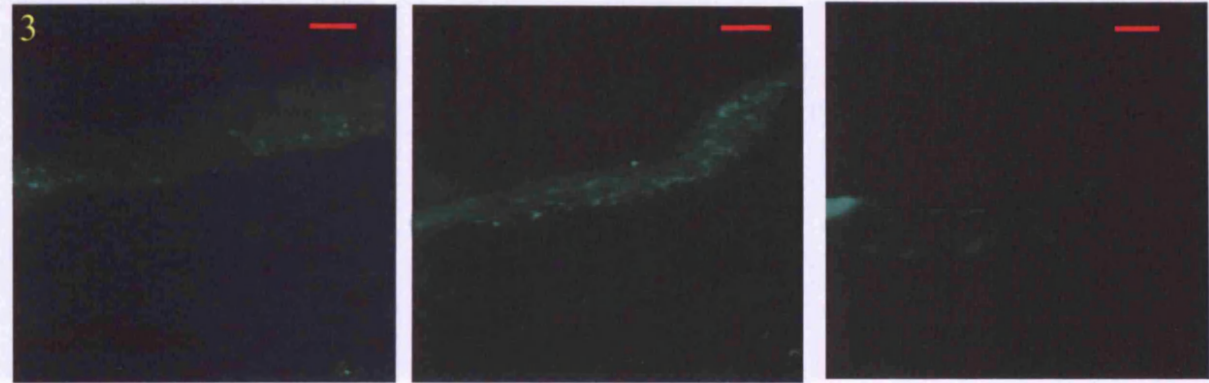
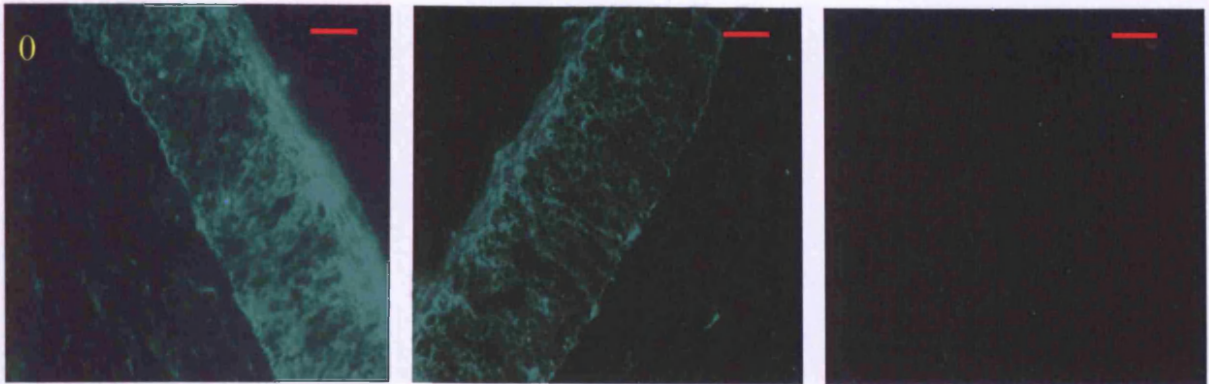
Immunostaining of frozen sections of porcine cornea for ZO-1 during organ culture storage (Fig 4.5) showed linear suprabasal staining in the central, peripheral and limbal regions of epithelium at day 0. After 3 days of organ culture the intense staining could be observed in the superficial cells of the epithelium both in the central (d) and peripheral (e) regions. After 6 days of organ culture the staining was absent in the peripheral, central and limbal regions of epithelium. At day 9 diffuse staining was observed across the basal and suprabasal layers in the central (g) and peripheral (h) regions and punctate staining was detected in the peripheral (wing cells) and central (superficial cells) regions of epithelium after 12 days of organ culture (j and k).

Fig 4.4 Immunofluorescent staining of Cx50 in cryosections of porcine corneas after 0, 3, 6 and 9 days of organ culture storage (Bisbenzimidazole nuclear staining of these images were shown in the following page). At day 0 strong superficial staining (arrow) was observed in the epithelial peripheral (b) and central (a) regions. Diffuse basal (arrow) and suprabasal staining was displayed in both the peripheral (e) and central regions (d) at day 3. Cx50 expression seemed to be absent in both central and peripheral regions (g and h) at day 6. Some punctate staining was evident in epithelium at day 9 (j). No staining was evident in the limbal regions at any time points of organ culture (c, f, i and l). Bar represents 30 μm .

Central

Peripheral

Limbal



Central

Peripheral

Limbal

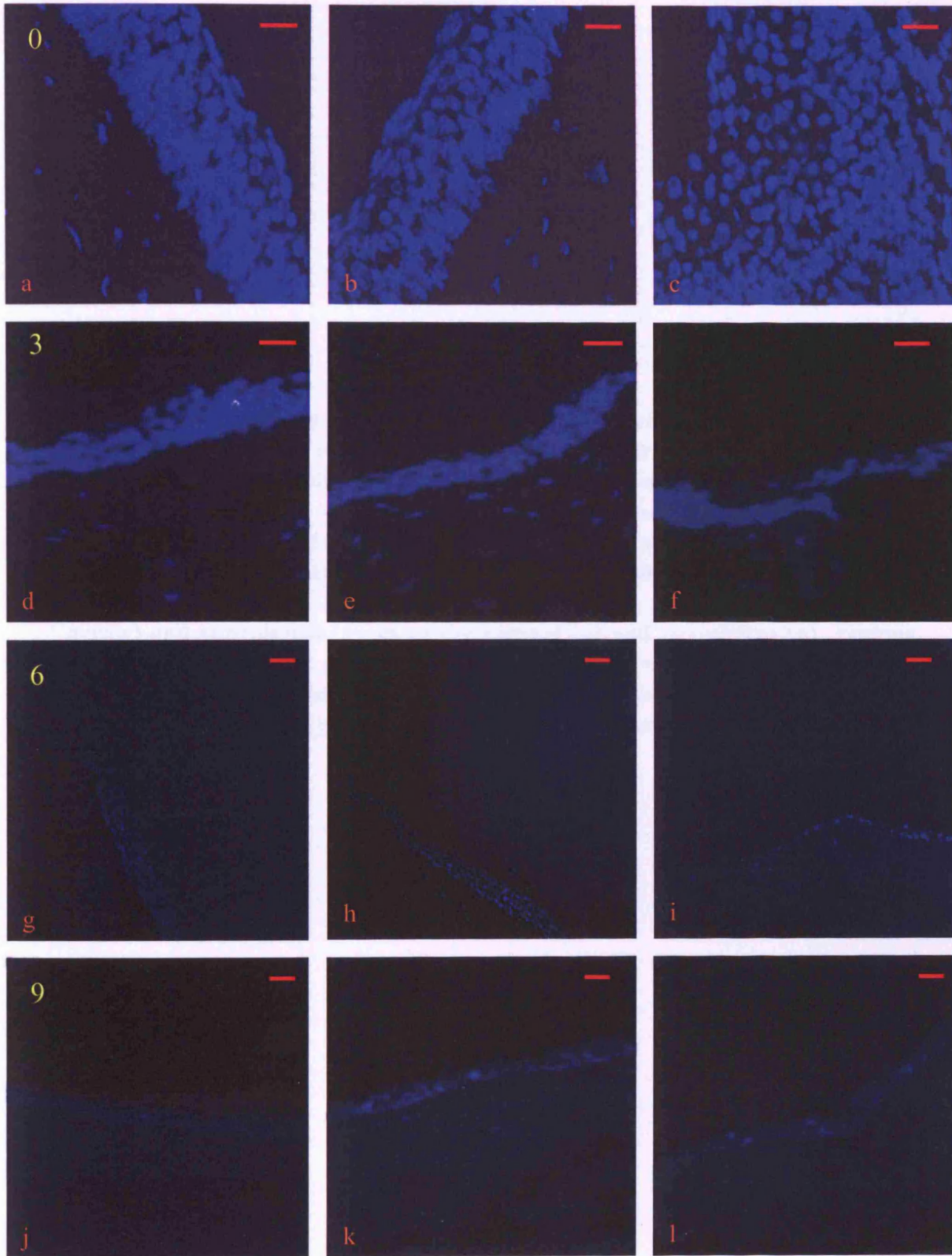
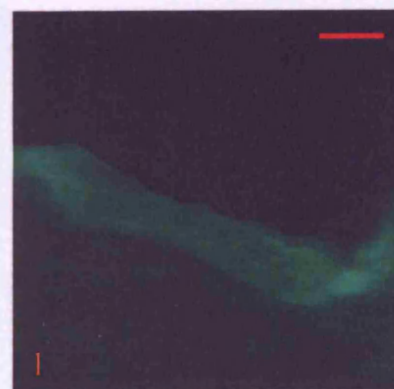
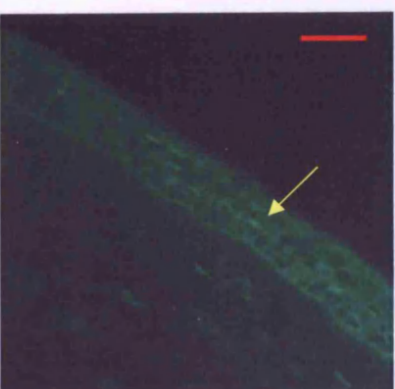
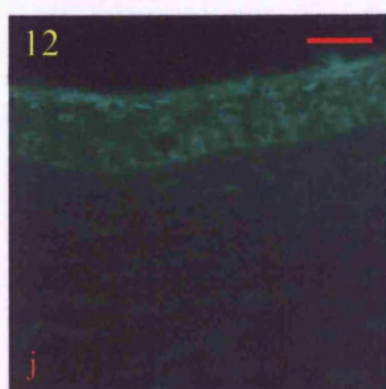
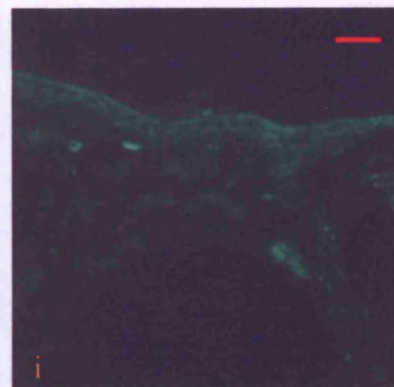
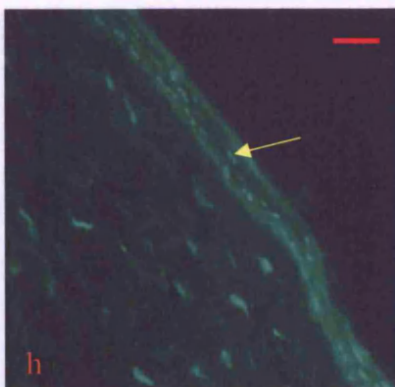
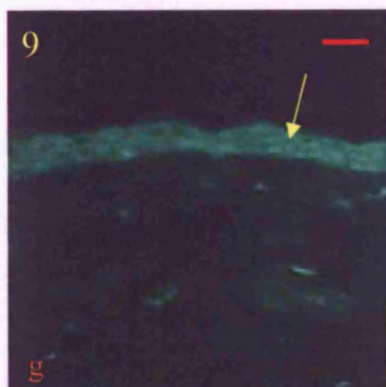
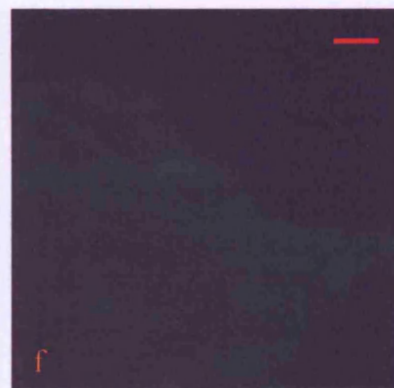
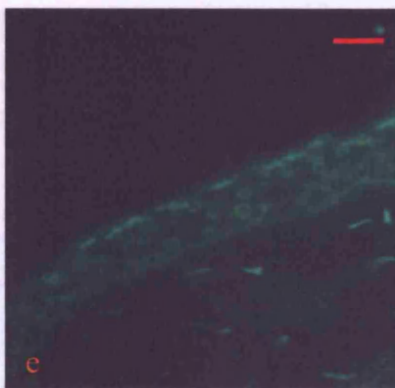
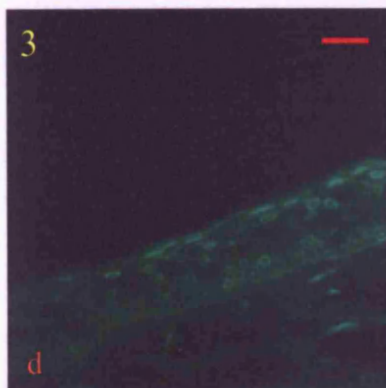
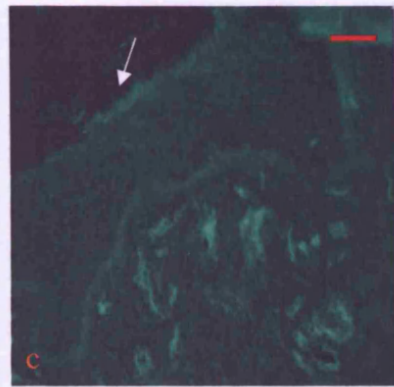
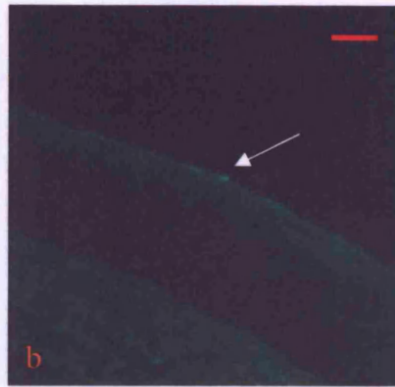
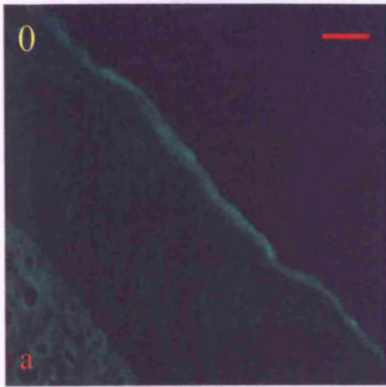


Fig 4.5 Immunofluorescent staining of ZO-1 in porcine corneal sections after various time points in culture (Bisbenzimidazole nuclear staining of these images were shown in the following page). Linear suprabasal staining is obvious (white arrows) in the central (a), peripheral (b) and limbal (c) regions of porcine epithelium at day 0. At day 3 the intense staining was in the superficial cells (white arrows) of epithelium both in the central (d) and peripheral (e) regions. At 9 day diffuse staining was all over the basal (yellow arrow) and suprabasal layers of the central (g) and peripheral (h) regions. Punctate staining was detected in the peripheral (wing cells, yellow arrow) and central (superficial cells, white arrow) regions of epithelium after 12 days of organ culture (j and k). Bar represents 30 μm .

Central

Peripheral

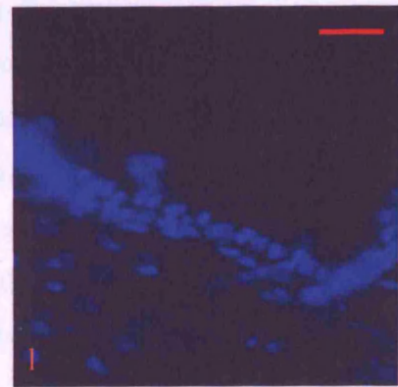
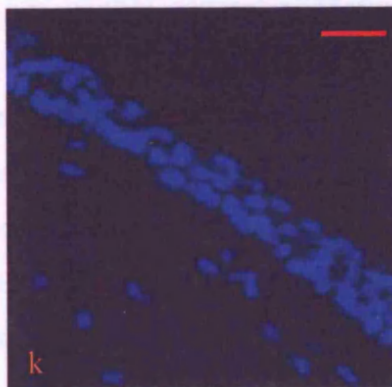
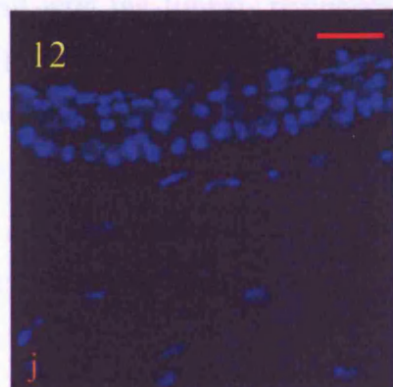
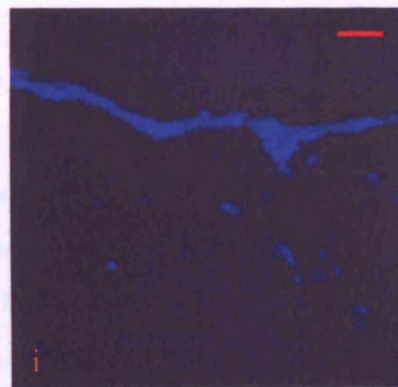
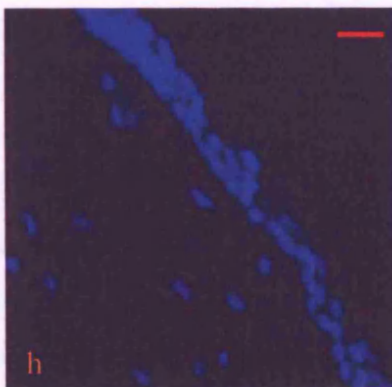
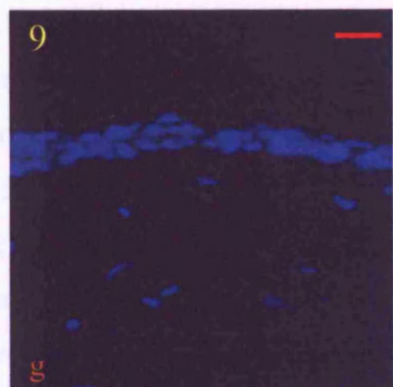
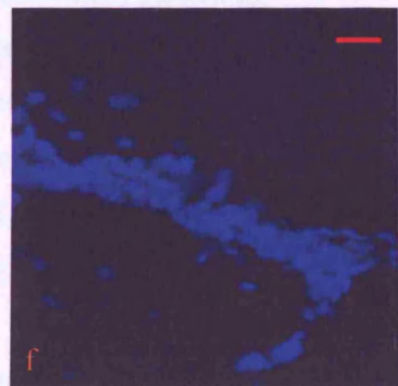
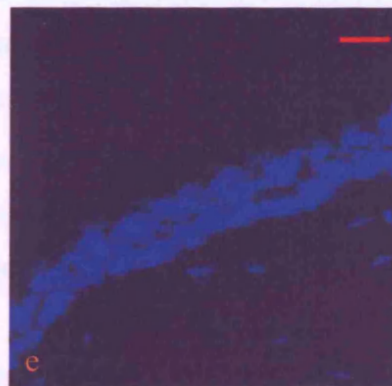
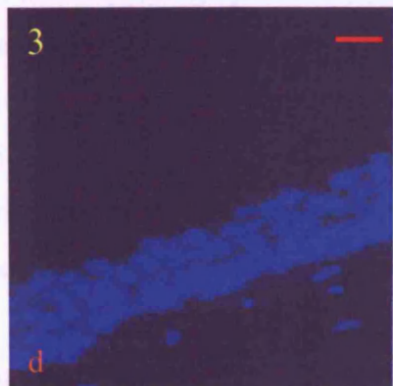
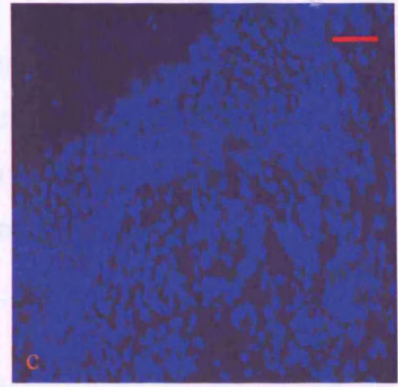
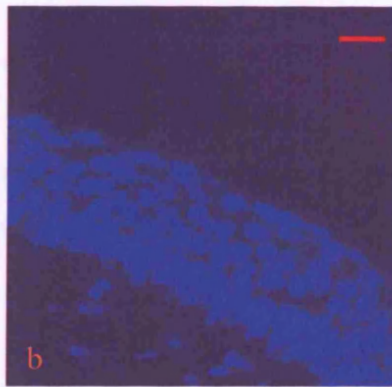
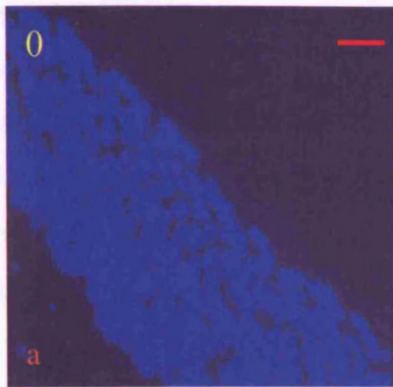
Limbal



Central

Peripheral

Limbal



4.4 Discussion

In this study in order to show if the barrier properties of corneal epithelium were preserved during organ storage time, alteration of cytoskeletal components of epithelium (cytokeratins), cell to cell communication (gap junctions: Cx43 and Cx50) and cell adherence to one another (tight junctions: ZO-1) were investigated in organ culture storage.

CK3 immunostaining by AE5 showed a homogeneous cytoplasmic distribution pattern in central and peripheral regions, in contrast to the more heterogeneous limited suprabasal staining in limbus. So the fact that epithelial basal cells of central cornea but not those of the limbus possess the 64K keratin, indicates that corneal basal cells are in a more differentiated state than limbal basal cells, and constant expression of more differentiated epithelial cells (CK3 positive) was demonstrated in culture by AE5 for up to 12 days.

Immunostaining with AE1/AE3 directed against a subset of acidic and basic cytokeratins revealed a homogeneous cytoplasmic staining pattern throughout the epithelium in organ culture. These keratins are expressed by cells in the suprabasal layers of epiderms, which are conventionally thought to be terminally differentiated (Sun et al., 1983; Woodcock et al., 1982). However Van et al. (1983) illustrated that all suprabasal cells, including some replicating cells (TACs) are AE3 positive in epidermis suggesting that the expression of this marker is position dependent and does not require the cells to be in the terminally differentiated compartment. In the cornea, it appeared that CK 6, 7, 8, 10 and 18 were not present with the limbal, peripheral and central corneal epithelium (Lauweryns et al., 1992). CK4 stained only the upper third of limbal, peripheral and central corneal cells (Lauweryns et al., 1992). CK13 was not expressed in the central cornea and it stained the superficial cell layer of the limbal and peripheral region (Krenzer et al., 1996; Lauweryns et al., 1992). CK19 exhibited a mosaic pattern in the peripheral cornea with a strong homogeneous reaction pattern on the small clusters of cells and on the limbal basal cell layer (Lauweryns et al., 1992). Sun and his collaborators (1983, 1984) observed that the basal cells covering the outer vascularised limbal rim expressed the CK5/CK14

cytokeratin pair seen in the basal cells of most stratified epithelia. Therefore the immunostaining detected by AE1/AE3 in this study demonstrated expression of cytokeratins in the central (CKs 3, 4 and 5), peripheral (CKs 3, 4, 5, 13 and 19) and limbal (CKs 3, 4, 13 and 5/14) regions of corneal epithelium.

The cytokeratin expression demonstrated by AE1/AE3 reveals cellular differentiation through all layers of epithelium in the peripheral, central and limbal regions and thus the cytoskeletal properties of the corneal epithelium is more likely to remain stable during organ storage.

Our second goal was to identify cell–cell interactions during corneal storage. Four types of intercellular junctions have been identified in the corneal epithelium: gap junctions, desmosomes, adherens junctions, and tight junctions (McLaughlin et al., 1985; Messent et al., 2000; Matic et al., 1997a, b; Wolosin et al., 2000).

Gap junctions mediate intercellular signalling and thereby allow functional synchronisation among neighbouring cells. They are composed of the proteins called connexinS (Kumar et al., 1996). Connexin 43 (Cx43) is a gap junction protein that is expressed in the basal layer of the cornea but not in the limbus suggesting that Cx43 expression is acquired during differentiation of transient amplifying cells (Matic et al., 1997a; Dong et al., 1994; Wolosin et al., 2000).

In this study in order to investigate the status of epithelial cell communication during organ storage, immunolocalisation of Cx43 and 50 for different times of culture were investigated on porcine corneal sections for different times of culture. Cx43 staining was very faint (nearly absent) at day 0 probably due to degradation of connexin caused by epithelial cell shut-down perhaps after postmortem trauma. After 3 days of organ culture the Cx43 and Cx50 staining was more diffuse in the basal and suprabasal layer of peripheral and central regions that suggests the presence of more differentiated cells in these regions. The diffused pattern of staining observed by staining with both Cx43 and

Cx50 reveals diffusible signals that are more likely to be due to release of a large variety of cytokines and growth factors at this stage. Karukonda et al. (2000) has shown that in some phases of wound healing various cytokines are produced, for example PDGF, TNF α , TGF β , and EGF which can also influence gap junctional intercellular communication (GJIC) in various cells and tissues (Rivedahl et al., 1996). From our results it was clear that at certain times of the storage the expression of Cx43 and 50 were down regulated. The staining of Cx43 and Cx50 seemed to be absent in both peripheral and central region of cornea after 6 days of organ culture. This weakness of intercellular communications may be a causal factor for cellular activation (Hendy et al., 1993). Hendy et al. (1993) has also showed that the elimination of cell to cell communications maybe be advantageous for cells undergoing rapid migration.

Matic et al., 1997b and Saitoh et al. (1997) revealed down regulation of Cx43 expression in epithelial cells of wounded tongue and cornea similar to down regulation of Cx43 and 50 in this study. Lack of their expression was observed at some time points of storage. This leads us to question the existence of functional epithelial cell re-epithelialisation process during storage also an important factor in corneal epithelial cell homeostasis.

Compatible with previous studies, neither connexin showed staining in the limbal region at any time point of organ culture. Matic et al. (1997a) speculated that the absence of Cx43 containing gap junctions and gap junction intercellular communication is one of the local intrinsic factors that promote a stem cell niche. The presence of gap junctions (Cx43) would make a cell vulnerable to insults affecting its neighbours. Absence of gap junctions would therefore confer a survival advantage to stem cells.

A loss of epithelial cell layers occurs during organ culture storage (refer to section 3.3.1.). This may have implications for the effectiveness of the epithelial barrier because of disruption of ZO-1. In the cornea, ZO-1 is found in the superficial layer of epithelium where tight junctions (TJs) are located. ZO-1 is also present between wing cells and basal epithelial cells that have no tight junction (TJ) structure (Surge 1997; Wang 1993). Loss

of ZO-1 and or ZO-2 would result in a disorganisation of TJs and loosening of TJs could increase paracellular permeability and therefore change epithelial barrier efficiency (Yi 2000). The discontinuity in the distribution of ZO-1 in corneal endothelium causes endothelium to be a leaky barrier (Petroll et al., 1999; Barry et al., 1995).

In this study, the localisation of ZO-1 in corneal epithelium altered during culture. The tight junctions seemed to be intact up to 3 days in culture. At day 6 a specific loss of ZO-1 staining was observed that was partially recovered at day 12. Petrol et al. (2001) has shown a decrease in the intensity of ZO-1 reveals cell spreading over the wound. They revealed a decrease in the intensity of ZO-1 with pericellular ZO-1 staining during the S phase, prophase, metaphase, anaphase and early G1 (after mitosis), with a partial but not complete breakdown of ZO-1 during telophase. Therefore this decrease in ZO-1 distribution reveals not only a loose epithelial barrier but also a high proliferating status of epithelial cells (see chapter 5).

Discontinuous distribution of ZO-1 in corneal storage reveals the existence of a leaky epithelial barrier at some time points of storage. This is inconsistent with Armitage et al. (2001) who demonstrated no fundamental change in distribution of ZO-1 in epithelial cells of cornea stored for up to 28 days.

In addition to the epithelium and the tear film, which are most important to the barrier function of the cornea, the avascular stroma provides additional barrier protection (Chakravarti et al., 2004). Positive labelling of keratocytes for ZO-1 was also observed during organ culture in punctate or elongate patterns. First disruption and then repair of these junctions was noticed during storage. As mentioned by Muller et al. (1995) and Watsky et al. (1995) in the stroma of the normal cornea, keratocytes communicate with one another by gap junctions and wounding disrupts these junctions. During repair, corneal fibroblasts and myofibroblasts re-establish this communication (Jester et al., 1994 and Wilson et al., 1998). Taliana et al. (2005) has also found that ZO-1 was a candidate for organising components of gap and adherens junctions of corneal fibroblasts during

repair. The appearance of ZO-1 at the leading edge of migrating fibroblasts makes it a candidate for a role in the initiation and organisation of integrin-dependent fibroblast adhesion complexes formed during migration and adhesion.

4.5 Conclusions

The studies reported in this chapter revealed that the cytoskeletal properties of epithelial cornea remained stable during storage. However, the integrity of the epithelium in terms of epithelial cell attachments (tight junctions and gap junctions) was compromised at some time points of culture. The absence of ZO-1, Cx 43 and 50 could contribute to an increase in paracellular permeability of epithelium and therefore change epithelial barrier efficiency. Thus, not only is there a decrease in epithelial thickness during organ culture but the integrity of epithelial cells remaining is also compromised.

CHAPTER FIVE

5. Chapter 5 - Determination of Proliferative Activity in Epithelial Cells of Porcine Stored Corneas

5.1 Introduction

The purpose of this study was to determine the effect of organ culture storage on porcine corneal epithelial cell proliferation. The corneal epithelium forms an integral part of the ocular surface and is necessary for maintaining a clear and proper corneal function. When disrupted, it is therefore important that it is rapidly regenerated to maintain the corneal transparency.

Cell proliferation is a fundamental biological activity of the corneal epithelium that plays an important role in both physiological and pathological processes (Jester et al., 1999b; Kruse 1996). A large number of studies on human cornea have shown that corneal epithelium undergoes continuous cell renewal and regeneration throughout adult life (Matsuda et al., 1996; Gan et al., 1998). Cells desquamated from the corneal surface are replaced by mitotic division of stem cells (Zieske et al., 1992). These small cells are located in the limbus of the cornea, the peripheral region between the cornea and the adjacent bulbar conjunctiva.

Under normal conditions these stem cells undergo unlimited self replication and have relatively slow turnover. They give rise to transient amplifying cells (TACs) derived from each stem cell mitosis. The TACs cells multiply by undergoing additional rounds of cell division, but are already committed to the cell differentiation pathway (Fig 5.1., Tseng et al., 1989; Kruse et al., 1994; Schermer et al., 1986).

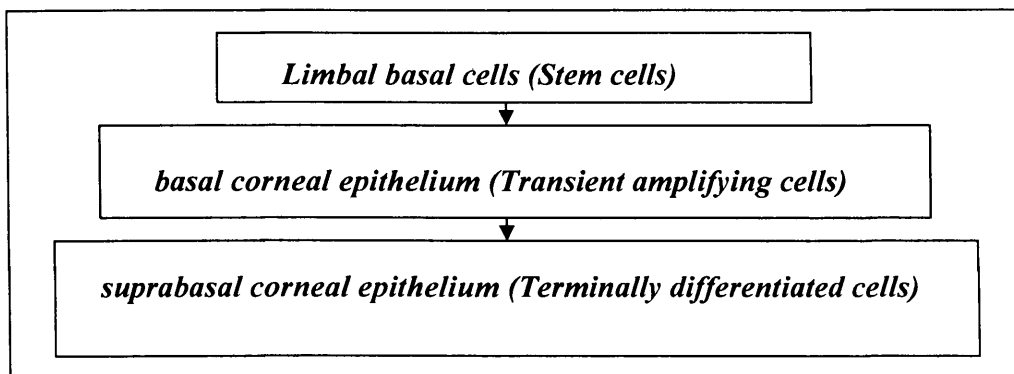


Fig 5.1 Diagram proposed by Schermer et al. (1986) demonstrating epithelial cell proliferation of the cornea.

The TACs have got a relatively long cell cycle time of about 72 hours. Early TACs in the peripheral cornea normally undergo at least 2 rounds of DNA synthesis, but as cells migrate toward central cornea (mature TA cells), their replicative capacity decreases. However they can be induced to replicate more, in response to wounding (Lehrer et al., 1998).

Proliferation plays an important role in epithelial cell homeostasis as excessive proliferation of corneal cells can lead to hyperplasia in the epithelium, excessive scar formation in the stroma or retro-corneal membrane formation on the endothelium (Gan 2001). On the other hand, the lack of proliferative ability of epithelial cells, which as in corneal stem cell deficiency, can cause corneal degeneration and abnormal wound healing, leading to replacement of the corneal epithelium by conjunctival tissue. This process is known as conjunctivalisation. While protecting the stroma, the problem is that conjunctival cells are not able to transdifferentiate into corneal epithelial cells and they express different proteins (Kruse et al., 1990; Dua 1998; Tseng et al., 1984b).

The mechanisms behind the regulation of proliferative activity are not fully understood. The evaluation of the proliferative activity of corneal cells is important in order to understand the cellular and biological aspects of corneal homeostasis.

In the previous chapter disruption, of the epithelial structure was shown to occur following the submersion of the cornea in the artificial environment of organ culture

storage medium. A huge decrease in epithelial thickness and keratocyte density was observed together with a significant increase in stromal thickness.

Disruption of this epithelial structure following storage requires a rapid and accurate response of the corneal stem cells to restore the normal multilayered structure of the epithelium. The re-epithelialisation involves the cellular processes of proliferation, differentiation and migration.

The aim of this chapter is to determine the proliferative rate of epithelial cells in central, peripheral and limbal regions of cornea stored at different times in organ culture.

5.2 Experimental design for proliferation assay:

In order to evaluate the possible effect of time on epithelial cell proliferation during organ culture, different markers were tested on porcine corneal sections to determine which one was most suitable.

a) The Ki67 marker, is used to identify a large (395 KDa) nuclear protein that is synthesised during the G1 phase of the cell cycle and is degraded at the completion of the M phase (Gerdes et al. 1984, 1991).

b) Proliferating cell nuclear antigen (PCNA) marker recognises an auxiliary protein of DNA polymerase, associated with the DNA replication during S phase (Krude 1999), the expression of this protein in the cornea has been used as an indicator for the activation of the corneal cells (Gan et al., 1999).

After finding that these two markers did not cross react with porcine corneal sections, the BrdU assay was performed on corneal wax sections. This marker could detect BrdU incorporation into the nuclear DNA and therefore could be used to discover a possible effect of time on epithelial proliferation during organ culture.

5.2.1. Organ culture storage

6 porcine corneas were stored following the procedure described in section 2.2.1.1, in triplicate for 0, 1, 3, 6, 9, 12 days in organ culture (n=18). Three, 10µm wax sections from each cornea were cut (section 2.2.4), floated on to the slides and left overnight at 56 °C.

5.2.2 Finding a suitable marker to measure corneal epithelial cell proliferation

Immunolabelling for Ki67 and PCNA was first performed on corneal sections (in duplicate) as described previously in sections 2.2.7.1 and 2.2.7.2, but as none of these markers was found to cross-react with porcine corneal sections, an alternative BrdU incorporation labelling assay was evaluated and modified.

5.2.3 Determination of proliferative activity of corneal epithelial cells

5.2.3.1 BrdU labelling of corneal wax sections

In order to determine the proliferative activity of corneal epithelial cells of different storage time of the corneas, immunolabelling of BrdU (Amersham kit) was coupled with methyl green on all corneal wax sections. Corneal wax sections (refer to section 5.2.1) were immunostained for BrdU (Amersham antibodies). Nuclear labelling was visualised by the two optimised methods (using peroxidase DAB for development of immunolabelling and Methyl green for all nuclei stain) described in section 2.2.7.3.

5.2.3.2 Images analysis of BrdU stained cells

To measure the number of BrdU stained cells in central, peripheral and limbal regions of porcine epithelial cornea, each BrdU stained corneal section (refer to section 5.2.3) was then imaged using a Leica image processing microscope (40 x objective). 4 pictures were taken from each periphery, 2 pictures from each limbal region and 4 pictures from each centre. The pictures for each corneal section were arranged and grouped using Corel Draw to form the complete images of the corneal section in the peripheral (500µm width), central (500µm width), and limbal (115µm width) region. 764 images were taken in total to quantify the epithelial cells proliferation rate in different regions of porcine cornea.

5.2.4 Quantification of labelling index

The labelling index of cycling cells as a percentage of total cells in each region was calculated according to the formula: $\frac{\text{Number of BrdU labelled cells}}{\text{Total numbers of cells}} \times 100$

Total numbers of cells

5.2.5 Statistical analysis

Statistical analysis was performed using Graph Pad Prism software. Prism normality test was also performed for deviations of data from Gaussian distribution using Kolmogorov-Smirnov (KS) test and $p > 0.1$ was considered as significant. One way ANOVA and unpaired t test was performed for parametric data, Kruskal-Wallis test and Mann-Whitney test for non parametric data. The statistical analysis of regression was also performed to analyse the relationship between proliferative activity of corneal epithelium and organ culture storage time. $P < 0.05$ was considered to be statistically significant in all analyses.

5.3 Results:

5.3.1 Proliferative potential of Ki-67 and PCNA in porcine corneal sections

To understand the proliferative activity of the porcine cornea during organ culture storage, Ki67 staining was first tested on human and porcine cornea. For porcine cornea only non-specific staining was detected (refer to methods chapter, section 2.2.7.1 and fig 2.3). For PCNA similar results were obtained. It is likely that the antibodies used to identify these two markers were specific for human tissue (refer to methods chapter, section 2.2.7.2, fig 2.2) and did not cross react with porcine cornea. Thus an alternative marker was needed to identify the proliferative activity of the porcine cornea.

5.3.2 Determination of the proliferative activity of epithelial cells of organ culture porcine stored corneas using the BrdU labelling assay

The immunocytochemical assay for the detection of BrdU incorporation into the cellular DNA was performed and modified to evaluate a possible effect of time on epithelial proliferation during organ culture. Limbal epithelium was distinguished from corneal epithelium by its subjacent blood vessels and loose stroma. Images were captured using a microscopic visual field, for each central, peripheral and limbal region. Proliferation was also detected in conjunctiva although no quantification was performed in this region. As shown in figs 5.2 and 5.3, an increase in the relative percentage of proliferative cells with respect to the total number of cells, was shown to occur with increasing time of culture from 1-12 days. Linear regression analysis showed an apparent increase in the peripheral ($r=0.79$, $n=18$) central ($r=0.62$, $n=18$) and limbal ($r=0.67$, $n=18$) regions ($p<0.05$, fig 5.4) of cornea with increasing time of culture.

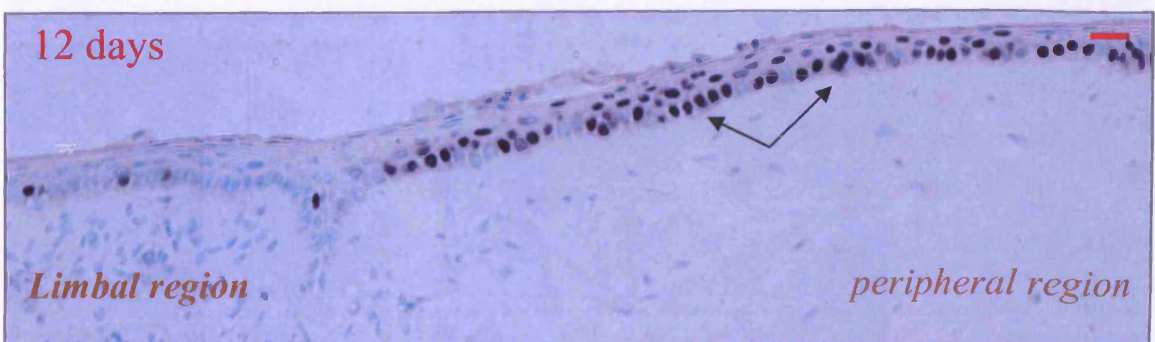
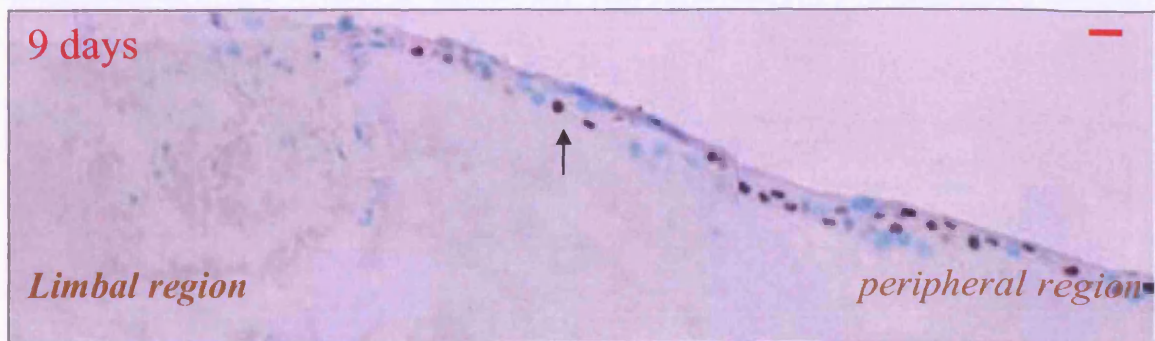
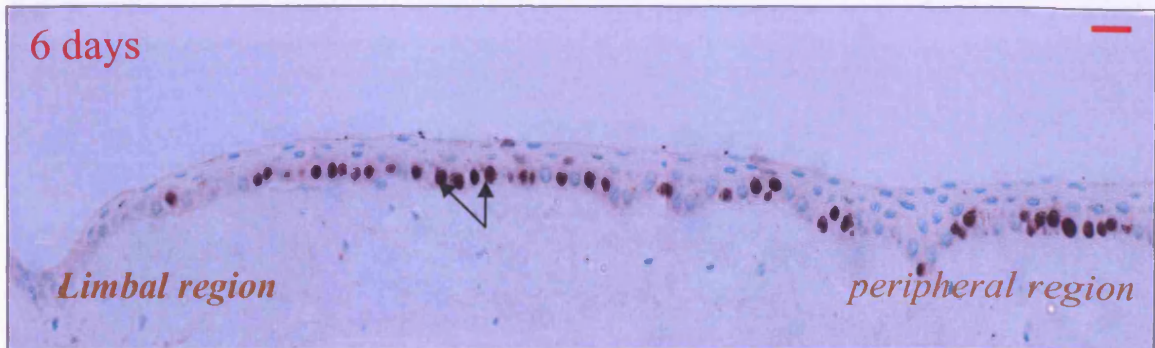
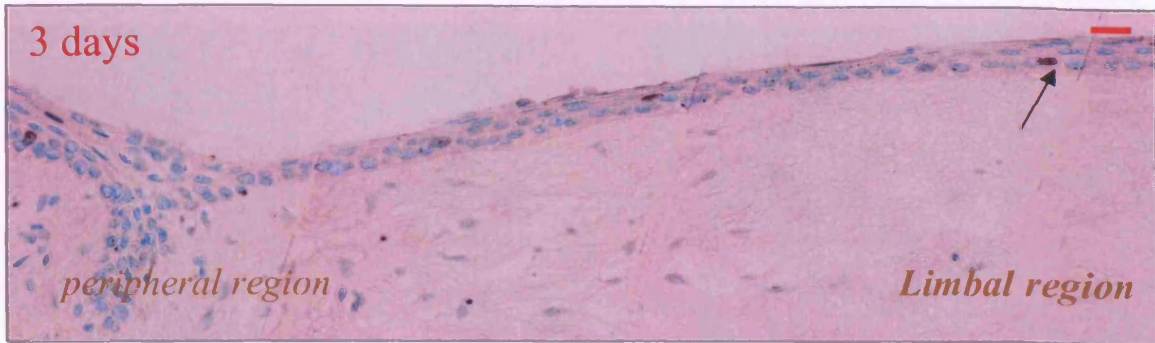


Fig 5.2 BrdU immunostaining (arrows) of wax sections of porcine corneas (limbal+periphery) following 1, 3, 6, 9 and 12 days of organ culture. An increase in the relative percentage of proliferative cells with respect to total, demonstrated with increasing time of culture from 1-12 days. Bar represents 20 μ m.

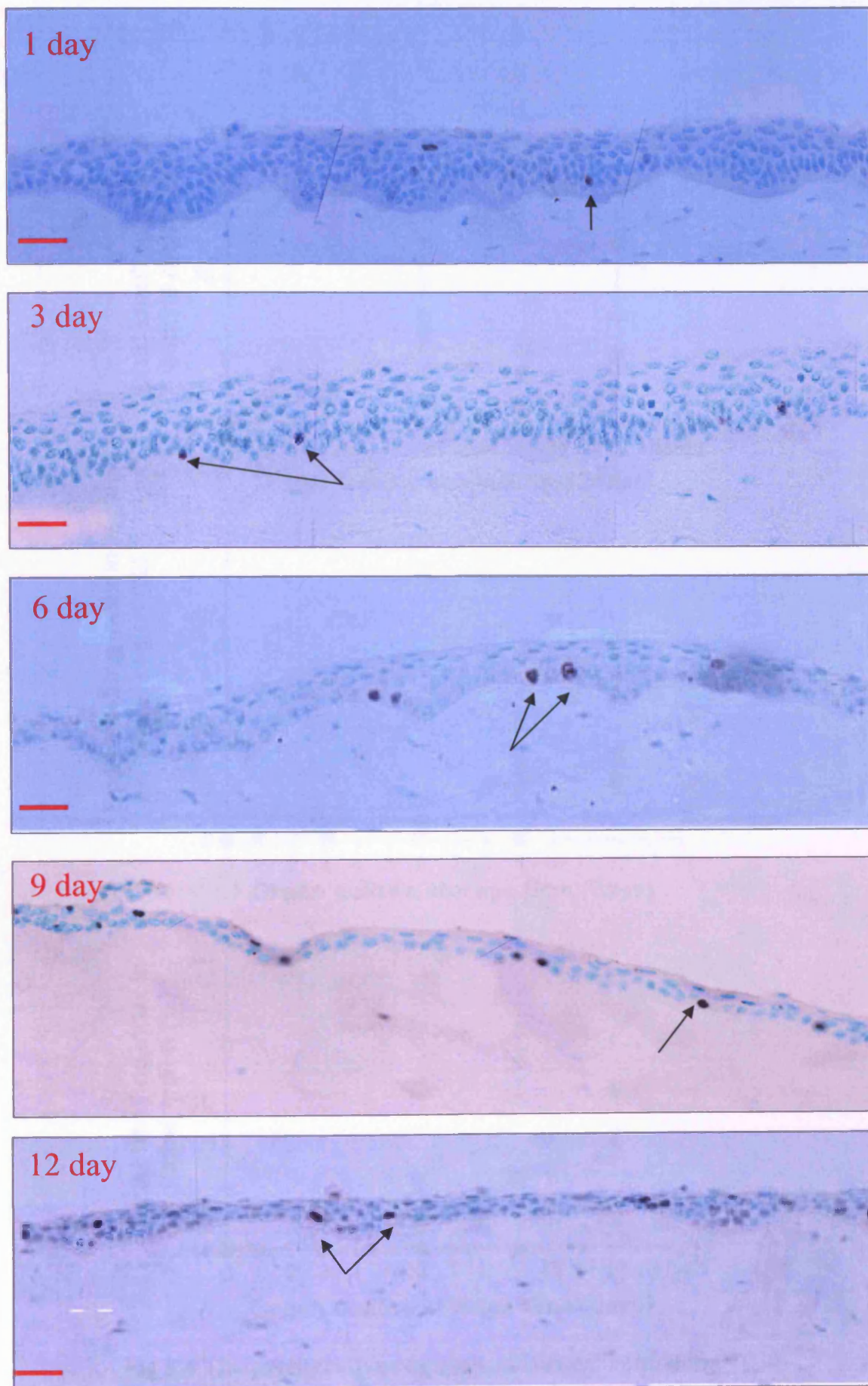


Fig 5.3 BrdU immunostaining (arrows) of wax sections of central region of porcine cornea following 1, 3, 6, 9 and 12 days of organ culture. An increase in the relative percentage of proliferative cells with respect to total, demonstrated with increasing time of culture from 1-12 days. Bar represents 20 μ m.

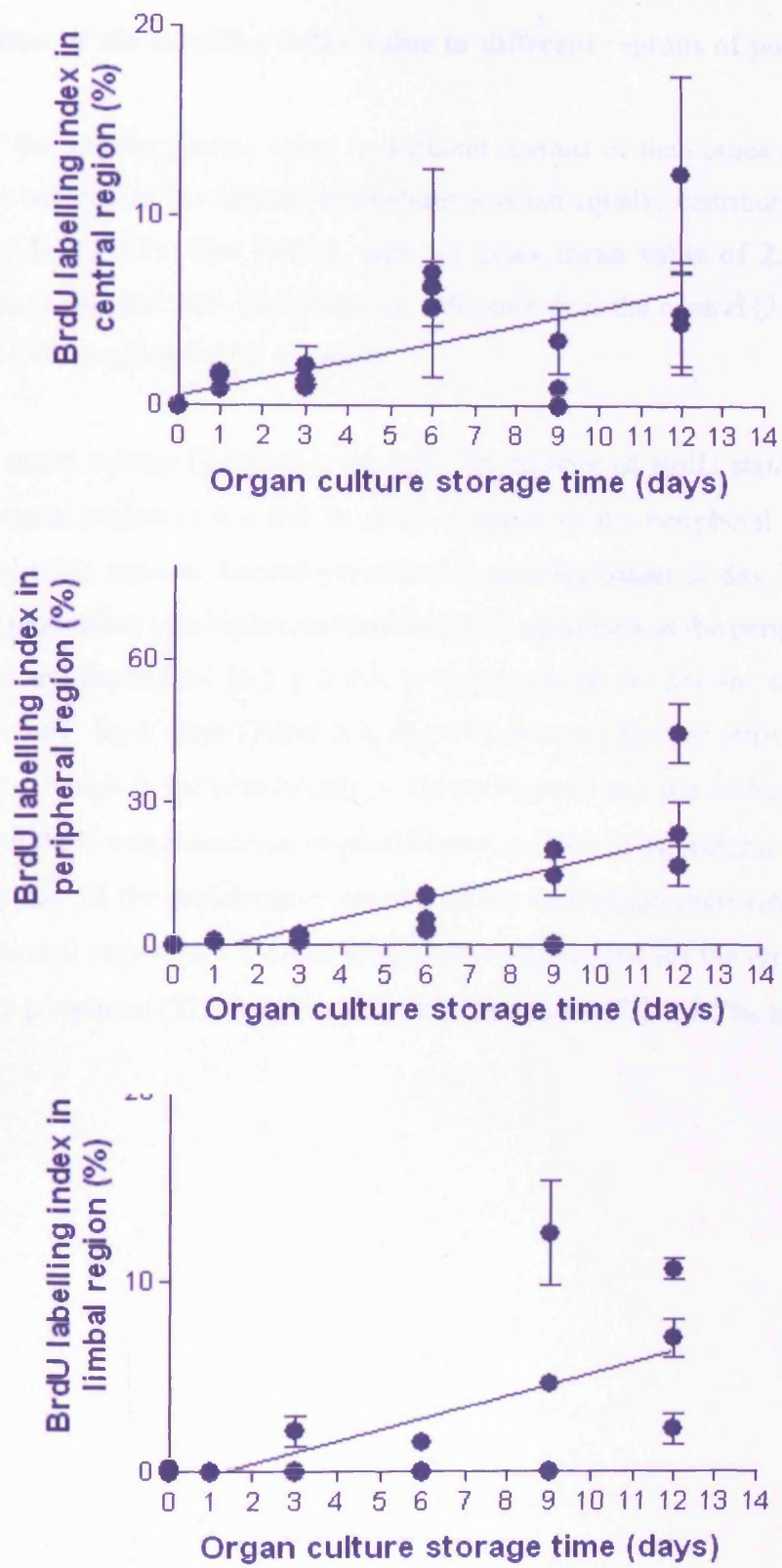


Fig 5.4 The proliferative response of corneal epithelium in peripheral, central, limbal region as a function of organ culture storage ($p < 0.05$). 3 corneas were examined for each time point of culture.

5.3.3 Comparison of the labelling index value in different regions of porcine stored cornea

Comparison of the labelling index value in different regions of the cornea revealed that the proliferation activity of the corneal epithelium was not equally distributed across the cornea (Table 5.1, fig 5.5). The limbus, with an index mean value of 2.3 ± 1.2 was significantly less populated with BrdU-labeled cell pairs than the central (3.0 ± 1.2) and peripheral (8.0 ± 4.3) regions (n=18 corneas).

After 1 day of organ culture (Table 5.1, fig 5.5), the number of BrdU stained cells was higher in the central region (1.4 ± 0.3 %, n=3) compare to the peripheral (0.9 ± 0.1 %, n=3) and limbal (0%) regions. Limbal proliferative activity began at day 3 (Table 5.1) resulting in the production of a higher number of TACs especially at the peripheral (6.345 ± 2.126 %, n=3) and the central (6.2 ± 0.5 %, n=3) regions of the porcine cornea after 6 day of organ culture. By 9 days (Table 5.1, fig 5.4), the proliferative activity of limbal epithelium was still high in the peripheral (11.5 ± 6.0 %, n=3) and the limbal regions (5.7 ± 3.7 %, n=3) but there was a decrease in proliferative activity in the central region (1.5 ± 1.0 %, n=3). By day 12 the proliferative activity of the epithelium increased (Table 5.1, fig 5.5), in the limbal region (6.7 ± 2.4 %, n=3) that is responsible for the greatest amount of activity in the peripheral (27.7 ± 8.6 %, n=3) and the central (7.2 ± 2.5 %, n=3) regions.

<i>BrdU LABELLING INDEX</i>	<i>Peripheral Mean ± SEM</i>	<i>Central Mean ± SEM</i>	<i>Limbus Mean ± SEM</i>
<i>0 DAY ORGAN CULTURE</i>	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
<i>1 DAY ORGAN CULTURE</i>	0.9 ± 0.1	1.4 ± 0.3	0.0 ± 0.0
<i>3 DAYS ORGAN CULTURE</i>	1.6 ± 0.5	1.5 ± 0.4	0.7 ± 0.7
<i>6 DAYS ORGAN CULTURE</i>	6.3 ± 2.1	6.2 ± 0.5	0.5 ± 0.5
<i>9 DAYS ORGAN CULTURE</i>	11.5 ± 6.0	1.5 ± 1.0	5.7 ± 3.7
<i>12 DAYS ORGAN CULTURE</i>	27.7 ± 8.6	7.2 ± 2.5	6.7 ± 2.4

Table 5.1 Mean percentage of BrdU labelling index (number of BrdU labelled cells / total numbers of cells × 100) in the peripheral, central and limbal regions of porcine stored cornea (Mean ± SEM).

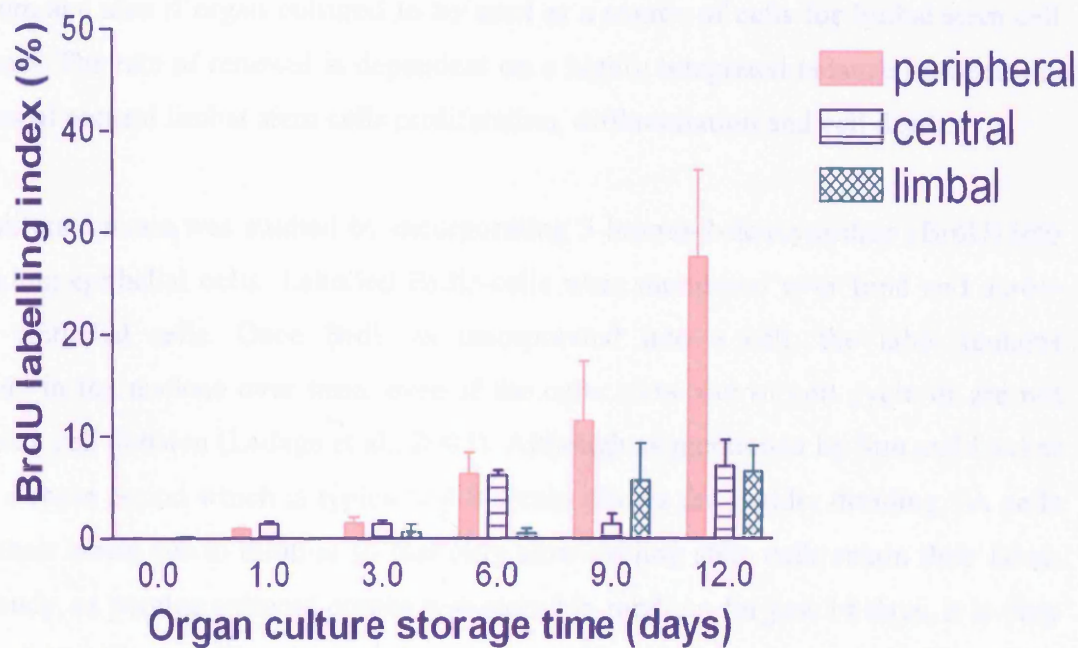


Fig 5.5 BrdU labelling index (number of BrdU positive cells/total numbers of cells ×100) in the central, peripheral and limbal regions of porcine corneas for 0-12 days storage in organ culture. The BrdU labelling index increased with storage time. Each Bar represents the mean ± SEM of 3 corneas.

5.4 Discussion

One of the important functions of the cornea is to maintain normal vision by refracting light onto the lens and retina. This property is dependent in part on the ability of the corneal epithelium to undergo continuous renewal. Epithelial renewal is essential during organ culture as it enables this tissue to act as a barrier that protects the corneal epithelium and also if organ cultured to be used as a source of cells for limbal stem cell transplants. The rate of renewal is dependent on a highly integrated balance between the processes of corneal limbal stem cells proliferation, differentiation and cell death.

The proliferation rate was studied by incorporating 5-bromo-2-deoxyuridine (BrdU) into the dividing epithelial cells. Labelled BrdU-cells were monitored over time and across corneal epithelial cells. Once BrdU is incorporated into a cell, the label remains detectable in the nucleus over time, even if the cells come out of cell cycle or are not undergoing cell division (Ladage et al., 2003). Although as mentioned by Sun and Lavker (2004), a chase period which is typically 4-8 weeks allows the rapidly dividing TA cells to lose their labels due to dilution so that only slow-cycling stem cells retain their label. In our study, as porcine cultured cornea was stored in medium for just 14 days, it is very unlikely that cells are diluted to such an extent. Therefore the cells that have been labelled in this study are both stem cells (only in limbal region) and TACs.

No proliferative activity was identified in porcine control corneas. Treatment with N-acetyl cysteine revealed only some proliferation in the limbal region of one of them. This lack of BrdU staining could be due to the shut down of epithelial cell proliferation (perhaps because of postmortem trauma and loss of cell survival factors) or inability of BrdU to incorporate into epithelial cells because of stratified epithelium.

The overall proliferative rate in the limbus was lower (~2%) than peripheral and central region during organ culture storage. This was expected as the limbus contains slow cycling stem cells along with transient amplifying cells. This study showed an obvious increase in proliferative index of epithelium especially at the peripheral region. Therefore it shows peripheral epithelial TA cells have a rapid proliferation response. This could be

either because of an increase in times of their replication (although double labelling procedure need to be applied to prove this), or due to the shortening of their cell cycle time. Limbal region demonstrated proliferative activity after 3 days suggesting that the limbus is sensitive to changes occurring in the epithelium and that it is capable of rapidly up regulating cell proliferation after epithelial cell disruption. The result of this activity was the production of lots of TACs especially at peripheral (6%) and subsequently central regions (6%) of porcine cornea at day 6 of organ culture. Therefore this data is comparable with finding of Gan et al. (1998) that revealed 50 % positive PCNA staining for human corneal epithelium after 3 days of organ culture storage. However they could not with certainty prove that all cells that express PCNA actually went into mitosis, as the cells also expressed PCNA during the late G1 phase (Linden et al., 1992).

An increase in proliferative activity has been identified in central region, but still a higher amount of BrdU-labeled cells was identified in the periphery of the porcine cornea (~8%) compared to central and limbal region. This reflects a higher overall proliferation rate in the peripheral epithelium than central. Lehrer et al. (1998) has also found that corneal TA cells in the central region can usually divide only once prior to becoming post mitotic even after TPA (topical application of 0.5% phorbol myristate to produce a significant increase in proliferation) stimulation. However in this study, another explanation may be the high level of epithelial cell death in the central region, that occurs after proliferation (especially at day 9), because of apoptosis or even early post mitotic differentiation. The confusion of epithelial cell apoptosis is fully investigated in the following chapter. By day 12, the proliferative activity of the epithelium seemed to increase, in the limbal region (~7%) that was responsible for the greatest amount of activity in peripheral (~28%) and central region (~7%). It was more likely that the layers of epithelium had been restored at this time point of culture.

Some proliferation was also detected in conjunctiva during organ culture storage, this supporting the existence of a healing response in the conjunctiva. Corneal epithelial wounds are known to stimulate a proliferative response in the perilimbal conjunctiva (Haaskjold et al., 1992) but under normal circumstances the limbal epithelial acts as a

barrier and is able to exert an inhibitory growth pressure preventing migration of conjunctival epithelial cells on the cornea (Tseng 1989). However, when the epithelial defect involves the limbus, this barrier is lifted and conjunctival migration on to the cornea occurs with the result that regenerated epithelium exhibit both corneal and conjunctival features without one changing the other.

The interaction between corneal epithelium and stroma plays an important role in proliferation. As shown by Herrmann and Lebeau (1962) removal of the corneal epithelium results in disturbance of the stromal cells. Further investigation has also shown loss of keratocytes as a result of apoptosis after removal of epithelium (Wilson et al., 1996b; Hanna et al., 1989). In response to different traumas, the usually quiescent keratocyte change from resting state, to proliferate and become an activated keratocyte to effectively repair the apoptotic area in the anterior stroma. In organ culture, the entire cornea is bathed in medium and, this artificial medium could cause different trauma and stress to cultured cornea. However in this observation no detectable staining for keratocyte proliferation was observed to support this idea. This observation is not therefore in agreement with Moller-Pedersen et al. (1996) who reported mitoses in keratocytes in the anterior half of the stroma (0.23% mitoses per 48 h) during the first 14 days of organ culture. Therefore this lack of proliferative activity in our porcine cornea could be either real and show lack of proliferative activity in keratocytes during organ culture or failure of the procedure to detect BrdU stained cells in the stroma. If this is the case, then further optimisation of the BrdU procedure could help to learn more about the effect of organ culture on keratocyte proliferation and consequently epithelial-stromal interactions.

5.5 Conclusions

The longevity and quality of a limbal stem cell transplant could not be achieved without a large number of highly proliferative cells. So far all these results show that proliferative activity does still exist, but is only partly successful (at 6 and 12 days of organ culture) in maintaining the stratified epithelium during organ culture storage. This may be explained

by immediate epithelial cell death after proliferation that will be investigated in full in the next chapter.

CHAPTER SIX

6. Epithelial Cell Death in Porcine Stored Cornea

6.1 Introduction

In this chapter, cell death by apoptosis as a possible mechanism responsible for the huge amount of epithelial cell loss occurring in organ culture was investigated.

Apoptosis is one of the most fundamental biological processes in mammals, in which individual cells die by activating an intrinsic suicide mechanism. It has become evident that a family of proteins known as caspases, comprising 14 members related to interleukin-1 β converting enzyme are typically activated in the early stages of apoptosis (Ahmad et al., 1998; Alnmeri et al., 1996).

The previous chapter (section 5.3.2) showed that epithelial cell proliferation increased with increasing time of organ culture. However the increased epithelial proliferation does not appear to be effective in completely restoring the multilayered structure of the corneal epithelium since a decrease in epithelial cell layer and thickness was demonstrated in section 3.3.1.

On the other hand differentiation of post mitotic corneal epithelial cells has also increased during organ culture (refer to chapter 5). The post mitotic differentiation could also be associated with apoptosis, or programmed cell death (Glaso et al., 1993).

Epithelial cell apoptosis has been shown to reflect physiologic epithelial turnover. A strict equilibrium can be observed; epithelial cells that die are thought to be replaced by the proliferation of activated epithelial cells (Bourcier et al., 2000). The number of viable epithelial, endothelial and keratocytes corneal cells have been shown to decrease at both 4°C (Van Horn et al., 1975; Stein et al., 1986; Means et al., 1995 and 1996; Komuro et al., 1999) and 34°C (Pels et al., 1983; 1985; Barisani et al., 1997; and Redbrake et al., 1998). Van Horn et al. (1975), Stein et al. (1986), Means et al. (1995) all demonstrated a loss of endothelial cells at 4°C with extending time of preservation. Means et al. (1996) also revealed a significant amount of epithelial cell loss for more than 10 days of storage

at 4°C. Pels et al. (1983, 1985), Barisani et al. (1997), and Redbrake et al., showed a limitation in the long term organ culture, because of endothelial cell loss. Komuro et al. (1999) concluded that both apoptosis and necrosis occur in keratocyte cells during corneal storage at 4°C with apoptosis appearing to predominate. Apoptosis in the corneal epithelium has been detected after mechanical injuries (e.g., corneal trauma, PRK), infection, and UV exposure (Gao et al., 1997, Ren et al., 1996). The gradual death of epithelial corneal cells during preservation may occur by two pathways: necrosis and apoptosis with apoptosis appearing to be more dominant (Komuro et al., 1999). It is possible to inhibit the apoptotic pathway by addition of molecules that inhibit apoptosis, such as interleukin 6 (Wyllie 1980, Linchtenstein et al., 1995, Kuo et al., 1998; Araki et al., 1998). Therefore an understanding of the death process in corneal cells and inhibiting that process during preservation could help us to prolong corneal storage time.

Apoptotic cells can be detected by various techniques. In this investigation, we examined each corneal section by TUNEL. This technique has been known to be subjected to misinterpretation because necrotic cells also generate fragmented DNA strands (Negoescu et al., 1996). Immunolocalisation of caspase-3, 8, and 9 was also performed to confirm apoptosis. Determination of caspase activation will identify by which mechanism the execution (extrinsic or intrinsic pathways) of apoptosis (if present) happens during corneal organ culture.

6.2 Experimental design for apoptosis

To investigate changes in epithelial cell death, apoptosis was investigated using two different techniques: 1) The TUNEL technique that labels DNA fragments, of 180 bp nucleosomal unit produced by apoptotic process and 2) Immunodetection of caspase-3, 8 and 9, all of which are characteristic enzymes of the apoptosis. Therefore 0, 3, 6, 9 and 12 days of porcine corneal organ cultured sections were examined, in duplicate and triplicate for caspase and TUNEL assay, respectively.

6.2.1 TUNEL Technique

Porcine corneas, that were previously organ cultured in triplicate (section 2.2.1.1) for 0, 3, 6, 9 and 12 days of culture, were processed for wax sectioning (section 2.2.4) and stained with Apoptag TUNEL kit (as described in section 2.2.8.1). The positive and negative control slides were also used to justify the system. Positive control slides consisted of sections of rat mammary glands obtained at the fourth day after weaning. Negative control was those slides in were TdT enzyme was omitted from the labeling reaction.

6.2.2 TUNEL index quantification

The apoptotic index was assessed in TUNEL stained sections (section 6.2.1) for each time point of culture and in different regions of the epithelium. Two random fields for each central, peripheral and limbal region of the epithelial cornea were examined under the light microscope using a 40X objective. The percentage of apoptotic cell numbers in each epithelial region was then calculated according to the formula:

$$\frac{\text{Number of TUNEL- positive cells}}{\text{Total number of cell nuclei}} \times 100$$

6.2.3 Statistical analysis of apoptotic index

Statistical analysis of the TUNEL assay was performed using GraphPad Prism software. The statistical analysis of regression was performed to analyse the relationship between the percentage of apoptotic epithelial cells and the organ culture storage time. One way ANOVA and unpaired t-test were applied to determine the significant differences in the number of apoptotic epithelial cells at different times of organ culture. $P < 0.05$ was considered to be statistically significant.

6.2.4 Caspase-3, 8 and 9 staining

Immunolabelling for activated caspase-3, 8 and 9 were performed on corneal sections (in duplicate) as described previously in sections 2.2.8.2-4.

6.3 Results:

6.3.1 Determination of cell death in corneal epithelial cells using TUNEL labelling

The immunohistochemical assay of TUNEL was performed and modified to evaluate epithelial cell death during organ culture storage. Positive stained cells were observed at all levels of the epithelium for porcine stored cornea. Some TUNEL positive stained cells were observed in cells that are releasing from the surface of the multilayer epithelium (Fig 6.1). Keratocyte apoptosis was also detected in different regions of the stroma, especially the anterior region. TUNEL labelling of porcine epithelial corneal sections stored for 0, 3, 6, 9 and 12 days revealed an increase in the percentage of apoptosis, with increasing time of organ culture of organ ($p < 0.05$, fig 6.3) in the central ($r = 0.79$, $n = 18$), peripheral ($r = 0.62$, $n = 18$) and limbal ($r = 0.67$, $n = 18$) regions.

6.3.2 Comparison of TUNEL labelling index in different regions of porcine stored corneas

The comparison of TUNEL labelling index across the peripheral, central and limbal regions of porcine cornea, for 0, 3, 6, 9 and 12 days of organ culture revealed an un equal distribution of labelling index for different times of culture (Table 6.1, fig 6.4). Low levels of epithelial cell death were observed in the central: 11.5 ± 5.7 , peripheral: 12.9 ± 4.6 , and limbal: 16.3 ± 9.0 regions at day 0. After 3 days of organ culture, the limbal region with a mean of 3.4 ± 1.2 % (Table 6.1), demonstrated significantly less epithelial cell apoptosis than the peripheral (22.3 ± 10.2 %) and the central epithelium (21.6 ± 11.5 %). This limbal resistance to cell death was shown to continue up to day 6 (15.5 in the limbal region compared to 27.2 ± 6.5 % and 32.7 ± 9.5 % in the peripheral and the central region respectively). A significantly high level of epithelial cell death was also observed at day 9 in all regions of corneal epithelium, central: 79.3 ± 19.5 %, peripheral: 86.3 ± 12.1 % and limbal region: 95.2 ± 2.9 %. These values decreased by day 12 (peripheral: 57.9 ± 13 , central: 54.0 ± 6.7 , and limbal 64.9 ± 14.1 regions respectively), although not as low as levels of apoptosis identified in days 0-6 of organ culture (Table 6.1).

CENTRE

PERIPHERY

LIMBUS

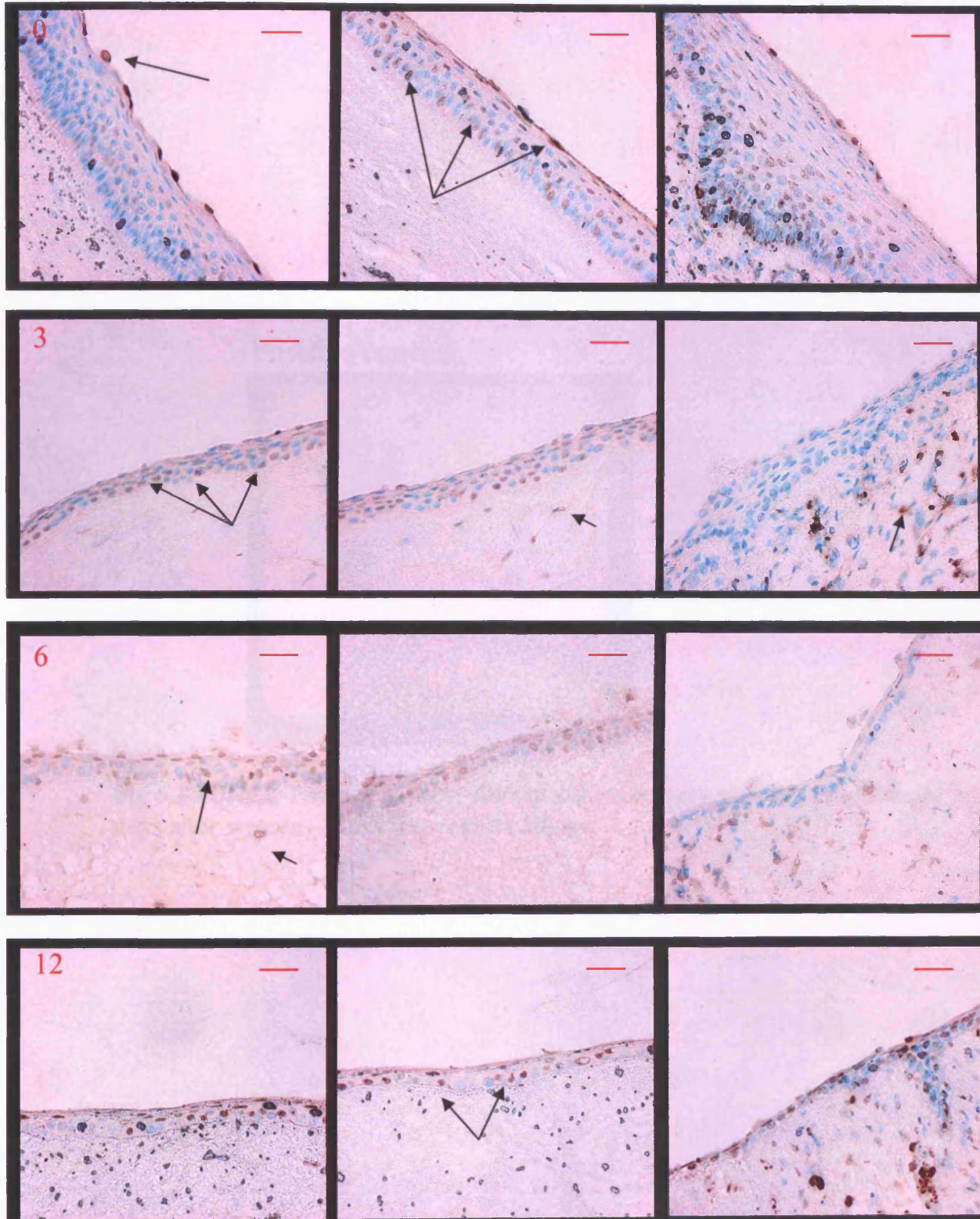


Fig 6.1 Epithelial cell and keratocyte apoptosis: TUNEL-positive cells (arrows) in wax sections of central, limbal and peripheral regions of porcine corneas following 0, 3, 6, and 12 days of organ culture storage. Bars represents 20 μ m.

Positive control

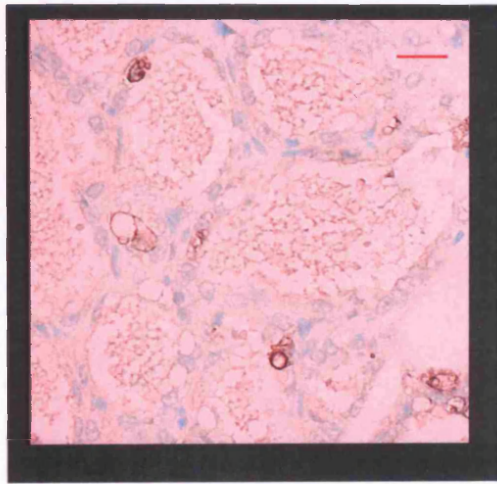


Fig 6.2 TUNEL-Positive control slide of rat mammary glands at the fourth days after weaning. Bars represents 20 μ m.

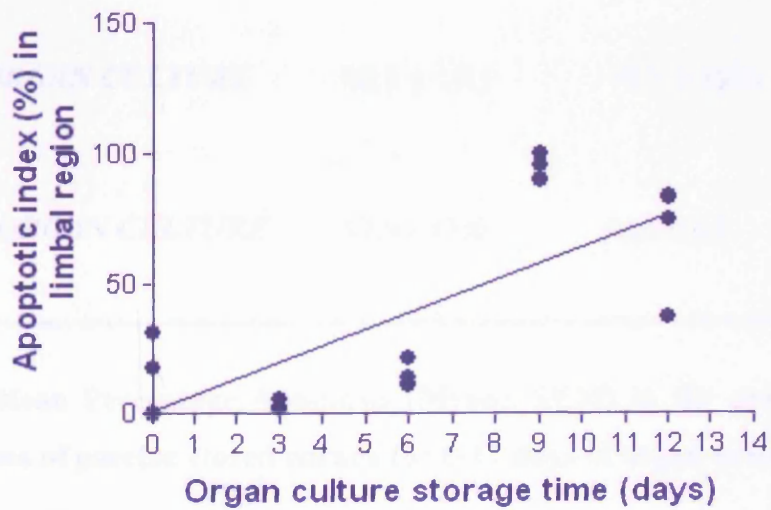
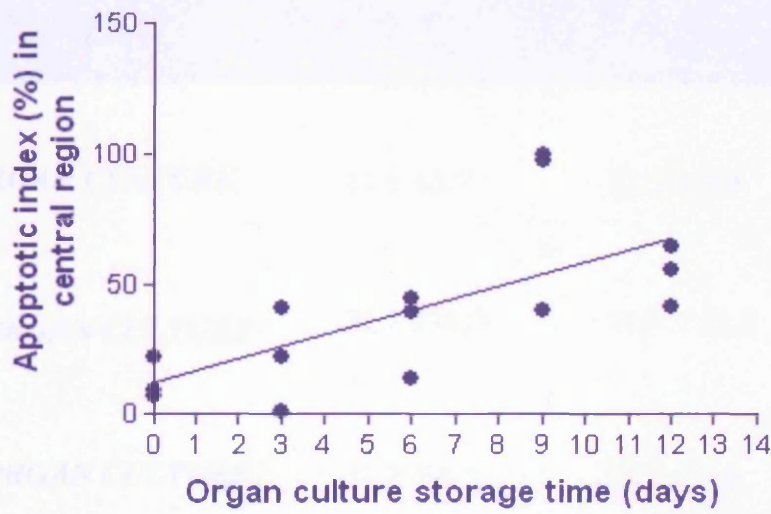
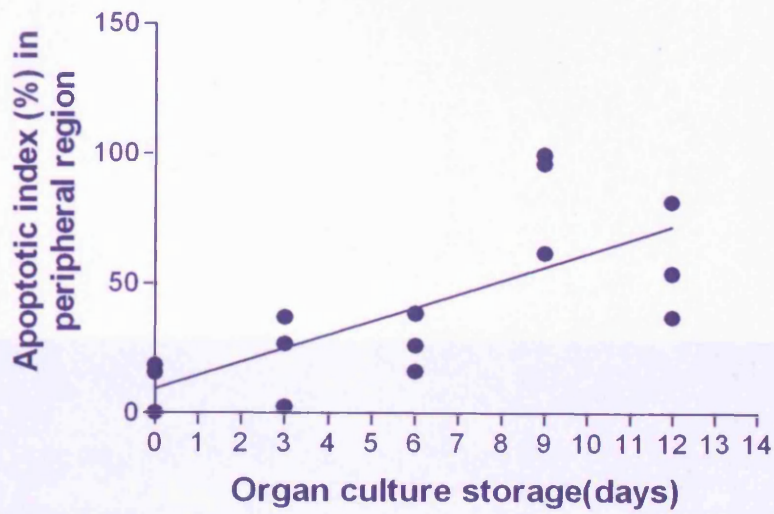


Fig 6.3 Graphs representing the percentage of corneal epithelial cell apoptosis as a function of storage time in the peripheral, central and limbal regions ($p < 0.05$).

TUNEL LABELLING INDEX	Peripheral Mean±SEM	Central Mean±SEM	Limbus Mean±SEM
0 DAY ORGAN CULTURE	11.5 ±5.7	12.9 ± 4.6	16.3 ± 9.0
3 DAYS ORGAN CULTURE	22.3 ±10.3	21.6 ± 11.5	3.4 ± 1.2
6 DAYS ORGAN CULTURE	27.2 ± 6.5	32.7 ± 9.5	15.5 ± 3.0
9 DAYS ORGAN CULTURE	86.3 ± 12.1	79.3 ± 19.5	95.2 ± 2.9
12 DAYS ORGAN CULTURE	57.9± 13.0	54.0± 6.8	64.9 ± 14.1

Table 6.1. Mean Percentage Apoptosis (Mean± SEM) in the peripheral central and limbal regions of porcine stored cornea for 0-12 days of organ culture.

TUNEL positive epithelial cells

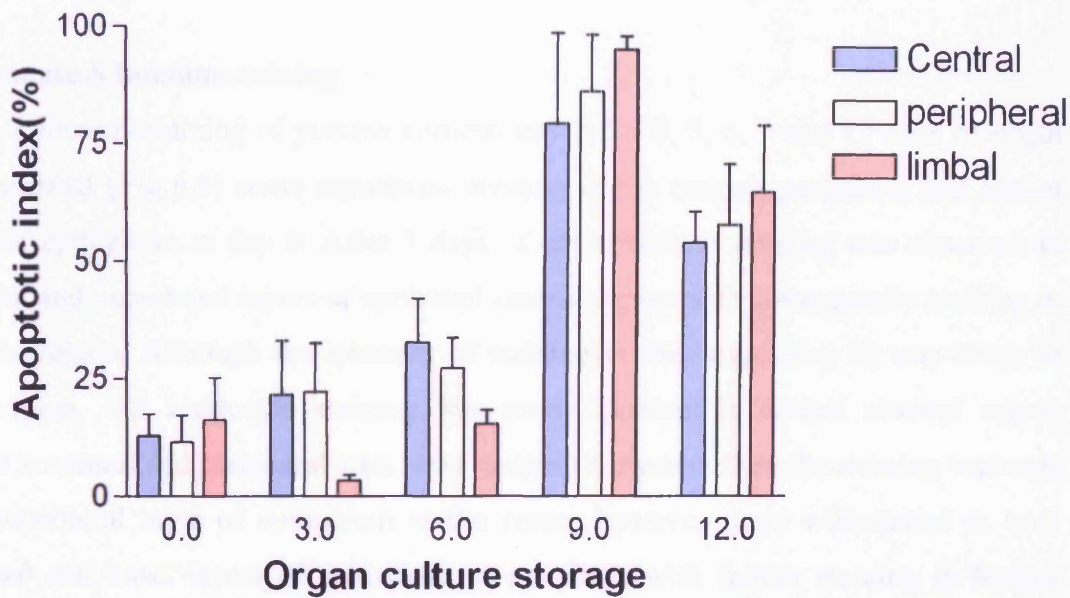


Fig 6.4 TUNEL index (number of TUNEL positive cells/total number of cells \times 100) in central, peripheral and limbal regions of porcine corneas stored in organ culture for 0-12 days. The labelling index increased with storage time in the peripheral ($r = 0.72$), central (0.68) and limbal (0.73) regions respectively ($p < 0.05$, $n = 15$). Each Bar represents the mean \pm SEM of 3 corneas.

6.3.3 Caspase-3 immunostaining

Caspase-3 immunostaining of porcine corneal sections for 0, 3, 6, 9 and 12 days of organ culture did not reveal any specific staining (refer to section 2.2.8.2).

6.3.4 Caspase-9 immunostaining

Non-specific immunostaining of porcine corneal sections with caspase-9 was observed for 0, 3, 6, 9 and 12 days of organ culture storage (refer to section 2.2.8.4).

6.3.5 Caspase-8 immunostaining

Caspase 8 immunostaining of porcine corneas stored for 0, 3, 6, 9 and 12 days of organ culture showed (Fig 6.5) some suprabasal staining in the central, peripheral and limbal regions of epithelium at day 0. After 3 days of organ culture staining was observed in both basal and suprabasal layers of epithelial central region with some patchy staining in peripheral region. Although the intensity of staining at this stage (day 3) was lower in limbal region, but keratocyte staining was more apparent in limbal stromal region compare to central and peripheral area. After 6 days of organ culture the staining was only in the superficial layer of epithelium at the centre however more widespread in both suprabasal and basal layers of epithelium at periphery with fainter staining in limbus (both basal and suprabasal layer). At day 9 all epithelium at centre, limbus (epithelium and keratocyte) and the greatly thinned epithelium at periphery revealed caspase 8 staining. All parts of the epithelium were stained after 12 days of organ culture.

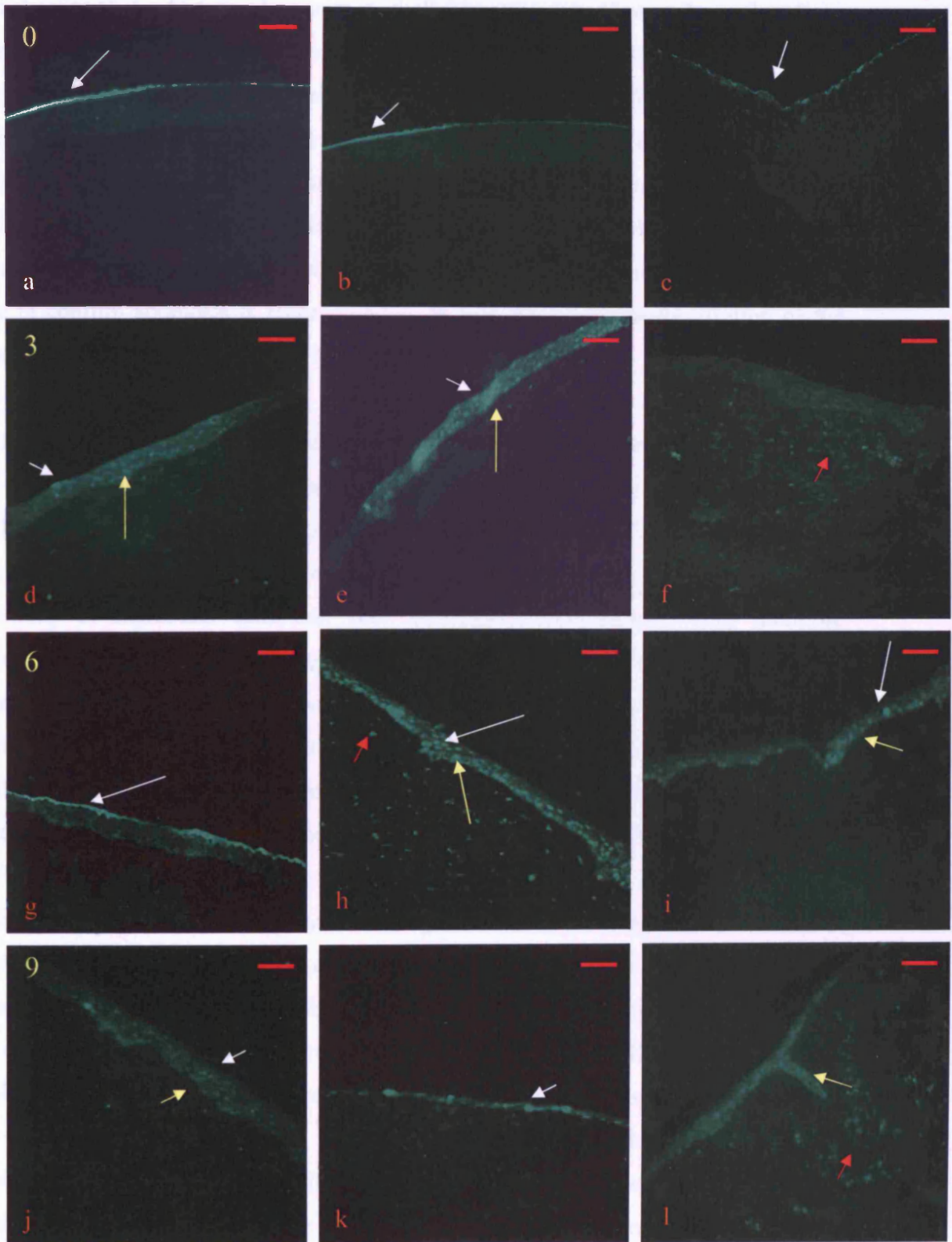
Fig 6.5. Immunofluorescent localisation of caspase-8 in porcine corneal wax sections for various time points of culture. Picture shows some suprabasal (white arrow) staining in the central (a), peripheral (b) and limbal (c) regions of epithelium at day 0. Obvious patchy staining was in the peripheral region (e) at day 3, and fainter staining in the limbal region (f).

At day 6 widespread intense staining observed in the basal (yellow arrow) and suprabasal layers (white arrow) of the epithelium in the peripheral region (h) and more in the superficial cells in the central region (g) compare to fainter staining in the limbus (i). All the central (j), limbal (l) and the greatly thinned epithelium at periphery (k) showed staining after 9 days of culture. Red arrows show keratocyte staining in stromal regions. Bar represents 30 μm .

Central

Peripheral

Limbal



6.4 Discussion

Epithelial renewal is a feature of corneal epithelial homeostasis. How cells are lost from the corneal epithelium is not fully understood, although Ren and Wilson (1996) believe that one of the mechanisms involved is apoptosis. In this study the determination of epithelial cell death was investigated during organ culture storage. Epithelial cell death was studied by TUNEL labelling, and the apoptotic index was monitored over time throughout the corneal epithelium. However since Kim et al. (2000) has shown that, in some situations, necrotic cells are falsely detected by the TUNEL assay, an alternative method to confirm apoptosis is recommended. In this study immunolocalisation of the apoptotic enzymes, activated caspase 3, 8 and 9 was also performed.

TUNEL positive apoptotic cells were localised in all regions of the corneal epithelium during organ culture storage. One interesting finding in this study is the evidence of TUNEL positivity in a few cells that appeared to be about to be shed from the surface of the multilayer epithelium. However Lomako et al. (2005), observed a lack of apoptosis in the majority of suprabasal cells that had undergone desquamation. They therefore proposed that epithelial cells were mainly shed by a non-apoptotic desquamation process. In contrast Estil et al. (2000) found that 57% of shed cells demonstrated TUNEL positivity and postulated the existence of a nonapoptotic pathway as well. Taken together it would be reasonable to propose that both apoptosis and a non-apoptotic process are involved in the process, however further research is needed to clarify this issue.

Apoptosis was minimal up to 6 days after organ culture that suggests the limbus is resistant to changes occurring in the epithelium up to 6 days. In fact, limbal stem cells may have access to survival factors, because of their close proximity to a blood supply that is unavailable to the cells of the central cornea (Daniels et al., 2001). The underlying stromal cells may also modulate limbal stem cell function by the release of cell survival factors and it maybe these survival factors that make limbal cells more resistant to apoptosis than peripheral and central (Raff et al., 1993) cells. Cotsarelis et al. (1989) has also mentioned that basal cells in the limbal region are heavily pigmented and thus are

well protected from changes in their environment. Hogan et al. (1971) has shown that the limbal epithelium is extremely resistant to shearing forces. The limbal epithelium displays a highly undulating epithelial stromal junction that gives a natural advantage for retention of stem cells in the face of environmental onslaughts. Taken together all these data reveal a degree of limbal epithelium resistance to apoptosis.

Previously a drop in proliferation at day 9 was observed (section 5.3.3). This is therefore consistent with the higher amount of apoptosis detected at this time point in this study. On the other hand the increase in epithelial cell death in response to organ storage, could explain why the proliferative response of the corneal epithelium (as discussed in chapter 5) was not enough to restore the multilayer structure of the epithelial cornea. The apoptotic values decreased by day 12, although not as low as levels of apoptosis identified in days 0-6 of organ culture.

In this study some keratocyte apoptosis was observed in cultured cornea with both TUNEL and caspase 8 staining. Keratocyte apoptosis occurs as a result of epithelial wounding in the cornea (Wilson et al., 1998b). We postulate that a similar process may occur in corneas during organ culture storage. Komuro et al. (1999) revealed that both apoptosis and necrosis occur in keratocytes during corneal storage at 4°C. Apoptosis appeared to predominate, and the study revealed that the TUNEL assay identified cells undergoing apoptosis, but not necrosis, in corneal tissue. Albon et al. (2000) have also demonstrated that corneal endothelial cell apoptosis occurs in the human cornea organ culture at 34°C. The decrease in keratocyte density reported in section 3.3.4. with increasing organ culture storage time supported this finding. Therefore further investigations need to be done to identify the mechanism of keratocyte cell death and their effect on epithelial cell homeostasis during organ storage.

Apoptosis is a morphologically stereotyped form of cell death, prevalent in multicellular organisms, by which single cells are deleted from the midst of living tissues. Recognition of the cellular corpses and their removal by phagocytosis occurs without disturbance to

tissue architecture or function and without initiating inflammation. Apoptosis is of fundamental importance to tissue development and homeostasis. Cellular susceptibility to apoptosis is determined by a variety of signals, of both extracellular and internal origin, including proliferative status of the cells (Bellamy et al., 1995).

In the organ culture model, the cornea is in a closed system that has a limited oxygen supply resulting in glucose depletion and lactate accumulation (Redbrake et al., 1999; Hjortdal et al., 1989). This may cause epithelial cell injury and the release of IL-1 that is one of the triggers of apoptosis (Pflugfelder et al., 1998, 1999). There are a variety of extrinsic and intrinsic signals to induce apoptosis. These include death receptors such as Fas/APO system, growth factors or their absence, oxidative damage, viral infection, chemotherapeutic agents and mechanical stress (Thompson et al., 1995). Among these, Fas ligand is a key physiologic inducer of apoptosis (Nagata et al., 1995), because when Fas ligand binds its receptor Fas, the target cell undergoes apoptosis. All three corneal cell types expressed Fas (Wilson et al., 1996a) whereas Fas ligand has only been identified in epithelial and endothelial cells, or in keratocytes when stimulated by interleukin-1 (Mohan et al., 1997). Fas-FasL interaction leads to cleavage of procaspase-8 to caspase-8. Activated caspase-8, the most upstream caspase in the Fas apoptotic pathway, promotes caspase-3 activation and DNA fragmentation (Stennicke 2000). However Jones et al., (2001) revealed the activation of caspase 8 by a Fas-independent mechanism induced by cytotoxic drugs or δ radiation in lymphatic leukemia.

One of the important concerns in this study was to determine by which pathway the activation of caspases occurred in the corneal organ storage. In this study, Caspase-8 is indeed activated during organ culture storage. Immunolocalisation for activated caspase 8 demonstrated in the suprabasal epithelial cells at day 0 and 3 which changed to include staining of both suprabasal and basal cells by day 6 and 12. The latter implies that TACs also induced to apoptose in corneas during organ culture storage. None of the other caspases antibodies (caspase-3 and 9) demonstrated any cross reactivity with porcine corneal section. Although the neoepitope of cleaved caspase-3 and 9 should be fairly

ubiquitous and of sufficient levels to be detected in most cells, the cross reactivity of these antibodies with this neoepitope in porcine cornea has not been tested yet.

The relative amounts of initiator (caspase-8 and 9) and effectors (caspase-3) determine whether a cell uses a mitochondrial dependent (Type II cells) or independent (Type I cells) pathway. The lack of positive activated caspase-9 staining with the high intensity of immunorelated caspase-8 detected and the previously observed Fas expression (Wilson et al., 1996a) could lead us to propose that the activation of caspases in the corneal organ storage model may occur by extrinsic death receptor-mediated pathway.

Therefore this data is inconsistent with Kurpakus et al. (2003) who found that upregulation of caspase-9 activity occurs in the epithelium, stressed by hypoxia. However Khurana et al. (2002) showed both caspase-8 and 9 activation in the cerebral cortex. The hypoxia model could be comparable with this model of organ culture to some extent, as hypoxia happens during organ culture storage. Therefore further investigation needs to be undertaken to clarify the caspase pathway responsible for apoptosis during organ culture storage. It is likely that different pathways are activated due to different conditions to which the cornea is subjected to.

6.5 Conclusions

The conclusion of this investigation is that the level of epithelial cell loss appears to be greater than that restored by epithelial cell proliferation. There appears to be a lack of balance between epithelial cell proliferation and apoptosis, such that the demand for tissue regeneration due to epithelial shedding and apoptosis can not be met by the proliferative activity of the stem cells and TACs during organ culture storage. This results in a disruption in homeostasis of the corneal epithelium. It is likely that the huge loss of epithelial cells especially at the limbus may limit the use of such corneas for corneal stem cell transplantation.

CHAPTER SEVEN

7. Chapter 7 - Changes identified in human organ culture stored corneas

7.1 Introduction

This chapter aims to investigate the alterations in human corneas that occur during organ culture. Furthermore these data will be compared with previous results from porcine corneas identified in this thesis (chapter 3).

The UK Corneal Transplant Service (CTS) Eye Banks provide organ-cultured donor corneas for over 2500 corneal transplants per annum. The use of organ culture for corneal storage as opposed to other short-term storage media (e.g. Optisol) is due to the short supply of corneas available for transplantation in the United Kingdom and also the benefits of organ culture. The extended storage allowed in organ culture for up to 28 days, allows routine surgical procedures, virological and microbiologic screening, and tissue-typing (if necessary) (Pels et al., 1983). Therefore, a high quality of donor tissue is supplied for transplantation. However, during organ culture, corneal endothelial cell deterioration due to endothelial cell insufficiency results in the discard of 30% of donor corneas stored in the Corneal Eye Banks in the UK (Armitage et al., 1997).

In this chapter several morphological changes of human corneas will be investigated. The effect of preservation time on the epithelium and stroma will be determined. Changes in epithelial cell proliferation and thickness, stromal thickness and keratocyte density will be determined as a function of storage.

7.2 Experimental design

Twenty-eight human donor corneas from twenty donors, aged between 1.5 and 87 years were obtained from the Manchester Eye Bank. Human corneo-scleral discs were stored in organ culture at 34°C for up to 30 days. Corneas were then bisected and prepared for wax or frozen sectioning.

7.2.1 Organ culture storage

Human corneas were stored, according to standard CTS Eye Bank operating procedures. After thorough cleansing of the whole eyes (four rinses in sterile 0.9% [wt/vol] NaCl, a 3-minute immersion in 0.5% polyvinylpyrrolidone iodine, neutralisation in 0.2% sodium thiosulphate, and a final wash in 0.9% [wt/vol] NaCl), corneoscleral discs were cultured as described for porcine corneas (see section 2.2.1.1) except that organ culture medium did not contain chondroitin sulphate. Corneas were stored in a standard incubator at 34°C for varying lengths of time, up to 30 days, with medium remaining unchanged throughout storage. Corneal endothelial cell density and viability were assessed according to the protocol described in section 2.2.2. Each cornea was graded from 1-5 (1=excellent, 5=very poor <1000 cell/mm²), as determined by endothelial cell count, number of dead cells and the level of folding. The corneas were then bisected and prepared for wax (section 2.2.4) and frozen sectioning (section 2.2.3).

7.2.2 Quantification of epithelial thickness in human stored cornea

7.2.2.1 Haematoxylin and Eosin staining

In order to measure epithelial thickness in human stored cornea, wax sections (refer to section 7.2.1) were stained with haematoxylin and eosin as described in section 2.2.5.1.

7.2.2.2 Image analysis for epithelial thickness

Images of the haematoxylin stained sections were captured using ultra-violet illumination and a digital camera attached to an Olympus IX-70 microscope. Scion image analysis software was used to measure epithelial thickness.

7.2.3 Quantification of corneal stromal thickness and keratocyte density in human stored cornea

7.2.3.1 Bisbenzimidazole nuclear staining of human organ stored cornea

In order to measure stromal thickness and keratocyte density in human stored cornea, wax sections were first stained with the nuclear dye (bisbenzimidazole), as described in section 2.2.5.2.

7.2.3.2 Image analysis of stromal sections for stromal thickness assessment

In order to measure stromal thickness in central stroma, first human corneal central Hoechst stained sections (section 7.2.3.1) were imaged using Leica QFluoro image analysis software. Then Scion image analysis software was used to measure stromal thickness.

7.2.3.3 Image analysis of stromal sections for keratocyte density assessment

In order to measure keratocyte number and density, Hoechst-stained nuclei of keratocytes were counted in the stroma of the central area of each cornea (section 7.2.3.2). Then the Scion image program was used to measure the areas in each part of anterior, middle and posterior stromal regions. Finally Image Proplus software was used to count the number of keratocytes in each area. Keratocyte density was calculated using the formula:

$$\text{Keratocyte density} = \frac{\text{No of ks}}{\text{area}} \times \frac{\text{STo}}{\text{STn}}$$

No of ks= Number of keratocyte in cornea area

STo=Stromal thickness at 0 day STn=stromal thickness at appropriate period of time in organ culture

7.2.4 Determination of proliferative activity of corneal epithelial cells

In order to determine the proliferative activity of human corneal epithelial cells in different regions of stored cornea, immunostaining of Ki-67 was performed on human frozen sections (section 7.2.1) as described in section 2.2.7.1.

7.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Prism normality test was also performed for deviations of data from Gaussian distribution using Kolmogorov-KS test. $P > 0.1$ was considered as significant. One way ANOVA and Unpaired t test was performed for parametric data and Kruskal-Wallis test and Mann-Whitney test for non parametric data. $P < 0.05$ were considered to be statistically significant. Regression analysis was also performed to analyse the correlation of different parameters.

7.3 Results:

7.3.1 Epithelial thickness of human corneas during organ culture storage

In order to identify epithelial morphological changes during human organ culture, haematoxylin and eosin stained sections of wax cornea were investigated over 0 to 40 days of storage. In organ culture, the human corneal epithelium was composed of 5-6 cell layers. After 28 days of organ culture the ocular surface epithelium was markedly reduced in thickness, sometimes consisting of no more than one layer in limbal and two cell layers in the central and peripheral regions (Fig 7.1).

This study has also demonstrated a significant negative correlation (Fig 7.2) between storage time and epithelial thickness ($P < 0.0001$, Spearman correlation $r_s = -0.510$). Epithelial thickness significantly decreased from approximately $30\mu\text{m}$ to $2\mu\text{m}$ by 30 days after organ culture.

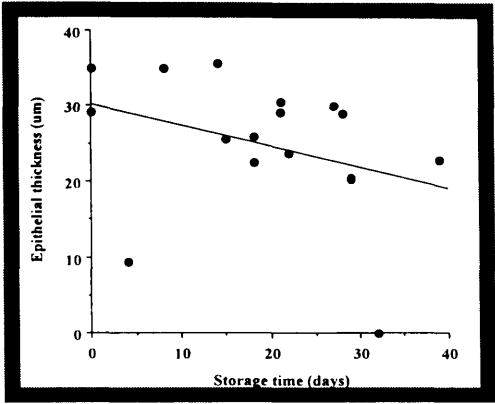


Fig 7.2 Epithelial thickness as a function of storage time ($p < 0.05$, $r_s = -0.510$).

Epithelial thickness was decreased inversely with the deterioration of the corneal endothelium as determined by assessment grade, where Grade 1: excellent, 2: very good, 3: good, 4: not suitable for transplantation, 5: very unsuitable ($r = -0.540$, $p < 0.05$, fig 7.3).

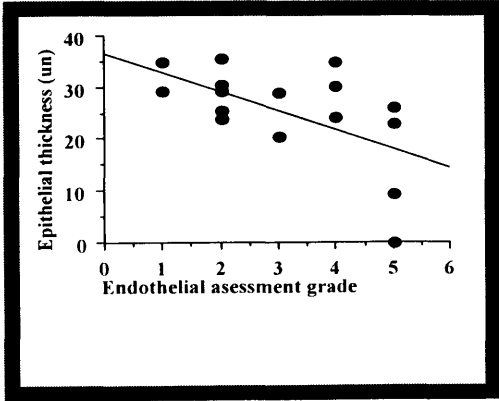


Fig 7.3 Epithelial thickness as a function of endothelium assessment grade. Epithelial thickness decreased with deterioration of endothelium ($p < 0.05$, $r_s = -0.540$).

Stromal thickness also appeared to significantly correlate with the endothelial assessment grade. However this correlation was not significant ($r=0.334$, $p=0.19$, fig 7.4).

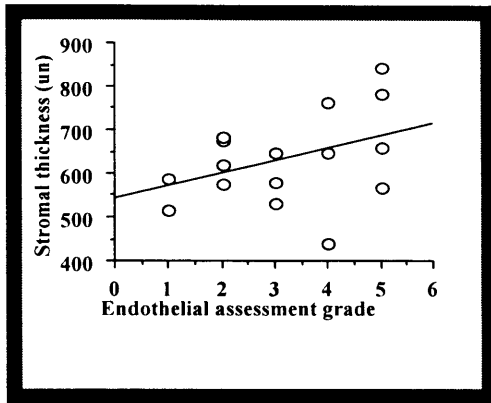


Fig 7.4 Stromal thickness as a function of endothelial assessment grade ($p=0.19$, $r_s=0.334$).

7.3.2. Keratocyte density in human stored cornea

The overall keratocyte density of human cornea remained unchanged with storage time. However a significantly decreased keratocyte density was observed in the mid-stroma region (Fig 7.5) as a function of increasing storage time. ($r_s=0.46$, $P<0.05$). Decreased keratocyte density in the middle stroma was also correlated to deterioration of corneal endothelium ($r=-0.485$, $p<0.05$, fig 7.6).

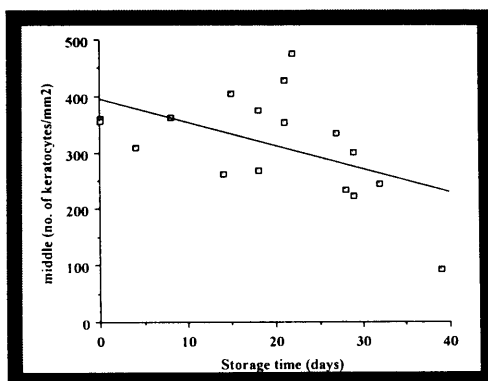


Fig 7.5 Keratocyte density in the mid-stroma as a function of storage time ($p<0.05$, $r_s=-0.46$).

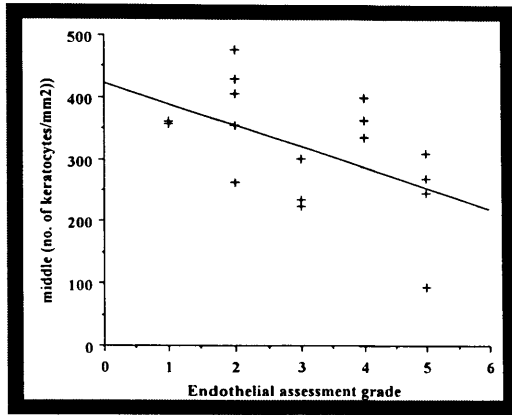


Fig 7.6 Keratocyte density in the mid-stroma as a function of endothelial assessment grade ($p < 0.05$, $r_s = 0.485$).

7.3.3 Determination of proliferative activity of corneal epithelial cells using Ki-67 immunostaining

In order to determine the proliferative activity of the human corneal epithelium during organ culture storage Ki67 immuno-staining was performed. Cycling cells are identified by Ki67 immuno-reactivity. Ki67 positive labelling of cells was demonstrated in the central, limbal and peripheral regions of corneal basal epithelium. At day 0 labelled proliferating cells were observed in the central, peripheral and limbal regions with the greatest number in the peripheral region. The labelled proliferating cells only remained in the peripheral and limbal regions after 29 days of organ culture with the greater quantity in the peripheral region (Fig 7.7).

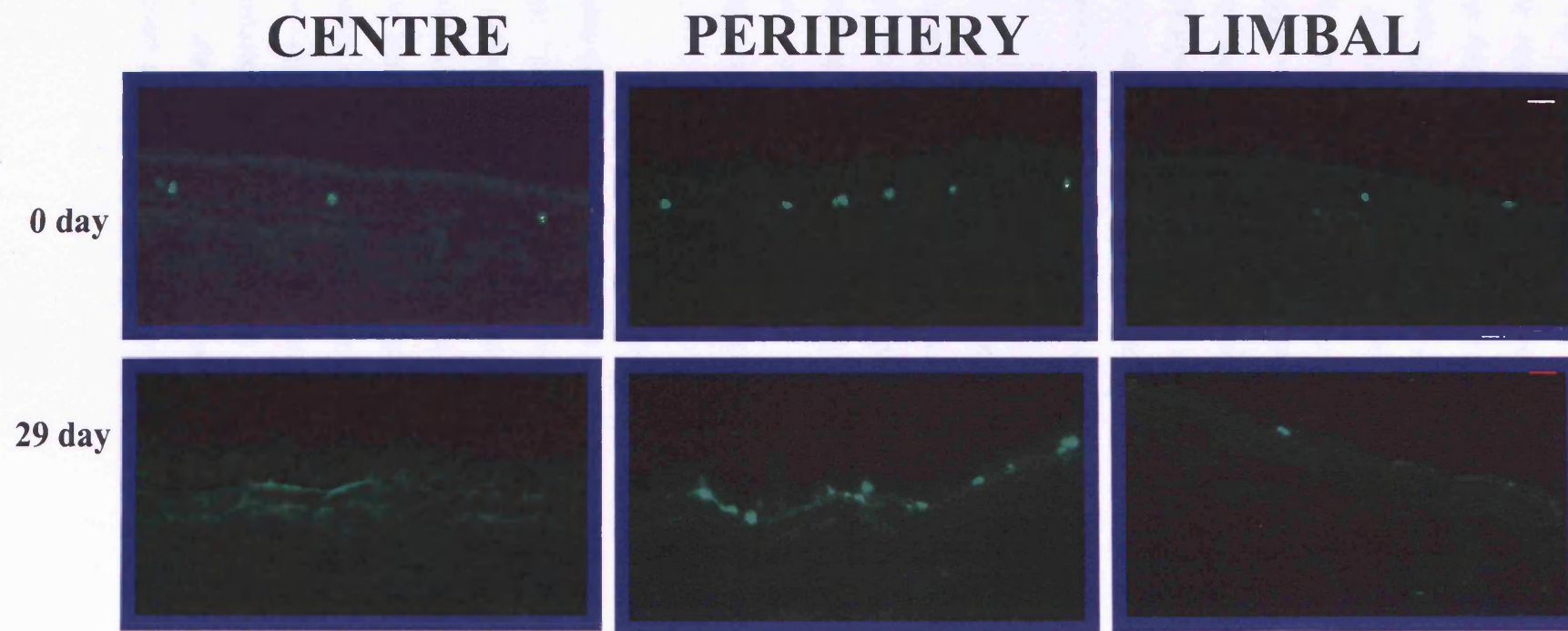


Fig 7.7 Immunolabelling for Ki67 positive proliferating corneal epithelial cells during storage in organ culture for 0 and 29 days. At day 0 all regions of epithelium shows proliferative activity with the greatest amount in the peripheral region. After 29 days of culture an increase in the proliferative activity was observed in the peripheral region, with lack of proliferation in the central region and decreased proliferative in limbal area. Bar represents 30 μ m.

7.4 Discussion

Organ culture is currently the most common corneal storage method used in Europe. Although the *in vitro* viability of the endothelium in organ culture storage is longer than with other storage techniques, endothelial cell loss does occur with increasing time of storage. In addition the endothelium alone does not reflect the viability of the whole cornea: the epithelium and stroma are also of major importance. This investigation not only documents the changes in corneal morphology and cellularity of the human cornea during storage but it also reveals a detailed comparison of human data with porcine cornea and other investigators. Different studies have also shown changes in epithelial layers (Crewe & Armitage 2001; Borderie et al., 1995; Neubauer et al., 2002), stromal thickness (Redbrake et al., 1997; Neubauer et al., 2002) and keratocyte number (Moller et al., 1998; Komuro et al., 1999) during human corneal storage.

The results in this chapter have demonstrated a decrease in epithelial thickness with increasing time of storage. There was also an increase in stromal thickness with increasing time of culture. Keratocyte density has only decreased in the middle stromal region with increasing time of culture. Peripheral, but not central or limbal regions showed increased proliferative activity with increasing time of culture. These data can be compared to that of porcine stored cornea described in chapter 3 and 5.

This investigation revealed that by 2 weeks in culture the human corneas exhibit prominent signs of epithelial cell loss in agreement with the porcine corneal storage model (refer to chapter 3). Previous studies in agreement with our findings undertaken using human corneas have all shown a considerable decrease in epithelial thickness with organ culture storage time (Crewe & Armitage 2001; Borderie et al., 1995; Pels et al 1983). Pels et al. (1983) reported a profound reduction in the epithelial layers in organ culture of human cornea. Crewe & Armitage (2001), using confocal microscopy demonstrated that the number of epithelial cell layers reduced from 5-7 layers in human corneas of eyes in moist-chamber to <5 after organ culture storage. They also showed a reduction in the depth of the epithelium; from an epithelial thickness in the moist-chamber cornea of 54µm to 42µm thick after 14 days in organ culture in the contralateral

corneal epithelium of the same donor. These results are all compatible with the result described in this thesis.

Another study by Neubauer et al. (2002) used a new fast and easy non-contact method for sterile measurement of structure and thickness of donor cornea (27 cornea) called optical coherence tomography (OCT). The results showed that epithelium, stroma and posterior curvature, as well as thickness, can be measured by standard OCT while the cornea remains in its storage bottle. The results showed that corneal epithelial thickness, decreased from $57.6 \pm 10.4 \mu\text{m}$ at 0 day to $32.8 \pm 8.8 \mu\text{m}$ thick after 10 days of culture. This is consistent with our data where corneal epithelial thickness was $\sim 36 \mu\text{m}$ after 14 days in organ culture.

Overall, other studies agree with the finding that a decrease in epithelial thickness occurs with increasing time of organ culture. However the difference between various experiments could be due to individual variation in human tissue, donor age, history and different methods of assessment (light versus confocal microscopy versus optical coherence tomography).

Epithelial changes leading to stromal swelling were also visualised in this study. Human stromal corneal thickness increased with increasing time of culture. Our data was also consistent with Redbrake et al. (1997) who showed an increase in human corneal thickness from 652.5 at day 0 to 1509.7 μm after 28 days of organ culture storage. Neubauer et al., (2002), using optical coherence tomography (OCT), also demonstrated an increase from 488 μm at day 0 prior to organ culture to 1300 μm thick after 10 days of culture. Therefore all these investigations revealed a swollen stroma, possibly due to both epithelial and endothelial dysfunction. The corneal epithelium possesses the Cl^- secretory mechanism that has the potential to participate in the stromal volume regulation process (Klyce et al., 1985). All Cl^- uptake within this system is coupled to the Na, K-activated ATPase associated with the Na pump (Friedman et al., 1978). Stiemke et al. (1995) have also revealed an increase in corneal hydration and a decrease of sodium activity within the stroma of corneas stored in Optisol for 21 days. Corneal swelling appear to be

important in the viability of the corneal endothelium, as endothelial cell loss occurs with increasing length of storage time, partly due to Descemet's folding as a consequence of stromal swelling (Pels et al., 1983). This is a very most important issue regarding donor cornea selection for transplantation.

Organ culture storage of human corneas, before grafting, causes cell death (Albon et al., 2000, Crewe and Armitage 2001) in the endothelium, which reduces endothelial cell density during storage (Frueh et al., 1995; Means et al., 1995; Armitage et al., 1997, 2001). Stromal thickness was also significantly correlated with the endothelial assessment grade that reflects the effect of the endothelial barrier dysfunction on stroma and vice versa. However the exact mechanisms of the endothelial cell death during organ culture has not been fully understood. The endothelial cell death may be linked to necrosis, apoptosis or a combination of the two (Albon et al., 2000; Crewe and Armitage 2001). Albon et al. (2000) showed a high incidence of apoptosis in the endothelium by TUNEL, with confirmation of Hoechst 33345 and caspase staining. The in situ TUNEL technique on whole corneas used by Albon et al. (2000) could specifically detect localisation of apoptotic cells in the human corneal endothelium. However Crewe and Armitage (2001) found that apoptosis was not a significant mechanism of endothelial cell loss during storage. The difference between Albon and Armitage could be due to methods used by these authors. The TUNEL method that was used to detect DNA fragmentation, a marker for apoptosis and sometimes a feature for necrosis cell by Albon versus caspase 3 activation (an early event in apoptosis) which could therefore not be detectable at all times.

Now the critical question that has to be answered is to what extent organ culture and swelling have an effect on the density and functional integrity of the keratocytes. During human organ culture the overall keratocyte density in the upper and lower central stromal regions remained unchanged. However the keratocyte density in the middle stromal region decreased with storage time. Therefore one explanation for the reduction of keratocytes in the middle stroma could be that putting cornea in organ culture medium may cause epithelial injury, therefore keratocyte in the anterior part of the stroma close to

the injury could go through apoptosis, then the remaining keratocyte in the middle stroma will begin to proliferate and migrate to repopulate the anterior stroma. Wilson et al. (1998b) revealed that keratocyte apoptosis was the first observable stromal response following epithelial injury. Some keratocyte apoptosis has also been observed during storage of porcine cornea (see chapter 6). Komuro et al., (1999) also demonstrated keratocyte apoptosis in the human cornea stored at 4°C.

Keratocyte density in porcine cornea appeared to decrease in all levels of the stroma, and not just middle stroma (section 3.3.4.) and is in agreement with Salla et al. (1995) who revealed a decrease in keratocyte density with increasing storage time. In contrast Moller Pedersen et al. (1994) found no changes in keratocyte density with storage time. Keratocytes have been shown to play a crucial role in maintaining the organisation of extracellular matrix because of the synthesis and secretion of collagen, chondroitin sulphate and glycoproteins (Birk 1981; Schittny et al., 1988). The extensive network of keratocyte gap junctions are arranged to maintain synchronised metabolic homeostasis of the corneal stroma (Watsy et al., 1995). Further studies are needed to investigate whether the decrease in keratocyte number could actually influence the outcomes of transplantation.

The proliferative activity of human corneas examined by Ki67 immunostaining increased in the peripheral regions of the cornea with increasing time of storage, in agreement with porcine findings in section 5.3.2. In both human and porcine stored corneas the proliferative activity of the epithelium was greatest in the peripheral region compared to the central and limbal regions with increasing time of storage. This is consistent with upregulation of TACs in injured epithelium (Lehrer et al., 1989). The pattern of proliferative activity peripheral>limbal>center is also consistent with previous studies. However the amount of proliferation in the corneal epithelium was not fully successful in restoring the epithelial layer, as a huge decrease in epithelial layers were demonstrated with increasing time of culture.

7.5 Conclusions

The relative similarity of porcine to human cornea makes the porcine organ culture method a good model to study organ culture related changes. In addition, for highly reproducible measurements, the average of several corneas must be used to reduce the standard deviation and consequently standard error. This is more achievable with porcine corneas because of their availability in great numbers at a similar age therefore more flexibility to work compared to human cornea.

In this study epithelial thickness, decreased with storage time. Stromal thickness showed a huge increase with increasing time of storage. Considering the amount of cell loss in the endothelium, it would be reasonable to say that the epithelial dysfunction (cell loss or permeability dysfunction) and endothelial dysfunction contribute to stromal swelling and consequently the corneal deformation of the swollen cornea could provide a stimulus for mechanical disruption of cell-cell and or cell-matrix interaction (Albon et al., 2000), and thereby consequently more damage to the cornea.

CHAPTER EIGHT

8. Chapter 8 - Discussion

8.1 Corneal changes in organ culture

The integrity of donor corneas following storage is critical to recipient graft survival. For successful corneal transplantation, the quality of the donor material is of vital importance. Placing a cornea in organ culture causes increased stromal thickness and consequently a decrease in endothelial cellularity due to cell death (Albon et al., 2000). Besides endothelial cell viability, epithelial cell integrity should also be preserved to ensure a good quality of donor cornea for transplantation, as breakdown of the epithelial barrier may also contribute to stromal swelling and thus endothelial deterioration. The latter is of particular importance if the donor cornea is to be a source of cells for limbal stem cell transplantation.

The overall purpose of this project was to improve the quality of donor corneas and thus increase the availability of tissue for corneal and limbal stem cell transplantation. The specific aim was to determine the mechanisms responsible for impaired epithelial cell homeostasis during organ culture. In essence, the extent to which organ culture was able to sustain epithelial corneal cell homeostasis was questioned. Understanding such processes as proliferation, apoptosis and the effect of the organ culture preservation technique on the porcine cornea was the first aim of this study.

Furthermore this study also aimed to assess the impact of the preservation technique on corneal epithelial cell homeostasis. In particular both epithelial cell-cell junction and the cytoskeletal organisation of the epithelium in addition to its cell proliferative ability and viability are critical in the maintenance of a stratified corneal epithelium to provide a competent barrier.

8.2 Corneal epithelial changes in organ culture

8.2.1 Epithelial cell stratification

The stratified corneal epithelial cell layer can be defined as a tight ion transporting functional system which serves both as a protective barrier to the ocular surface, as well as adjunct fluid secreting layer assisting the corneal endothelium in the regulation of stromal hydration (Klyce et al., 1985). The primary role of the corneal epithelium is that of a high resistance barrier to both solute and water flows to maintain stromal hydration and preservation of their own cell volume. Corneal epithelium possesses the Cl^- secretory mechanism that has the potential to participate in the stromal volume regulation process (Zadunaisky et al., 1966; Marshall et al., 1982 and 1983). However the fluid transport of the epithelium is many times less than that of endothelium. The endothelial fluid pump appears to function at fixed capacity, whereas that of the epithelium is under neural control (Klyce et al., 1985). Although the fluid transporting capability of the corneal epithelium in terms of the maintenance of corneal hydration is small compared to endothelium, epithelial function can be modulated by activation of specific receptors under neural control that have not been demonstrated for the corneal endothelium (Klyce 1982 and 1985).

The endothelial integrity is essential for maintaining corneal transparency as the tendency of stroma to absorb water and swell, is also controlled by the endothelium and if disrupted, water will enter the stroma and stromal thickness will increase. Stromal thickness showed significant correlation with the endothelial assessment grade in preserved human (see chapter 8) corneas. Cell death is known to occur in the endothelium, during storage, thus reducing endothelial cell density (Frueh et al., 1995; Means et al., 1995; Armitage et al., 1997). The mechanisms of endothelial cell death during organ culture are not fully understood and are believed to be linked to necrosis or apoptosis, or a combination of the two (Albon et al., 2000; Crewe and Armitage 2001).

In organ culture, the entire cornea is bathed in medium and this artificial situation is likely to cause trauma and stress to the cornea. In this study, the submersion of the cornea in the

artificial environment of organ culture storage medium caused a significant increase in epithelial damage as a function of time. A huge decrease in epithelial thickness was identified with increasing time of storage, in agreement with Crewe and Armitage (2001).

8.2.2 Epithelial cell-cell communication

The corneal epithelium forms a barrier that isolates the eye from the outside environment and regulates passive movement of fluid, electrolytes, macromolecules and cells through the paracellular pathway. Tight junctions (TJs) create this paracellular permeability barrier (Mclaughlin et al., 1985; Wang et al., 1993) which to a great extent is generated by zonula occludens (ZO). In epithelia and endothelia, ZO-1 acts as a scaffolding molecule that localises in adherens junctions, tight junctions, and gap junctions early in the assembly of actin-based junctional complexes (Itoh et al., 1997; Imamura et al., 1999). ZO-1 has also been shown to associate directly with connexins, facilitating their lateral aggregation before intercellular junction formation, and regulating their role in facilitating epithelial differentiation (Imamura et al., 1999). Gap junctions have also been implicated in the control of cell proliferation, differentiation and regeneration (Donaldson et al., 1997). Therefore the integrity of epithelial cells would be disrupted if dissociation of these intercellular junctions (ZO-1) occurred as these components are responsible for the low permeability of the corneal epithelium (Klyce et al., 1972).

During organ culture accelerated loss of corneal epithelial cells, as is shown in this study, is likely to disrupt the barrier function of the epithelium. In addition the loss of tight junctions would also result in passive movement of fluid and macromolecules through the paracellular pathway of the epithelium (Sugrue et al., 1997). In organ storage, although the cytoskeletal properties of corneal epithelium seemed to remain stable during storage, the lack of ZO-1, Cx43 and 50 expression in cornea at some time points of culture suggests the existence of a leaky or impaired epithelial barrier. This leaky epithelial barrier is also likely to contribute towards the severe stromal swelling observed during cornea storage (both human and porcine).

8.2.3 Proliferative activity and epithelial cell death in organ culture

Disruption of the epithelial structure following storage requires a rapid and accurate response of the corneal stem cells and TACs to restore the normal multilayered structure of epithelium. This property is partly dependent on the ability of the corneal epithelium to undergo self-renewal. The corneal epithelium constantly renews its cell population with cells added through mitosis in the limbus and basal cell layer loss due to cell shedding from the surface (Bowman et al., 1894; Hanna et al., 1961). The rate of renewal is dependent on a highly integrated balance between the processes of corneal TAC limbal stem cell proliferation and epithelial cell differentiation and cell death. Although the regulatory mechanisms of epithelial cell renewal are not clear, one of the determining mechanisms involved is believed to be apoptosis (Ren and Wilson 1996).

One other important concept is that cell proliferative and apoptotic pathways are coupled, even though the processes appear to be mutually contradictory (O'Brien et al., 1998). In this study, both epithelial cell apoptosis and proliferation were investigated to see if one process could affect the other. To date, there are no reports in the literature that clearly evaluate the proliferative activity of the corneal epithelium during organ culture storage.

The overall proliferative activity of the corneal epithelium revealed that cell proliferation increased with increasing time of organ culture in the central, peripheral and limbal regions of corneas (28% at day 12 in the peripheral region). However the TUNEL labelling of corneal epithelial cell stored for different time of storage, also revealed an increase in the central, peripheral and limbal region (58% at day 12 in peripheral region). Therefore the level of epithelial cell loss appears to be greater than that restored by epithelial cell proliferation.

8.2.4 Caspase activation during organ culture storage

To confirm that TUNEL positivity was in fact apoptosis-related and also the mechanism of initiation of apoptosis i.e. involved caspase-9 and/or caspase-8 activation. These caspases were evaluated in corneal storage. The lack of positive activated caspase-9

immunostaining, together with the high intensity of immunoreactivity for caspase-8 detected in the porcine cornea suggests that the epithelial apoptotic pathway during organ culture is initiated by an extrinsic death receptor-mediated pathway. Previous studies has suggested that Fas-FasL interaction to cleave procaspase-8 to generate caspase-8 (Stennicke et al., 2000) is one mechanism by which this pathway can be initiated.

8.2.5 Corneal stroma changes during organ culture storage

The structural and functional integrity of the epithelium and endothelium does not fully guarantee the viability of the whole cornea. Keratocytes are also of great importance to corneal transparency (Redbrake et al., 1995). Komouro et al. (1999) showed a greater percentage of apoptotic keratocytes in corneas that were swollen during 4°C storage. The storage time related decrease in keratocyte density of human (only in middle region of stroma) and porcine cornea suggests that organ culture at 34°C also induces keratocyte cell death. Evidence of keratocyte apoptosis in cultured porcine cornea was supported with both TUNEL positivity and caspase 8 immunostaining. However, further investigations need to be done to identify the mechanism of keratocyte cell death during organ culture. Correlations between apoptosis in stroma, epithelium and endothelium shown here in organ culture and 4°C are consistent with other studies that describe the importance of epithelial-stromal interactions in the maintenance of corneal integrity (Komouro et al., 1999; Wilson et al., 2001).

8.3 Relevance of changes in porcine cornea to human changes

In the present study, the focus was on the corneal epithelium. Our studies document that by 12 days in culture, both human and porcine corneas showed prominent signs of deterioration. By 28 days of culture, the epithelium of human corneas showed major defects in agreement with Crewe and Armitage (2001), Pels et al. (1983) and Richard et al. (2001) that reported a profound reduction in the epithelial layers in organ cultured human corneas. Deterioration of epithelial cells, together with loss of keratocytes, occurred in both human and porcine corneas during organ culture storage. Epithelial cell

restoration by proliferation was found to be inefficient in both porcine and human corneas.

The clinical outcome after grafting of corneas stored by hypothermic (at 4°C) and organ culture storage is different (Pels et al., 1997; Bohnke 1991). Organ culture is the preferred method in Europe as apposed to 4°C storage in US, as during organ culture the integrity of the endothelium can be maintained, for a longer period. The storage time of 4 to 5 weeks allowed by organ culture is long compared with the generally accepted period of 3 to 5 days for hypothermic storage (Doughman et al., 1976). Organ culture storage extends the epithelial storage lifetime longer compared to Optisol-GS, where central epithelial integrity is only maintained up to 6 days in cold storage (Means et al., 1996). During organ culture, the passenger leukocytes are lost that cause less immunological reaction and the donor epithelium becomes hypocellular (Pels et al., 1984).

8.4 Potential impact of changes identified on corneal integrity and function during culture

This study clearly demonstrated the alterations in the morphology, integrity and the proliferative capability of the corneal epithelium during organ culture storage. It has also revealed that although the corneal epithelial barrier deteriorated during organ culture the proliferative activity of epithelium which appears to follow a healing response, leads to the partial restoration of epithelium at some time points of culture (especially at day 12). The consequence of this response results in the maintenance of the structural integrity of the epithelium at these points. The numbering below highlights some of the changes during organ culture that results in partial restoration of corneal epithelium:

1. The limbal region has clearly shown proliferative activity after 3 days of organ storage. This shows that the limbus is sensitive to changes occurring in the epithelium and that it is capable of upregulating cell proliferation in response to epithelial cell disruption.

2. The loss of ZO-1 resulted in TJs disorganisation and possibly an increase in paracellular permeability. However cell attachment (ZO-1) seemed to be intact up to 3 days of organ culture. Therefore the structural integrity of epithelium seems to be preserved up to this time point of culture.

3. Although the overall proliferative activity of epithelium was increased by increasing time of storage, the peripheral region revealed a higher amount of proliferative activity compared to central and limbal region. This increase in proliferative activity of corneal epithelial cells especially in peripheral regions suggests that the peripheral epithelial TA cells undergo a rapid proliferation response during storage. However, this increase did not appear to be effective in restoring the multilayered structure of corneal epithelium, since the decrease in epithelial cell layers and thickness was still high (~ 10 μm at day 12 compared to 45 μm at day 0). This could be explained by immediate epithelial cell death after proliferation, as the TUNEL assay also revealed an increase in epithelial cell death (especially at peripheral regions) with storage time. Cell death was not evident until 6 days following organ culture storage, indicating a limbal resistance to changes, possibly because of their access to survival factor before this time. The presence of survival factors in the limbal niche has previously been postulated by many researchers (Daniels et al., 2001).

4. The proliferative activity of the corneal epithelium seemed to be rapidly increased with time. The lack of Cx43 expression at day 6 supports functional corneal re-epithelisation at this time point. Matic et al. (1997b) has suggested that the presence of gap junctions (Cx43) would make a cell vulnerable to insults affecting its neighbours and therefore the absence of gap junctions would therefore confer a survival advantage to stem cells at day 6.

5. Petrol et al. (2001) discovered a decrease in the intensity of ZO-1 in cells spreading over a wound (epithelial cell re-epithelisation), and in cells during S-

phase, prophase, metaphase and early G1. The specific loss of ZO-1 intensity could also support epithelial cell re-epithelialisation. Therefore the reduction in ZO-1 distribution demonstrated during organ culture may not only contribute to a loose epithelial barrier, but also be indicative of the high proliferating status of the epithelial cells.

6. By day 12, the superficial layers of the corneal epithelial cells appeared to be restored (most apparent in the peripheral region). TUNEL-labelled cells also showed a decreased apoptotic index at this time point, although, not at apoptotic levels identified prior to six days of organ culture. On the other hand, the specific loss of ZO-1 staining observed at day 6 seemed to be partially recovered by day 12. Therefore the reappearance of superficial cells, together with the positive staining of ZO-1 at this stage, denotes the corneal epithelium attempt to repair the deficient epithelial barrier. Therefore future work to modulate epithelial cell activity during organ culture could be helpful to reduce the discard rate of human donor cornea.

8.5 Importance of such impact on corneal storage for limbal stem cell and corneal transplantation

Despite the numerous studies published in this area, a precise therapeutic approach to modulate epithelial cell function while in the organ culture storage has not been determined. Therefore the results of this study could be used to suggest ways to modulate epithelial cell function and stem cell population in organ culture storage and thus improve the quality of corneas for transplantation, and consequently reduce the discard rate of human donor cornea as well as providing a new source of graft material for limbal stem cell transplantation. However the latter procedure (stem cell transplantation) due to the depletion of the stem cells population during organ culture still is not possible.

- Therefore the deterioration of corneal epithelium and endothelium reported in this study, caused by apoptosis, could be suppressed by manipulating culture medium

with factors such as fibroblast growth factor and vascular endothelial growth factor that can inhibit apoptosis (Seigel et al., 2000).

- Since the activation of caspases occurs in corneal epithelium by extrinsic death receptor-mediated pathway, factors that are able to inhibit that pathway could also reduce apoptosis e.g thymosin- β 4 (Sosne et al., 2004).
- Lack of enough proliferation in corneal epithelium to restore epithelium during storage, found in this investigation could be increased by adding stimulatory factors such as epidermal growth factor EGF, or by blocking the proliferative inhibitory factors, such as TGF β (Nihimura et al., 1998).
- Since differentiation appears to promote epithelial cell apoptosis, adding factors that could inhibit terminal differentiation (e.g inhibitors of heparan sulphate proteoglycan) could also improve the quality of corneal epithelium for transplantation (Wang et al., 1997, 2001; Fannon et al., 2003).
- Keratocyte apoptosis could indirectly contribute to barrier decrease due to the correlations between apoptosis in stroma, epithelium and endothelium (Komouro et al., 1999). Therefore further investigations into on keratocytes would clarify the keratocyte role in epithelial proliferation, apoptosis and stratification of cornea during storage.

8.6 Conclusion

Organ culture preservation of human donor corneas still offers advantages compared to hypothermic storage. Although hypothermic storage is less complicated than organ culture storage, the extended storage time by organ culture facilitates tissue typing, controlling for microbial contamination and extending the epithelial and endothelial storage lifetime for a longer period of time. However, during organ culture storage, a loss of epithelial cell layers results in disruption of the epithelial barrier. Therefore the

epithelial barrier becomes somehow compromised in its ability to prevent the swelling that is likely to contribute to the endothelial cell damage. In addition, epithelial cell loss appeared to be greater than that restored by epithelial cell proliferation. The increase in proliferative index is likely to be an attempt to restore the normal multilayered structure of epithelium, triggered by the large level of cell loss during storage.

This loss of epithelial cells and the lack of complete restoration of the corneal epithelium, due to a homeostatic imbalance demonstrate either the lack of epithelial stem population or the functionally impaired limbus. The huge loss of epithelial cells, especially at the limbus may limit the use of such corneas for corneal stem cell transplantation. However it is likely that, by modulation of the epithelial cell function during storage, a greater supply of high quality donor corneas could be available for penetrating keratoplasty and limbal stem cell transplantation.

8.7 Future work

Our results suggest the need for future investigations focused on improving the longevity and integrity of systems for organ culturing of human corneas. Advances in preserving the structural and functional properties of corneas during organ culture will be of great importance.

1) Deterioration of corneal epithelium and endothelium caused by apoptosis would be suppressed by manipulating culture medium, with insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) which can inhibit apoptosis, and offers the possibility of a higher cell density (Azuara-Blanco et al., 1999; Seigel et al., 2000; Tosi et al., 2005).

2) Lack of sufficient proliferation would be increased by adding stimulatory factors such as epidermal growth factor EGF (Kitazawa et al., 1990; Kawaba et al., 1984; Kandarakis et al., 1984), platelet-derived growth factor (PDGF) (Knorr 1990), cyclins (Hunter et al., 1994; Massague et al., 1995) and blocking the inhibitory factors of $TGF\beta$ (Nihimura et al., 1998), cyclin dependent kinase inhibitors p15 (Polyak et al., 1994) and p27 (Hannon et al., 1994; Zieske 2000).

3) Factors that could inhibit terminal differentiation like inhibitors of epithelial cell migration (e.g. inhibitors of heparan sulphate proteoglycan, heparin, EGF and HGF) could be added to retard this process (Fannon et al., 2003).

APPENDIX I: CHEMICALS AND MATERIALS

Reagent	Company
Acetone	BDH
Amphotericin B	Gibco
APES	Sigma
Betadiene	Seton Healthcare, AAH
Calcium Chloride	BDH
Chloroform	BDH
Chondrotin Sulphate	Sigma
Diamino Benzidine	Sigma
Disodium Hydrogen Orthophosphate	Gibco
Eagle's Minimal Essential Medium	Gibco
EDTA	BDH
Embedding molds	BDH
Embedding rings	BDH
Ethanol	BDH
Eosin	GIBCO
Fetal Calf Serum	GIBCO
Formaldehyde	BDH
Formalin	BDH
Glutamine	Gibco
Glycerol	Sigma
Haematoxylin (Mayers and Harris)	BDH
Hoechst H33345	Sigma
Hydrochloride acid	BDH
Hydromount	BDH
Methanol	BDH
Microtome disposable blade	Lamb
Paraffin wax	Lamb
Penicillin /streptomycin	Gibco
Potassium Chloride	BDH

Potassium	BDH
Orthophosphate	
Scalpel blades No 22/23	Fisher
Sodium Chloride	BDH
Streptomycin	Sigma
Super Frost Plus slides	BDH
Surgical blades	Fisher
Tissue-Tek OCT compound	Lamb
Triton X-100	Sigma
Xylene	BDH

APPENDIX II: BUFFERS AND SOLUTIONS

Phosphate buffered saline

(PBS) (1x)

40gr Sodium Chloride

1gr Potassium Chloride

5.75gr Disodium hydrogen orthophosphate

1gr Potassium dihydrogen orthophosphate

5000ml H₂O

Phosphate buffer

5.75g Na₂HPO₄

1.48 NaH₂PO₄·2H₂O

In 1 litre of distilled water

3% betadine solution

15ml of 10% Betadine solution

In 500ml of ddH₂O

Saline

0.9% NaCl

0.9 gr of NaCl in 100 ml ddH₂O

APPENDIX-III: HISTOLOGICAL SOLUTIONS

10% Neutral Buffered Formaldehyde

60ml of Na₂HP04 (1.5M)

40Ml of KH₂P04 (1.5M)

900ml of ddH₂0

Take 900ml of above and add

100ml of formaldehyde

9gr sodium chloride

PH to 6.8

Proteinase-K solution (mg/ml)

10mg of proteinase-k in 1 ml ddH₂0

Methacarn Fixative

60%methanol

30%chloroform

10%%Acetic acid

0.2% Triton-X-100

2ml of Triton-X-100 in 100ml of PBS

0.2% Alizarin red

0.2% Alizarin red in 0.9% NaCl

adjust Ph to 7.2

10. References:

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