UNIVERSITY OF CAPE TOWN

Eviscerated Corneas as Tissue Source for Ex Vivo Expansion of Limbal Epithelial Cells on Platelet-Rich Plasma Gels

MASTER OF MEDICINE (MMED) IN OPHTHALMOLOGY

University of Cape Town

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DECLARATION PAGE

I, Leonard Goussard Heydenrych, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

This work has not been reported or published prior to registration for the abovementioned degree.

2 August 2016

ABSTRACT

Purpose/Aim of the study: To assess if corneal epithelium can be cultured ex-vivo from corneas eviscerated due to irretrievable trauma, according to a cell culture method which made use of autologous platelet-rich plasma (A-PRP) as culture substrate. To compare corneal epithelium cultured ex vivo from corneas eviscerated following trauma using A-PRP combined with DMEM (Dulbecco's modified Eagles medium), versus DMEM alone.

Materials and Methods: This was a laboratory case controlled study of human corneal cells cultured in a mixture of A-PRP and DMEM, versus DMEM alone from 6 eviscerated corneas. A hundred explants were created of which fifty explants were plated on A-PRP-gel construct combined with DMEM and fifty controls were placed in serum free DMEM alone. Donor patients received systemic antibiotics prior to evisceration.

Results: Confluent epithelium in mono-layers could be cultured when donor limbal biopsies were placed in a mixture of A-PRP culture medium and DMEM. No growth were observed when corneas were placed in serum-free DMEM medium only ($p<0.05$). No bacterial infection was observed in cultures.

Conclusions: This study demonstrated that autologous platelet rich plasma is a viable and effective alternative to bovine serum for the ex-vivo expansion of limbal epithelial cells. It also shows that eviscerated corneas are a viable source of donor tissue for this purpose in South Africa where access to tissue banks is limited.

KEY WORDS: Culture, Epithelium, Eviscerated cornea, Platelet-Rich Plasma, No animal products

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Prof. Colleen Aldous served as third co-author in the eventual publication in *Current Eye Research* and assisted in the layout and editing of the manuscript.

In this publication, I served as first author, du Toit D.F. as second author and Aldous C.M. as third author.

CURRICULUM VITAE OF RESEARCH SUPERVISOR: DONALD DU TOIT, FRCS EMERITUS, 2016.

Professor D.F. Du Toit: MBChB (Stell), FCS (SA), FRCS (Ed), D.Phil (Oxon), PhD (Stell), FICA (USA), CERT BUS Admin (formerly based at University of Stellenbosch Medical School)

Collaborative human corneal research between University of Cape Town and Stellenbosch University, within an ethically approved human research protocol. D.F. du Toit provided an approved super-specialist laboratory human tissue-culture infrastructure as well as expertise and guidance at the University of Stellenbosch Medical School Campus. He also provided direct technological supervision regarding tissue-culture methodology, quantification, morphology, cell-viability and cell-culture statistics.

Past Academic Head and Chairman: Department of Anatomy and Histology, Biomedical Sciences, University of Stellenbosch Medical School, formerly Associate Professor in Surgery, University of Stellenbosch and Tygerberg Academical Hospital, South Africa.

Biomedically-rated scientist and senior specialist scientist at the Medical Research Council (MRC)/Diabetes Discovery Platform Unit, Head Office, Parow, Cape Town 2011. Past advisor to the MRC President's Office.

Post-graduate researcher at the University of Oxford and honorary consultant surgeon, Nuffield Department of Surgery, John Radcliffe Hospital, University of Oxford, Headington, OX39DU, United Kingdom, 1980. Qualified FRCS and DPHIL (Oxon).

Performed first pancreas transplant in Southern hemisphere, 1988.

Wide experience in academic medicine, teaching, management, curriculum reform and specialist hospital patient care. Running large divisions of teaching and research, including research programmed directorship and MRC funding strategies. Involved in under -and postgraduate surgical and basic science education and research publications. Past examiner in surgery and anatomy at University of Stellenbosch, University of Cape Town and Colleges of Medicine of South Africa.

Former Head of Biotechnology Tissue Culture Laboratory, Department of Anatomy and Histology, Stellenbosch University Medical School. Extensive hands-on and theoretical experience within special university ethical protocols, regarding laboratory tissue-culture (skin, keratinocytes, myocytes, cartilage).

Promoter of ten postgraduate doctorate research-based degrees, two MSc, four MMed and two BSc (Hons) degrees at the University of Stellenbosch. External examiner for doctoral PhD candidates, as well as for postgraduate clinical specialist examinations in anatomy.

More than 140 publications in peer reviewed journals.

Recent publications:

- Heydenrych LG, du Toit DF, Aldous CM, Eviscerated Corneas as Tissue Source for Ex Vivo Expansion of Limbal Epithelial Cells on Platelet-Rich Plasma Gels. *Curr Eye Res.* 2016 May 23:1-5. [Epub ahead of print]
- Don F. du Toit, Benjamin Longo-Mbenza, , Benedict J. Page, , Venant Tchokonte-Nana. Efficiency of co-expression of transcription factors Pdx1, Ngn3, NeuroD and Pax6 with insulin: A statistical approach. *International Journal of Diabetes Mellitus,* 2015-05-01, Volume 3, Issue 1, Pages 57-64

Referee: Professor Sir Peter Morris, President Royal College of Surgeons, England, and former Head: Nuffield Department of Surgery. University of Oxford, 1982.

LIST OF FIGURES

Figure 1. Epithelium (blue arrow) is seen expanding from the “hill” or forming face (red arrow). 3D Proliferation is demonstrated here where the “hill” is out of focus due to proliferation in the vertical axis, whereas the “valley” is in focus, representing epithelium in a horizontal layer.

Figure 2. The corneal explant (blue arrow) is surrounded by a lucid area of epithelium (red arrow) in PRP.

Figure 3. Epithelium (red arrow) is seen proliferating in a monolayer from the limbus (blue arrow).

Figure 4. Hematoxylin and eosin (H&E) stain demonstrating squamous epithelial cells at the proliferating edge (green arrow). Notice the dense configuration of epithelial cell nuclei at the proliferating edge (blue arrow).

ABBREVIATIONS

Abbreviations appear chronologically as they are encountered in the text. Abbreviations are explained within the text as well.

LESCs: **limbal epithelial stem cells**

LSCD: **limbal stem cell deficiency**

KLAL: **keratolimbal lamellar allograft**

CLAU: **conjunctival-limbal autografts**

lr-CLAL: **living-related conjunctival-limbal allografts**

PRP: **platelet rich plasma**

A-PRP: **autologous platelet-rich plasma**

SLET: **simple limbal epithelial transplantation**

DMEM: **Dulbecco's modified Eagles medium**

LECs: **limbal epithelial cells**

H & E: **Haematoxylin and Eosin**

COASTL: **The Clinical Outcome Assessment in Surgical Trials of Limbal Stem cell deficiency tool**

HIV: **human immunodeficiency virus**

MIV: menslike immuungebrek virus

SJS: Stevens–Johnson Syndrome

ARV: anti-retroviral medication

THT: tapered head tube

CO_2: carbon dioxide

HEPA: high-efficiency particulate air

TGF-β: transforming growth factor beta

b-FGF: basic fibroblast growth factor

IGF-1: insulin-like growth factor 1

PDGF: platelet-derived growth factor

VEGF: vascular endothelial growth factor

EGF: epidermal growth factor

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Eviscerated Corneas as Tissue Source for Ex Vivo Expansion of Limbal Epithelial Cells on Platelet-Rich Plasma Gels

Introduction

Conditions such as Stevens–Johnson syndrome, ocular pemphigoid, and chemical and thermal injuries may destroy the limbal stem cells of the cornea, and lead to scarring of the cornea.[1] When limbal epithelial stem cells (LESCs) are destroyed by trauma or disease, conjunctiva and peripheral blood vessels migrate on to the cornea.[2] This may lead to vision loss and discomfort.[2] Normal corneal epithelium needs to be restored in order for a normal refractive surface to be established. Corneal repair could be achieved through the ex vivo culture of corneal epithelium.[3,4]

The use of animal products in media does not accommodate for all religious and cultural beliefs in the multicultural society of South Africa, and donor corneas are too scarce for culturing to be efficiently rolled out. In order for us to be able to give our population state of the art stem cell transplants to save their sight, we need to design a resource-based protocol for culturing stem cells ex-vivo, which excludes the use of animal products, and find viable sources of stem cells.

Relevant Anatomy and Physiology

Limbal stem cells possess the inherent ability to differentiate into epithelial cells, which cover and thus maintain the integrity of the cornea. Stem cells are situated in the limbus and in the limbal palisades.[2] Shortt states that there is still uncertainty regarding the exact distribution of stem cells in the limbus.[2] The corneal epithelium is constantly reproduced by the limbal stem cells of the cornea. Deficiency of limbal stem cells prevents the formation of new epithelial cells and thus epithelial defects and conjunctivalization of the cornea may develop. LESCs are situated within the basal cell layer of the limbal epithelium and have characteristics of a stem cell phenotype.[2] To date no specific cell markers exist to define corneal limbal stem cells.[2]

The corneal epithelium is responsible for maintaining a clear cornea and regular refractive surface.[2] As seen in the skin, desquamated corneal epithelial cells are replenished by LESCs located in the limbus.[2] Shortt et al. state that limbal stem cell deficiency (LSCD) is associated with the loss of limbal palisades suggesting that LESCs reside within the limbal palisades.[2] Currently there is uncertainty regarding the precise distribution of stem cells within the limbus.[2] Chen et al. demonstrated that the basal cells of the superior limbus have characteristics of a stem cell phenotype.[2,5] Dua on the other hand, described limbal epithelial crypts, which is a niche structure where the LESCs are located.[6] This study reported an average of six limbal crypts per cornea.[6]

Diagnosing limbal stem cell deficiency (LSCD)

Shortt and colleagues mention that LSCD is characterized by the ingrowth of conjunctiva onto the cornea with a resultant epithelial haze, sub-epithelial vascularization, persistent or recurrent epithelial defects and loss of the limbal palisades of Vogt.[2] LSCD may also be classified as total or partial.[2] Partial LSCD is characterized by localized loss of LESC with the ingrowth of conjunctiva in these areas of stem cell loss.[2]

Management of LSCD

Conservative management of LSCD includes lubrication, bandage contact lenses, and autologous serum eye drops.[2] Shortt mentions that only the use of autologous serum is supported by evidence in the literature.[2,7]

Surgical means to manage LSCD include debridement of migrating conjunctival epithelium and the transplantation of amniotic membrane.[2,8,9] Shortt et al. mention that a variety of methods may be used to transplant limbal stem cells, which include keratolimbal lamellar allograft (KLAL), oversized or eccentric penetrating keratoplasties, conjunctival-limbal autografts (CLAU) and living-related conjunctival-limbal allografts (lr-CLAL).[2,10–14] Shortt states that the removal of limbus is not without risk, and that partial stem cell deficiency may develop following limbal biopsy for CLAU in a previously normal eye.[2,15]

Summary and interpretation of literature, and its implications for the research

Historically corneal epithelium has been cultured ex-vivo with the use of lethally irradiated 3T3-J2 feeder cells and a fibrin-substrate combined with fetal-calf serum to facilitate expansion of limbal corneal stem cells and holoclones.[3] Rama et al. state that this culture method has been used worldwide since the 1980s.[3] The first pivotal article on the expansion of corneal epithelium was published by Pellegrini et al. in 1997.[16] Following the ground-breaking research of Graziella Pellegrini, similar methods have been used to expand corneal epithelium.[4,17] Most of these methods all use exogenous products in culture media, such as lethally irradiated feeder fibroblasts and fetal-calf serum.[4,16,17] In this research we are aiming to establish the growth of corneal epithelium without the use of any animal or exogenous products. This will potentially eliminate the transmission risk of blood borne diseases.

Other researchers have succeeded in culturing corneal epithelium without the use of murine 3T3 feeder cells.[18–21] Cell sheet carriers used included denuded amniotic membrane, Matrigel, Myogel and stromal extract,[18] as well as soft contact lenses.[20,21] Di Girolamo also succeeded in culturing epithelium with the use of autologous serum.[20,21] In our research project epithelial cells will be cultured on platelet rich plasma (PRP), using the REGEN PRP® system.[22,23] The PRP will be created from the patient's own blood and is therefore also called autologous platelet-rich plasma or A-PRP.[23] We plan to 'activate' the plasma with calcium since this enhances cell proliferation and -migration.[23]

The transplant of corneal epithelium onto stem cell deficient corneas has been attempted with success by previous researchers, e.g. Tsubota et al. (1999), Koizumi et al. (2001) and Shimazaki

et al. (2002).[1,4,24] Nakamura et al. demonstrated that it is possible to produce sufficiently stratified, well differentiated, autologous cultivated corneal limbal epithelium on amniotic membrane from a minimal biopsy of the donor eye and to transplant it onto the injured eye.[25] At 19 months post-transplantation the corneas were stable with no epithelial defects.[25]

Koizumi also showed that cultivated corneal epithelium had four to five layers of stratification and was well differentiated.[4] Corneas which received stem cell transplants provided evidence of the persistence of donor epithelial cells up to 3.5 years after limbal allograft transplantation.[26]

A new method of corneal limbal epithelial transplantation is termed SLET (simple limbal epithelial transplantation) and involves the creation of an in vivo culture environment on the patient's cornea.[27,28] Our culture method, using A-PRP, can also be applied in SLET. After sufficient epithelium has been cultured this can then be transplanted onto amniotic membrane 'epithelial side up' and sandwiched between an amniotic membrane covering it 'epithelial side down'. The newly cultured epithelium should then eventually cover the whole amniotic membrane and cornea. Eventually only epithelium is left to cover the corneal stroma, as the amniotic membrane degrades.

Substrates other than amniotic membrane may also be used as carriers. Francis et al. compared different substrates to grow corneal epithelial cells on.[18] Substrates included denuded amniotic membrane, Matrigel, Myogel and stromal extract.[18] The most rapid growth was observed on Myogel and denuded amniotic membrane and these two cell carriers also provided the most reliable substrata for achieving confluent epithelial growth.[18] Myogel may be obtained from an autologous muscle biopsy.[18] Nakamura et al. and Francis et al. used autologous serum as

substrate to culture corneal epithelium on to amniotic membrane and it is this method that we will follow in our protocol, but without the use of amniotic membrane or any other exogenous products.[18,25] This makes our proposed culture method novel.

Corneal stem cells were cultured with different mixtures of culture media. Utheim et al. used mixtures which contained dispase, Dulbecco's modified Eagles medium (DMEM), sodium bicarbonate, Ham's F12 solution, fetal bovine serum, dimethyl sulphoxide, human epidermal growth factor, insulin, transferrin, selenium, hydrocortisone, cholera toxin, gentamicin, amphotericin B and vancomycin.[29] Pellegrini et al. used lethally irradiated 3T3-J2 cells to stimulate epithelial growth.[16] The method that we will use differs from previous research in that we will not use murine irradiated feeder fibroblasts or any other exogenous or animal products in our cultures. We will solely rely on growth factors released from plasma to stimulate epithelial growth.

A medline search was performed to review the literature for any malignancy incurred following corneal epithelial stem cell transplantation. None was found.

Strategy

Corneal stem cells are usually cultured on a denuded amniotic membrane carrier and then attached to epithelial defects.[4] Corneal stem cells were obtained from cadaver corneas as allografts, as living related conjunctival limbal allografts or they were obtained as autografts from the same patient via corneal button biopsies on the limbal area from the contralateral eye.[30,31] In contrast to the normal methods used for harvesting corneal limbal stem cells, we

aim to harvest corneas from eyes which obtained irretrievable loss of vision due to trauma. In our search of the literature no studies could be found which investigated the culturing of corneal epithelium from eviscerated corneas. What makes our study novel is the fact that corneal cells will be grown from eviscerated corneas obtained due to trauma and not from cadaver corneas or from limbal biopsies as in previous research.[18,21]

Ex vivo cultured limbal epithelial cells

The ex vivo expansion of corneal epithelium is based on the pioneering research of epithelial expansion of skin by Rheinwald and Green.[2,32–34] Limbal cells are usually harvested from the contralateral healthy eye in the form of a small 2×2mm^2 biopsy.[2] If the patient has bilateral total LSCD, then Shortt et al. advises that allogeneic limbal epithelium be harvested from living related donors or from fresh cadaveric tissue.[2] The limbal biopsy contains a population of LESCs, which are cultured and expanded in a laboratory to produce a sheet of LECs (limbal epithelial cells) that is suitable for transplantation onto the diseased cornea following removal of the abnormal conjunctivalized epithelium.[2]

Shortt et al. state that the advantage of the ex vivo expansion of epithelium is twofold.[2] Firstly the required limbal biopsy is smaller than biopsies used in CLAU and lr-CLAL and this reduces the risk of stem cell failure in the donor eye.[2] The smaller sized biopsy also provides the option of taking a further biopsy if needed.[2] Secondly, due to the absence of antigen-presenting Langerhan's cells in ex vivo cultured LEC grafts, there is a theoretically reduced risk of allograft rejection.[2]

Researchers have also used oral mucosal epithelium as a source of stem cells for the ex vivo expansion of epithelium.[2] Both corneal epithelium and mucosal epithelium express keratin 3 and mucosal epithelium is thought to be at a lower stage of epidermal differentiation than corneal epithelium.[2] Since the mucosa from the same patient is autologous there is no risk of immune mediated rejection.[2] For the purpose of this research we will focus on the expansion of epithelium from corneal tissue.

Objectives

- To assess if corneal epithelium can be cultured ex-vivo, in a tissue culture laboratory from corneas eviscerated due to irretrievable trauma according to a novel cell culture method, with the exclusion of animal products and without the use of 3T3-feeder fibroblasts.
- To establish if A-PRP is essential for cultivating epithelium.
- The results of this study will create a pool of standardized data on culturing corneal epithelial cells from several centers specializing in the treatment of severe ocular surface disease.[35]

Quality criteria

The results will be presented to a large degree with statistical analysis of cell counts evolving from each explant within A-PRP culture-medium. Cells will be confirmed as epithelium by means of Haematoxylin and Eosin (H & E) staining and Masson-Trichrome staining and by means of K3 staining and K12 staining.[4,17,25,36,37] Inverted phase contrast microscopic digital photographs at magnifications of 100 times and 200 times will be taken at weekly intervals to monitor the growth of cultured mono-layers.[37] Success will be classified as the generation of at least one complete mono-layer of cells ex-vivo on a substrate. Cell proliferation in the cell sheets will be photographically counted using a micrometer and eye-piece grid, which will be focused on explants.

Clinical Application

Corneal stem cells may also be harvested from damaged or traumatized corneas, since only a small portion of the limbus is required to culture new cells. Due to the very high incidence of irreparably damaged corneas following trauma, we currently have a vast source of limbal cells in South Africa.[38] Corneas which have to be eviscerated due to trauma, may now also be used as a source of limbal stem cells to culture normal corneal epithelium from. We would like to see if we can successfully cultivate limbal stem cells from corneas obtained from irreparably damaged eyes that are being eviscerated as part of standard care.

The benefit of ex vivo transplantation of limbal stem cells

Limbal stem cell transplantation may reduce inpatient stay in hospital wards by expediting the healing process of persistent epithelial defects. Inevitably this will reduce the burden on our over full wards and will also lead to considerable financial savings for the state sector, since inpatient stay will be shorter.

Traditionally, patients with persistent epithelial defects require a much longer stay in hospital due to the chronicity of these defects, compared to other ophthalmological conditions. These defects are usually treated with lubricants, punctal occlusion, pressure patching, debridement of the epithelial defect and tarsorrhaphy of the eyelids.[39] A temporary ptosis by means of injecting Botulinum toxin into the upper eyelid may also be considered. These defects may also be covered with a bandage soft contact lens which aids the regeneration process by protecting the advancing epithelium from being sloughed off.[39] Amniotic membrane is useful in resistant cases since the growth factors released may stimulate epithelial growth and reduce inflammation.[39] Topical autologous serum drops, created from the patient's own blood, may also be used to promote epithelialization through the release of essential growth factors.[39] Scleral contact lenses may promote enhanced oxygenation and promote epithelialization.[39] Limbal stem cell transplantation or ex vivo cultivated epithelium may also be considered if persistent epithelial defects develop due to limbal stem cell deficiency.[39]

Inclusion criteria

Patients included in this study must have obtained irreparable eye trauma and may only partake in this study if they sign informed consent for evisceration. Clear guidelines for the indication of evisceration will be followed, which includes (i) no perception of light, (ii) gross damage to an eye due to trauma not salvageable with suturing, (iii) total afferent pupillary defect and (iv) auto-eviscerated eye following trauma.

Exclusion criteria

Patients who are not willing to partake and who have not signed informed consent.

Patients not exposed to eye trauma.

Patients still experiencing perception of light in their traumatized eye.

Identification of gaps or needs for further research

Shigeto Shimmura and Kazuo Tsubota mention an urgent need for more research regarding the transplant of corneal stem cells in the American Journal of Ophthalmology.[35] They state that 'techniques for cultivating epithelial sheets vary greatly from one report to another, all of which seem to show clinically successful patients.' [35] They also state that the overall number of patients reported with this surgical technique is relatively small, and that further reports are anticipated to determine the advantages of cultivated sheets over conventional limbal

transplants.[35] We will be using a standardized method for cultivating corneal epithelial cells as developed by our laboratory team and obtained from the relevant literature. The Clinical Outcome Assessment in Surgical Trials of Limbal Stem cell deficiency [COASTL] tool, as developed by Shortt and coworkers, could be used to assess the efficacy of our novel method on recipient corneas.[40] This method grades limbal stem cell deficiency and the response to treatment with ex vivo cultured limbal epithelial stem cells on a macroscopic and numerical basis.[40] Corneal haze, epithelial irregularity, epithelial defects and vascularization are graded using this method.[40]

Shimmura and Tsubota mention that the next several years may see a paradigm shift in the way LSCD patients are treated.[35] They state that cultivated sheets may be used as temporary grafts for acute phase LSCD, or perhaps for optical surgery to be repeated every several years.[35] They also mention that epithelial sheets may be engineered from ectopic epithelium (as is planned in our research project) or from stem cell banks.[35] They concluded with the following statement, 'Because randomized controlled studies are difficult under these circumstances, we completely agree with the concluding remarks by Cauchi and associates suggesting the need for a register to pool standardized data from several centers specializing in the treatment of severe ocular surface disease.' [35]

This emphasizes the importance of performing corneal stem cell research at Groote Schuur Hospital, and reporting on our experience. These results will then contribute towards a register to pool standardized data globally.

This study will help to provide further details for optimal culturing conditions in our setting and to refine our culturing technique. Eventually we will be able to transplant epithelium on to limbal deficient corneas with the necessary ethical approval. The long term goal of this research would be to construct a cornea consisting of ex-vivo cultured epithelium, endothelium and stroma. Due to the severe shortage of corneal tissue in South Africa as well as globally, this could help to reduce the demand for corneal tissue from cornea banks globally. Ultimately ex-vivo cultured corneal tissue will reduce waiting times for corneal transplants, particularly in Africa. The ultimate goal of this research will be to improve patient care in South Africa for patients waiting for corneal grafts.

Addendum

An observation of our research was that bacterial growth was absent from cultures, even though topical antibiotics were not applied in cultures. The researchers assume that this is due to the use of prophylactic systemic antibiotics in donor patients. We would like to postulate that researchers could consider this method, should they wish not to apply topical antibiotics in cultures.

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CHAPTER 2: PUBLICATION-READY MANUSCRIPT

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Eviscerated Corneas as Tissue Source for Ex Vivo Expansion of Limbal Epithelial Cells on Platelet-Rich Plasma Gels

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ABSTRACT
Purpose/Aim of the study: To assess if corneal epithelium can be cultured ex vivo from corneas eviscerated due to irretrievable trauma, according to a cell culture method that made use of autologous platelet-rich plasma (A-PRP) as culture substrate. To compare corneal epithelium cultured ex vivo from corneas eviscerated following trauma using A-PRP combined with Dulbecco's modified Eagles medium (DMEM), versus DMEM alone.
Materials and methods: This was a laboratory case-controlled study of human corneal cells cultured in a mixture of A-PRP and DMEM, versus DMEM alone from six eviscerated corneas. A 100 explants were created, of which 50 explants were plated on A-PRP-gel construct combined with DMEM and 50 controls were placed in serum-free DMEM alone. Donor patients received systemic antibiotics prior to evisceration.
Results: Confluent epithelium in monolayers could be cultured when donor limbal biopsies were placed in a mixture of A-PRP culture medium and DMEM. No growth was observed when corneas were placed in serum-free DMEM medium only ($p < 0.05$). No bacterial infection was observed in cultures.
Conclusions: This study demonstrated that A-PRP is a viable and effective alternative to bovine serum for the ex vivo expansion of limbal epithelial cells. It also shows that eviscerated corneas are a viable source of donor tissue for this purpose in South Africa where access to tissue banks is limited.

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Culture; epithelium; eviscerated cornea; platelet-rich plasma; no animal products

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Introduction

Donor corneas are too scarce for the culturing of corneal epithelium to be efficiently rolled out in South Africa. The authors therefore utilized fresh corneal tissue in patients who have obtained irretrievable damage to their corneas and required eviscerations, since this was more readily available, and then developed a resource-based protocol devoid of animal products, to culture corneal epithelium from. These eviscerated corneas would normally be discarded.

Corneal trauma is common in South Africa. Du Toit et al. state that there were 249 open globe injuries over a 2-year period at Groote Schuur Hospital, of which 24.5% required eviscerations.[1] Around 64.9% of these patients with open globe injuries were younger than 35 years of age.[1]

The management of limbal stem cell deficiencies of the cornea remains a big challenge in South Africa, due to the prevalence of the human immunodeficiency virus (HIV), which is 11%.[2] HIV-positive patients may present with Stevens–Johnson Syndrome (SJS) due to anti-retroviral medication (ARV) and patients with SJS have a 20% risk of serious eye manifestations.[3,4] In our setting SJS associated with anti-retroviral therapy is the leading cause of limbal stem cell deficiency.

It has become accepted practice in the developed world to transplant cultured epithelial limbal stem cells onto corneas with stem cell deficiencies such as are seen in chemical burn wounds, SJS, and in ocular cicatricial pemphigoid, where visual acuity can improve up to two or more lines post transplantation.[5,6,7,8]

In our study we investigated (i) the use of a culture medium that contained autologous platelet-rich plasma (A-PRP) as a culture substrate for corneal epithelial cell generation, obviating the need for 3T3-feeder fibroblasts and (ii) the viability of using eviscerated corneas, which are unfit for transplant, to culture epithelium from limbal explants.

Materials and methods

Ethical considerations

The study was approved by the Groote Schuur Hospital Ethics committee and adhered to the principles of the Declaration of Helsinki of 1964. Patients could withdraw from this study at any stage.

Study subjects

The participants in this study were six males with a median age of 28 (range 21–65). Two cases were HIV positive.

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Patients received intravenous cefazolin and oral ciprofloxacin preoperatively on admission.

Clear guidelines for the indication of evisceration were followed, which included (i) no perception of light, (ii) gross damage to an eye due to trauma not salvageable with suturing, (iii) total afferent pupillary defect, and (iv) auto-eviscerated eye following trauma.

Corneal harvesting

Limbal corneal stem cells were obtained from six corneas harvested from eviscerated eyes at the Division of Ophthalmology of Groote Schuur Hospital. Patients received blood tests prior to harvesting, which included Hepatitis B and C, HIV, and Syphilis serology.

Corneas were excised in theatre, during evisceration, with at least a 2 mm rim of sclera and stored in Optisol solution. After harvesting, corneas were kept at room temperature and delivered to the Department of Anatomy of the University of Stellenbosch. Corneas were accompanied by 8 ml of the patient's venous blood in REGEN PRP® blood-collection tubes.

Laboratory processing and limbal epithelial cell culture

Tissue cultures were set up within 12 hours of arrival at the laboratory. Venous blood was collected from the same corneal donor; 8 ml of venous blood was injected into a REGEN Lab™ sterile THT glass tube containing an anticoagulant (REGEN Lab™, Switzerland). Venous tubes were centrifuged at 4000 revolutions per minute (rpm) and plasma was separated from red blood cells above the buffy layer. Platelet-rich plasma (PRP) was aspirated, activated with calcium chloride, and 2ml aliquots used to coat the base of each sterile petri-dish, the procedure being conducted aseptically in a vertical-type laminar flow hood (Labotec™, Cape Town). Gel formation occurred within a few minutes after PRP activation with calcium.

Corneas were carefully orientated on a glass tablet and diced into explants containing cornea and limbus, devoid of donor conjunctiva, and measuring about 2 × 2 mm. Careful clearance of conjunctiva and excess sclera was important to avoid competitive overgrowth with conjunctival epithelium and fibroblasts.[9] Six-well petri dishes were used and each explant was suspended in PRP gel and matched with an explant in serum-free Dulbecco's modified Eagles medium (DMEM) in an adjacent well (control).

Explants were placed in PRP with the epithelial surfaces facing upward. One drop of DMEM, not supplemented with fetal-calf serum, was placed on each explant with a pasteur pipette within the laminar flow hood. No antibiotics were used in cultures. Control explants without PRP base were placed epithelium side up on the inside base of petri dishes and each explant was covered with one drop of serum-free DMEM. The two sets of petri dishes, PRP-coated and PRP-free plates, were placed in a sterile Nuaire™, carbon dioxide (CO_2), HEPA-incubator™. Epithelial cell proliferation was conducted in an atmosphere of 95% oxygen and 5% CO_2 throughout the study at 37°C. DMEM was changed three times a week in PRP and PRP-free control cultures.

Cultures were inspected three times weekly under an Olympus™ GX41 inverted phase-contrast microscope. Digital images of monolayers and cell clusters were captured with a Sony™ combination Carl-Zeiss™ camera at 7.2 megapixels. Inverted microscopic digital photographs at magnifications of 100 times and 200 times were taken at weekly intervals to monitor the growth of cultured monolayers. Cell dynamics and cell proliferation on the forming and maturing face of the monolayers were photographically recorded and counted using a micrometer and eye-piece grid, which were focused on explants (see Figure 1). Cell locomotion (speed of migration) was reviewed at 6 weeks and assessed daily by an inverted transmission microscope.

Figure 1. Epithelium (blue arrow) is seen expanding from the "hill" or forming face (red arrow). 3D Proliferation is demonstrated here where the "hill" is out of focus due to proliferation in the vertical axis, whereas the "valley" is in focus, representing epithelium in a horizontal layer.

For the purpose of this study we confirmed the characteristics of epithelium by means of its characteristic three-dimensional morphological in situ appearance on high-resolution inverted phase-contrast microscopy as did Shimazaki et al.[8] Epithelial cell counts were determined on a hemocytometer cell-counting chamber (Neubauer™) and viable cell staining was achieved applying 0.4% trypan blue solution and viewing cells at 100 times magnification (Zeiss™). Enhancement was selectively applied with the use of a Zeiss Image Analyser™ with specification written macroprogram. Nonparametric, descriptive statistics were selected (Mann-Whitney U test) to compare the two types of tissue-culture parameters evaluated, those in PRP and those without PRP. Results were considered statistically significant if $p < 0.05$ for that parameter.

Immunofluorescent staining was observed through a dark-field inverted microscope. K3 and K12 staining were performed with monoclonal antibodies, obtained from Gen Way Biotech Inc. A standard technique was used as recommended by the manufacturers. Histological analysis of the epithelium was also performed.

Results

At day 21 corneal explants were surrounded by a clear lucid area in the PRP substrate, which represented an epithelial cell

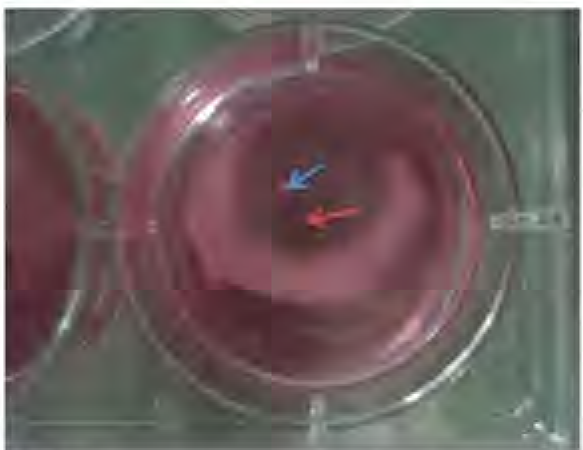

Figure 2. The corneal explant (blue arrow) is surrounded by a lucid area of epithelium (red arrow) in PRP.

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proliferation zone (see Figure 2). Epithelium was confirmed by means of phase contrast microscopy, histological studies, and immunostaining. Epithelial proliferation was only noticed in dishes containing PRP. Success was classified as the generation of at least one complete monolayer of cells ex vivo on a substrate. Growth disc sizes of 9 × 9 mm, 9 × 10 mm, and 9 × 12 mm could be cultured in the platelet-rich gel.

Primary cultures were not established in dishes consisting of DMEM alone ($p < 0.05$). Visible, proliferating cells were confirmed by inverted microscopy and trypan-blue staining on day 4 in PRP-based culture in all six wells. On day 14 epithelial cells formed a confluent monolayer of cells, similar to that reported by Shimazaki.[8,9] Primary cultures consisting of confluent 15 mm-diameter multilayered epithelium were established in 70–80% of PRP cultures by day 21. Cultures were devoid of conjunctival epithelium and more than 80% of cells were viable with trypan-blue test staining. Occasional fibroblasts were morphologically identifiable.

Mid-corneal epithelium proliferated at 0.47 mm/day and limbal-based epithelium at 0.59 mm/day. Growth at the limbus was faster than elsewhere in the cornea ($p < 0.05$) and slowest at the center of the cornea. When present, corneal fibroblasts communicated through strands with each other.

Phase contrast microscopy

Phase contrast microscopy revealed small round cells of two or more layers as described by Shimazaki et al., which were flat with no papillary structures.[8,10] These cells were squamous and cuboidal with prominent nuclei and clear cell borders and had the appearance of epithelial cells (see Figure 3). On low magnification, epithelium was seen proliferating in an "anticlockwise nebula" around the explant.

Histology

Donor cultured corneal monolayers were confirmed as epithelium after being stained with hematoxylin and eosin (H&E) at 200x magnification as performed by several other researchers[6,8] (see Figure 4). The epithelium was confluent, squamous, regular hexagonal cells of cuboidal appearance, and cell borders were clearly demarcated.[6,11,12]

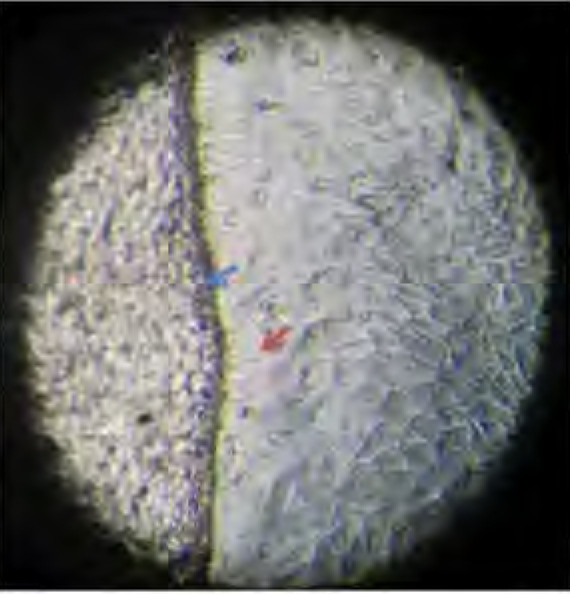

Figure 3. Epithelium (red arrow) is seen proliferating in a monolayer from the limbus (blue arrow).

Immunostaining

Cells were confirmed as epithelium by means of K3 and K12 staining.[5,13] Keratin expression was confirmed by immune staining of cultured corneal epithelial cells with cytokeratin 3 and cytokeratin 12 monoclonal antibodies (GWB-4E84C0, Gen Way Biotech Inc., USA).

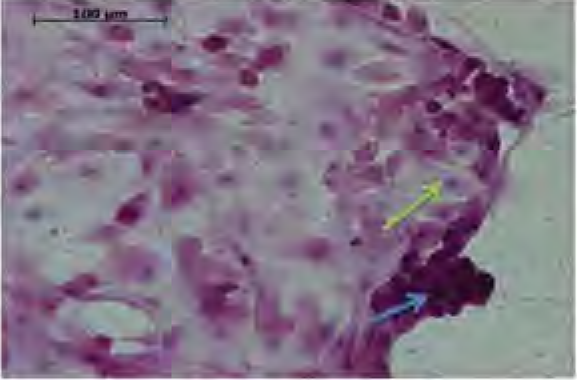

Figure 4. Hematoxylin and eosin (H&E) stain demonstrating squamous epithelial cells at the proliferating edge (green arrow). Notice the dense configuration of epithelial cell nuclei at the proliferating edge (blue arrow).

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Discussion

The key findings of this study are that epithelium could be cultured from eviscerated corneal tissue and that epithelial growth could only be initiated from corneas placed in autologous PRP.

Our culture method differs from conventional methods used by other researchers such as Pellegrini et al., Rama et al., and Koizumi et al., since we did not use any animal products, such as fetal-calf serum, mouse-derived irradiated fibroblasts, or amniotic membrane in our cultures.[5,14,15] Traditional methods used irradiated fibroblasts, which surrounded the explants, to initiate epithelial growth by releasing growth factors.[7] This method is currently still used worldwide since the 1980s and no adverse effects have been reported.[14] Media containing animal products unfortunately have the risk of transmitting zoonotic infections.[6]

Although cultures by Shortt et al. and Shimazaki et al. did not contain any irradiated 3T3 feeder fibroblasts, Shortt et al. used 10% irradiated fetal bovine serum, and Shimazaki et al. used denuded amniotic membrane.[8,11] Di Girolamo et al. moved away from the traditional culturing method as set out by Pellegrini et al. and only used autologous serum as culture medium, but combined with topical antibiotics, to culture epithelium on to contact lenses.[15,16] Researchers usually add antimicrobials containing penicillin, streptomycin, and amphotericin B.[5,8,11] We did not use any antibiotics in our cultures, and only used PRP as the culture medium. Our patients did receive systemic antibiotics before harvesting.

Shortt et al. mention that corneal epithelium can be cultured using an "explant culture system" or a "suspension culture system".[17] We used the explant system by placing the limbal biopsy (epithelium and limbus) directly in the culture medium consisting of autologous, donor-specific A-PRP gel. The advantage of this method is that limbal epithelial stem cells are not disrupted, but retained within their anatomical microenvironment during culture, which may favor the efficient formation of colonies of the epithelium.[16] The adhesive nature of fibrin in plasma also enables transplanted epithelial sheets to be attached without sutures, following which the fibrin matrix naturally degrades.[18] Satake et al. state that a carrier-free method of transplanting cultured epithelium could lead to better graft survival, less postoperative neovascularization, and better visual acuity.[7] Our culture method was devoid of amnion and consequently was carrier free.

Shortt et al. also describe a suspension culture method to separate epithelium from stroma by means of dispase, followed by the addition of trypsin to create an epithelial cell suspension, which is then placed into culture medium.[11,17] Cells were seeded onto amniotic membrane and cultured at 37°C in a humidified incubator with 5% CO_2 in air, as were done in our research, although we did not use amniotic membrane.[5,11] We did not disrupt the epithelium by means of enzymatic modulation.

Our culture method aimed at minimizing exposure to toxic products and zoonotic products. The risk of the transmission of animal viruses and prions is eliminated in our culture method, should the initial limbal biopsies be clear of any disease. The patient's own venous blood was collected to generate A-PRP gel.

In our experience the optimal culture period when the monolayer was optimally dense was between 15 and 21 days. The graft became too thick and became detached form the fibrin scaffold after 21 days. Koizumi et al. state that they used a 4-week culture period.[5]

In most cases the fibrin biological construct formed a thickened, proliferating edge on average consisting of epithelium (95%) and fibroblasts (5%), followed by a thin layer of epithelium (see Figure 1). In most cases the epithelium proliferated concentrically around the explant with cells expanding from the "hill" or "edge" of the explant toward the periphery of the PRP.

Du Toit found that fibroblasts proliferate at about the same speed as corneal keratinocytes, but that eventually at 6 weeks, fibroblast overgrowth occurs.[9] Once epithelial monolayer formation occurs, fibroblasts become less prominent and in the minority.[9] The authors assume that this is because sufficient fibroblast growth factors have already been produced by natural fibroblasts instead of lethally irradiated fibroblasts, to stimulate epithelial growth.

Shimazaki et al. and Pellegrini et al. performed a histologic examination using H&E staining and Shimazaki et al. examined tissues with phase microscopy, as were performed in our study.[8,15] Epithelium stained for K3, which indicated corneal epithelium.[15,19,20] We performed both K3 and K12 staining with monoclonal antibodies. The cultured epithelium displayed a corneal phenotype through the process of immunostaining, which used antibodies against cytokeratins K3 and K12.[19] According to Shermer et al., the 55K acidic (HK12) and 64K basic (HK3) keratins indicate a "corneal-type" differentiation.[10] K3 and K12 keratins are located supra-basally in the corneal epithelium as well as in the limbus, but Shermer et al. state that the central corneal epithelium stains uniformly including basal cells for HK3, while the limbal epithelium stains supra-basally for HK3.[10] Our epithelial cultures stained uniformly for K3, which indicated basal cell staining and confirmed that cultures were older than 15 days and corresponds with the statement by Shermer et al. that supra-basal staining is not an absolute requirement for K3 staining.[10]

PRP contains up to 4.4 times more platelets than whole blood, usually more than 200,000 platelets/μl and contains all components of whole blood.[21,22] It can be produced by many commercial systems and in our research we used the REGEN®-PRP system.[9,23] Mazzucco et al. showed that REGEN displayed a higher recovery of transforming growth factor beta (TGF-β), basic fibroblast growth factor (b-FGF), and insulin-like growth factor 1 (IGF-1) and the highest amount of growth factors, compared to other PRP gel preparations, but REGEN had lower platelet concentrations, probably due to only one centrifugation step.[22]

After centrifugation of the whole, anticoagulated venous blood sample, the buffy layer of white blood cells is situated just above the densely packed red blood cells.[21] The platelets are at its highest concentration just above the buffy layer, and it is this layer that we use to generate autologous A-PRP.[21] This differs from the autologous serum, which has been used by researchers such as Di Girolamo et al. and Nakamura et al. as culture medium, where whole blood after being centrifuged is usually filtered to create serum.[6,16]

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PRP typically contains three to five times more growth factors than baseline, which are responsible for cellular proliferation.[21,23] The alpha granules in platelets and in PRP release platelet-derived growth factor (PDGF), TGF-β1, vascular endothelial growth factor (VEGF), b-FGF, and epidermal growth factor (EGF).[20,21] Calcium, which leads to gel formation of PRP, also enhances cell proliferation and is beneficial to the release of PDGF.[9,24]

In the literature, cadaver corneas or limbal biopsies are used to culture ex vivo epithelium.[15] The South African situation creates the opportunity to culture ex vivo epithelial cells from severely damaged corneas due to trauma. These corneas, which are usually discarded following evisceration, have in our experience proven to be a useful source for epithelial cell proliferation. In none of the six cases observed was the penetrating corneal trauma obtained, sufficient to prevent epithelial cell proliferation. Eviscerated corneas were used in our setting to establish our culture method. Clinicians following this method for transplantation purposes should make certain that donors have no blood-borne diseases, are HIV negative, and not in the window period of HIV. This same culture technique can now be used to culture epithelium from living related donors or from the contralateral eye of a patient with limbal stem cell deficiency.

We postulate a new method for culturing corneal epithelium by the application of donor-specific A-PRP-gel, devoid of any animal products, antibiotics, amnion, or irradiated feeder fibroblasts. This approach may well improve the safety of cell cultures if eventually destined for human application.

Conclusion

To our knowledge, this is the first study to establish the growth pattern, cellular proliferation, and characteristics of epithelium cultured from eviscerated corneal tissue. In our experience, traumatized eviscerated corneas render successful epithelial growth.

Declaration of interest

No commercial relationship exists in the form of financial support or personal financial interests of the authors.

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APPENDICES

Protocol

Title

Eviscerated Corneas as Tissue Source for Ex Vivo Expansion of Limbal Epithelial Cells on Platelet-Rich Plasma Gels: Reviewing the ex vivo growth pattern and characteristics of cultured corneal epithelium in platelet-rich plasma gels.

Aim of Study: To establish if epithelium can be cultured from corneas eviscerated due to severe trauma.

Full ethical approval was obtained from the ethics committee of Groote Schuur Hospital (February 2010).

Summary

- Corneas will be harvested from patients consenting for evisceration due to the history of trauma with irretrievable loss of vision and no perception of light.
- These patients will present with an afferent pupillary defect as well as irreparable damage to the normal architecture of the eye.
- Patients will sign informed consent for above mentioned procedures to be performed.
- Consent for evisceration will be obtained by at least two clinicians not participating in this research project.

- Patient information sheets in three official languages will be provided to patients explaining the full extent of this research project.
- Consent to participate in this research project will be obtained on specially designed consent forms, as indicated in this protocol. Consent will also be obtained from patients for storage of human tissue. The second page of the consent form will be initialed by the patient.
- Corneas will be stored in normal saline and transported to the laboratories of the Department of Anatomy of the University of Stellenbosch, where optimal conditions reside for the culture of corneal cells under the directorship of Professor D. F. du Toit.
- Patients will receive blood tests prior to the donation of their eviscerated corneas, which will include Hepatitis B and C, HIV, and Syphilis serology. Standard protocol will be followed in obtaining consent for HIV testing.
- 20 milliliter blood will be collected from each corneal donor and centrifuged to create platelet-rich plasma (PRP).
- Limbal stem cells will be obtained from the donated corneas through the process of button biopsies and micro-dissection of each cornea.
- Corneal explants will be plated in PRP gel.
- Cells from each patient's cornea will be kept separately, and cultured separately from other patient's corneas.
- The researchers will attempt to culture cells from approximately five to ten corneas.
- Epithelial cell growth will carefully be monitored by means of phase contrast microscopy and photographs of cultured cells at seven day intervals.

- Success will be defined as the growth of at least one monolayer of cells on a substrate.
- Corneas and epithelium will be stored for the duration of this research project and consent for storage will be obtained from patients.
- The results obtained will contribute towards the MMed dissertation of Dr. Leonard G. Heydenrych, under the directorship of his MMed promoter, Dr. N. Cockburn.[1]
- The clinical research supervisor for this study will be Professor D. F. du Toit, FCS (SA), FRCS (Ed), PhD (Stell), D.Phil (Oxon), FICA (USA), a researcher who has published extensively in the field of molecular biological engineering.[2]
- The data obtained during this research will be reported at a national congress and the paper will be submitted for publication in a peer reviewed journal.[3]

Clinical Application

Through the process of harvesting, limbal epithelial stem cells may be obtained and used to produce corneal epithelial cells ex vivo or outside the human body, through the process of biological engineering.[1,2,3,4] Corneal stem cells may also be harvested from damaged or traumatized corneas, since only a small portion of the limbus is required to culture new cells. Due to the very high incidence of irreparably damaged corneas following trauma, we have a vast source of limbal stem cells in South Africa.[5] Corneas which have to be eviscerated due to trauma, may now also be used as a source of limbal stem cells to culture corneal epithelium

[1] Dr. N. Cockburn resigned from UCT in 2011 and is consequently not my internal supervisor anymore.
[2] Please see attached CV of Prof. D.F. du Toit.
[3] Preliminary data presented at the 2011 OSSA congress, Port Elizabeth and runner-up in the registrar prize competition.

from. We would like to see if we can successfully cultivate corneal epithelial cells from corneas obtained from irreparably damaged eyes that are being eviscerated as part of standard care.

Ocular surface problems such as infective corneal ulcers and chemical burns of the eye may also lead to persistent epithelial defects, not responding to treatment. This may ultimately lead to vascularization and perforation of the cornea. Blood vessels tend to grow over epithelial defects, leading to opacification of the cornea and the loss of vision. These defects may also perforate and lead to the development of devastating infection of the eye, called endophthalmitis. If not treated promptly with intravitreal antibiotics, this could lead to blindness. Limbal stem cell deficiency as a potential cause for corneal decompensation and corneal perforation may be managed by the transplantation of ex vivo cultured epithelium.

The benefit of ex vivo transplantation of limbal stem cells

Limbal stem cell transplantation may reduce inpatient stay in hospital wards and will also reduce the risks of opacification and perforation of the cornea. Inevitably this will reduce the burden on our over full wards and will also lead to considerable financial savings for the state sector, since inpatient stay will be shorter.

Traditionally, patients with persistent epithelial defects require a much longer stay in hospital due to the chronicity of these defects, compared with other ophthalmological conditions. These defects are usually treated with topical antibiotics, vitamins, progesterone and tetracycline and monitored daily in a ward. These defects may also be covered with a bandage contact lens or

with amniotic membrane, in an attempt to accelerate healing and prevent perforation.[6] The natural growth factors present in amniotic membrane tend to accelerate healing of these defects as well.[6]

Researchers have attempted to transplant limbal allografts onto diseased eyes in an attempt to replenish the source of epithelial cell formation.[7] This has been achieved with different degrees of success. Due to the risk of graft rejection, limbal allograft transplants carry the burden of continuous immunosuppression. The procedure is also technically difficult and is dependent on the availability of corneal grafts. Currently the waiting list for corneal grafts in South Africa is approximately two years.

Lately researchers have attempted to transplant corneal cells onto amniotic membrane.[2,3,7] The cells were cultured ex vivo from corneal limbal cells, which were harvested from donor corneas.[2,3,7] These membranes with cells were then transplanted onto corneas with limbal stem cell deficiency.[2,3,7,8] Currently these transplants have shown the most promising results and this method is also far less invasive than limbal stem cell allografts, which involves the transplant of a full thickness circular piece of sclera including the limbal epithelial stem cells.[7] The advantage of transplanting ex vivo expanded stem cells rather than keratolimbal or conjunctivolimbal transplants is that this novel method is less invasive and that rejection and immunosuppression are less likely.[7] Defects have recovered fully without corneal scarring and without the loss of vision.[2] These methods of transplantation have not been attempted on the African continent as yet. We feel that this form of transplant can greatly improve the current management of our

patients and can prevent the loss of vision in patients with limbal stem cell deficiency, awaiting corneal transplants.

Method

We will be obtaining corneas from eyes with irretrievable loss of vision, which had to be eviscerated following severe penetrating eye trauma and the consequent risk of sympathetic ophthalmia developing.[9]

The corneas will be surgically removed from the eviscerated eyes and stored in normal saline and kept below 7 degrees Celsius. The corneas will be excised during the procedure of evisceration. This will include the excision of the cornea with at least a two millimeter rim of sclera. Only eyes with irretrievable damage will be eviscerated. This will be performed by surgeons other than the surgeons involved in this research project. Clear guidelines for the indication of evisceration will be followed by the appropriate surgeons performing the eviscerations.

A standardized method for cultivating corneal epithelial cells will be used as established by Professor D.F. du Toit, from the Medical Research Council and obtained from the relevant literature.[2,3,8]

Limbal corneal stem cells will be grown from corneas harvested from eviscerated eyes with irretrievable sight-loss. The reason for evisceration will only be due to irreparable damage sustained by trauma in an eye with no perception of light. These patients will give written

informed consent for corneal harvesting which will then take place during evisceration. A separate consent form will be signed for this purpose and consent will be obtained by at least two eye surgeons. This will be additional to consenting for the primary procedure of evaluation under anaesthetic and evisceration. Patients will also receive blood tests prior to harvesting, which will include Hepatitis B and C, HIV and Syphilis serology. Patients will give written informed consent for HIV testing according to standard protocol and will receive pre- and post-test counseling by a social worker. Any patients testing positive for HIV, Syphilis or Hepatitis, will be informed of the results and will be referred for further management of these diseases. Patients receiving pre-test counseling and who are not willing to receive the outcome of their blood tests will be excluded from this research.

Blood will also be obtained from each patient to create autologous platelet-rich plasma (A-PRP) to extract the necessary growth factors to promote corneal cell proliferation for each individual case.[10,11,12]

Patients will be fully counseled before signing consent for evisceration by two clinicians not participating in this research project. Patients will receive information about the indication, surgical procedure and risks of evisceration as well as information on culturing their corneal stem cells.

Indications for evisceration:

- No perception of light.
- Gross damage to an eye due to trauma, not salvageable with suturing. Not removing such an eye with the patient's consent, will expose the patient to the risk of sympathetic ophthalmia, which could lead to the loss of vision in the normal fellow eye.[9]
- Full afferent pupillary defect.
- Auto-eviscerated eye following trauma.
- Consent from the patient for the evisceration to be performed.

The eye will only be eviscerated if the patient complies with all of the indications as set out above.

The corneas with the associated blood samples will then be transported to the laboratories of the Department of Anatomy of the University of Stellenbosch, for the sole purpose of molecular biological engineering with the consequent growth of corneal epithelial cells. Prior to this a short letter will have to be issued by the Departmental Head of Ophthalmology at Groote Schuur Hospital to state that he/she approves of the transport of discarded tissue and blood samples to the research laboratories at the Department of Anatomy of the University of Stellenbosch.[4] This letter will also be provided to the Inspector of Anatomy at the University of Stellenbosch to state that the appropriate researchers have received donated tissue that was discarded from patients at surgery because of irretrievable sight-loss. The written ethical approval from the University of Cape Town and the protocol number will also be required. A

[4] See letter by Prof. Colin Cook attached.

letter will also be provided by Prof. Colin Cook, Head of the Department of Ophthalmology at Groote Schuur Hospital, to state that he approves of the collaborative research.

The corneas will be accompanied by the signed informed consent form of the patient stating that his/her cornea may be used for culturing corneal cells. The corneal epithelial cells will be cultured under supervision of Prof. D. F. du Toit. He has given permission for this research to proceed and to be performed at his laboratories at the Tygerberg Medical Campus where optimal infrastructure is residing for biomedical engineering of corneal stem cells.

During this research project the corneas and cultured cells will be studied microscopically and immuno-histologically and will not be used for transplantation purposes. The corneas and grown stem cells will not be used for any form of financial gain whatsoever. Publications may come from above-mentioned research with the appropriate mentioning of all the participants in this study.

The research, under directorship of me and Prof. D. F. Du Toit, will aim to establish the growth patterns of cultured epithelial cells as well as the media best suited for epithelial cells to grow upon. Epithelium will be identified morphologically by means of microscopy as well as immunohistochemistry.

What makes this study unique is the fact that corneal cells will be grown from freshly eviscerated corneas, and not from cadaveric corneas or limbal biopsies as done in previous research.[2,3,7,8] The media which will be explored will include fresh amniotic membrane, biodegradable membrane, fibrin, as well as contact lenses.

Limbal stem cells obtained from harvested corneas will thus be cultured ex vivo in appropriate culture media. The most suitable transport media and carrier media for the cultured stem cells will also be established during research. Culture media will be provided by the University of Stellenbosch and amniotic membrane will be provided by the Division of Ophthalmology of the University of Cape Town.

The study design will represent a case series of five to ten cases of cultured corneal cells. The setting will be the Division of Ophthalmology of Groote Schuur Hospital, Cape Town, South Africa and the laboratories of the Department of Anatomy of the University of Stellenbosch. The subjects for this study will be selected on the basis of the inclusion and exclusion criteria as follows. Outcome measures will demonstrate the growth and success of cultured corneal cells by using freshly eviscerated corneas for culturing purposes.

Exclusion Criteria

- Patients not willing to partake in the study.
- Patients not willing to sign informed consent.
- Patients not willing to receive the outcome of their blood tests.
- Patients not qualifying for evisceration.
- Patients not exposed to eye trauma.

Inclusion Criteria

- Patients qualifying for evisceration.
- Patients signing informed consent.

Time Line

Literature review: May 2009 to December 2009

Protocol development: July 2009 to February 2010

Record retrieval and data capturing: February 2010 to February 2011

Analysis of results: March 2011

Writing of paper for submission to peer reviewed journal: March 2011 to September 2014

Budget

Assistance from the Department of Ophthalmology of the University of Cape Town and the Department of Anatomy of the University of Stellenbosch.

Communication

Reporting and implementation.

This project may be submitted as a dissertation for a Masters degree (MMed), reporting on a pilot study of five to ten cases of ex vivo cultured corneal epithelial cells, cultured from eviscerated corneas.

Alternatively the results will be reported at a local congress (OSSA, March 2011) and the paper will be submitted for consideration for publication in a peer reviewed ophthalmic journal.

The results of this study will assist to register a pool of standardized data on culturing corneal epithelial cells from several centers specializing in the treatment of severe ocular surface disease.[13]

Analysis

The results will be presented to a large degree without any statistical analysis and will mainly report on the success of growth of corneal epithelial cells, cultured from eviscerated donor corneas on a fibrin scaffold. Several microscopic digital photographs will be taken at weekly intervals to monitor the growth of cultured cells. The different monolayers achieved during culturing will be closely monitored and photographed. Culturing of epithelium from patients with blood borne diseases will not be excluded from this study and their epithelial growth patterns will be observed compared to healthy patients.

Success will be classified as the growth of at least one monolayer of cells on a substrate. Should six layers of epithelial cells be established, this could closely represent the architecture of the normal human corneal epithelium. The confluence of layers of cells will be monitored as well as the smoothness achieved of superficial layers of cells. These cells with their connections will be photographed by phase contrast microscopy.[14]

Leonard Heydenrych, September 2010

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Declaration of Helsinki

September 2010

To whom it may concern

I hereby declare that the research conducted in this study, namely:

Eviscerated Corneas as Tissue Source for Ex Vivo Expansion of Limbal Epithelial Cells on Platelet-Rich Plasma Gels: Reviewing the ex vivo growth pattern and characteristics of cultured corneal epithelium in platelet-rich plasma gels,

adheres to the ethical principles for medical research as set out in the declaration of Helsinki of the World Medical Association (59th WMA General Assembly, Seoul, October 2008).

Yours faithfully

Dr Leonard G Heydenrych

Division of Ophthalmology

Groote Schuur Hospital, University of Cape Town

Patient Information Sheet and Consent Form

Patient Information Sheet: Corneal stem cell growth

February 2010

Dear Patient

Many thanks for being willing to partake in this research project. As you are aware, your surgeon has explained to you that your eye has to be removed surgically due to the severe damage that it obtained during your accident and the risks involved in not removing it. We would like to ask your permission to use your cornea, which would usually have been discarded, for research purposes.

Research has shown that it is possible to artificially grow new cells from the existing cells of the cornea. We as researchers will try to grow cells from your discarded cornea. The cornea has a group of cells situated at the corneal limbus, which have the inherent ability to multiply and produce normal corneal cells. We will make use of this ability of your corneal cells, by placing them in special solutions in laboratories and observing their growth. This will help us to understand how corneal cells can grow outside the body. This is a very exciting new development, since new treatment modalities are now possible!

We can e.g. use these cells to close big corneal defects and improve healing. Please note that you will also be requested to agree/disagree that your cornea and stem cells may be stored for an indefinite period of time for further research. Hopefully one day, we might even be able to grow complete corneas through these methods and transplant them!

Many thanks for allowing us to use your discarded cornea for this research project. Please note that none of these corneas or cells will be used for any financial gain whatsoever!

Please feel free to contact me on speed dial 76622 or telephone 021 404 3525, should you have any questions regarding the use of your cornea. You are also welcome to contact the Research Ethics Committee, as indicated below.

With thanks

Dr Leonard G. Heydenrych

Division of Ophthalmology

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences

Research Ethics Committee

E52- 23 Old Main Building, Groote Schuur Hospital, Observatory, 7925

Tel: +27 21 4066492 Fax: +27 21 4066411

Pasiënt Inligtingsvorm: Korneale stamselnavorsing

Februarie 2010

Geagte Pasiënt

Baie dankie vir u bereidwilligheid om aan hierdie navorsingsprojek deel te neem. Soos u deeglik bewus is, het u chirurg aan u verduidelik dat u oog verwyder moet word, vanweë die erge skade wat u oog opgedoen het tydens u ongeluk. U chirurg het ook verduidelik dat daar in u geval risikos aan verbonde is indien u oog nie verwyder sou word nie.

Ons wil graag u toesteming vra om u kornea, wat gewoonlik wegdoenbaar sou wees, te gebruik vir navorsings-doeleindes. Navorsing het getoon dat dit moontlik is om kunsmatig nuwe selle te kweek van die bestaande selle van u kornea. Ons as navorsers wil poog om nuwe korneale stamselle van u kornea te kweek. Die kornea bevat n groep selle wat geleë is by die korneale limbus, wat die inherente vermoë besit om te vermeerder en om normale korneale selle te vorm. Ons wil graag van hierdie biologiese vermoë van u kornea gebruik maak, deur u limbale korneale selle in laboratoriums te laat vermeerder in chemiese oplossings buite die liggaam. Ons behoort meer te leer oor die wyse waarop korneale stamselle groei deur u selle te observeer. Hierdie wyse om selle buite die liggaam te laat groei, is n opwindende nuwe ontwikkeling, wat baie nuwe behandelings-modaliteite kan laat ontvou! Ons kan byvoorbeeld groot korneale defekte met hierdie selle bedek en genesing versnel. Neem asseblief kennis dat u versoek sal word om aan te dui of u kornea en stamselle gestoor mag word vir verdere navorsing. Dit mag dalk selfs later moontlik wees om volledige korneas buite die liggaam te kweek en om hulle oor te plant!

Baie dankie vir die voorreg om u wegdoenbare kornea te gebruik vir hierdie navorsingsprojek. Neem asseblief kennis dat u kornea of stamselle hoegenaamd nie vir enige finansiële gewin gebruik sal word nie.

Kontak my gerus per spoed-bel 76622 of telefoon 021 404 3525, indien u enige vrae het in verband met hierdie navorsingsprojek.

By voorbaat dank.

Dr Leonard G. Heydenrych

Departement Oftalmologie

UNIVERSITEIT VAN KAAPSTAD

Fakulteit van Gesondheidswetenskappe

Navorsings Etiese Komitee

E52-23 Ou Hoofgebou, Groote Schuur Hospitaal, Observatory, 7925

Tel: +27 21 4066492 Faks: +27 21 4066411

Sikhankasa uphando malunga ne glasi yeliso

February 2010

Mguli obekekileyo

Siyabulela kakhulu ngokuthatha le ngxaxheba koluphando lwale Project sithi xa siyibiza Cornealstem (Glasi yeliso)

Ugqirha wakho kuqala uzaku cacisela malunga nale projethi kwakhona aphinde akuxelele malunga nokususwa kwale glasi yeliso ngoba yiyo eyonzakalisa amehlo akho ungaboni kakuhle ngamehlo akho.

Phambi kokuba enze yonke into, uzakuxelela ukuba wawukhewafumana ingozi? Ukuba kunjalo ugqirha uqala athethe nawe akucacisele umonakalo owenzekayo kuwe.

Koluphando ugqirha ngemvume yakho mguli uzakususa lento yonzakalisa iliso lakho ayisebenzise koluphando.

Koluphando ugqirha uzakusebenzisa uzenzele (we glasi yeliso) ukwenzela ukuba amajoni la afumaneka apha elisweni lakho akwazi ukusebenza ngokwesiqhelo

Oku kukuzama ukunceda wean mguli, bafake la majoni (cells) akhule abe maninzi apha elisweni. Ngoku sebenza kwawo apha ngemva kwi (cornea) I glasi yeliso ibe yimpumelelo enkulu, luphando lutsha kodwa incede abantu abaninzi.

Koluphando mguli unalo ilungelo lokuthi hayi ukubaawuqondi okanye uvume xa uyivile ingxelo kagqirha yokutyalwa kwajoni apha elisweni koluphando.

Ndiqinisekile ngenye imini, sakutyala (cornea) glasi phantsi kwalendlela sityala ngayo.

Ndiyabulela kakhulu ngokundivumela koluphando nceda uqonde ukuba ngezi glasi cornea sizakuzisebenzisa ngendlela ephucukileyo.

Tsalela umnxeba kule nombolo (speed dial) 76622 okanye nanku omnye umnxeba cofa amanani (021)404-3525 ubuze imibuzo wamkelekile.

Ozithobileyo

Dr Leonard G. Heydenrych

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences

Research Ethics Committee

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Tel: +27 21 4066492 Fax: +27 21 4066411

Consent Form

Consent taken by medical professional:

……………………………………………………………….. (Name)

……………………………………………………………….. (Signature)

……………………………………………………………….. (Date)

Consent taken by medical professional:

……………………………………………………………….. (Name)

……………………………………………………………….. (Signature)

……………………………………………………………….. (Date)

Consent given by patient and donor:

Date: ………………………………………………………………

I, ……………………………………………………………………. (Full name)

……………………………………………………………………. (Signature)

Hospital Sticker

or

…………………………………………………………………... (Date of birth)

…………………………………………………………………... (Hospital Number)

hereby consent for the use of my eviscerated cornea for the sole purpose of stem cell research.

I also consent that blood will be taken from me for further tests, including the HIV blood test.

I also agree/disagree that my cornea and stem cells may be stored for an indefinite period of time for further research in this field (Please circle as appropriate).

Should I so wish, I acknowledge that I have the right to refuse at any time that my donated cornea be used for this research project. I have been counseled that this research is voluntary and that I can withdraw my consent at any time without prejudice.

I have also clearly been informed by the researchers that my cornea and stem cells will not be used for any financial gain of any source whatsoever. I hereby also consent that research obtained through the use of my stem cells and cornea may be published without the use or mention of my name.

Please contact the research leader, Dr Leonard Heydenrych, on Ward D4, telephone 021 404 3525 or speed dial 76622, should you have any questions regarding this research.

Toestemmingsvorm

Toestemming geneem deur mediese praktisyn:

………………………………………………………………….. (Volle naam)

………………………………………………………………….. (Handtekening)

………………………………………………………………….. (Datum)

Toestemming geneem deur mediese praktisyn:

………………………………………………………………….. (Volle naam)

………………………………………………………………….. (Handtekening)

………………………………………………………………….. (Datum)

Toestemming verleen deur pasiënt en donor:

Datum: ………………………………………………….

Ek, ……………………………………………………… (Volle naam)

………………………………………………………… (Handtekening)

Hospitaal-plakker

of

…………………………………………………………. (Geboortedatum)

…………………………………………………………. (Hospitaalnommer)

verleen hiermee toestemming vir die gebruik van my ge-evisereerde kornea vir die alleenlike doel van stamselnavorsing. Ek verleen hiermee ook toestemming dat bloed van my getrek mag word vir verdere toetse, insluitend MIV-toetsing.

Ek aanvaar/weier dat my kornea en stamselle gestoor mag word vir 'n onbepaalde tyd vir verdere navorsing in hierdie veld (Omkring asseblief die toepaslike opsie).

Sou dit my wens wees, aanvaar ek dat ek die reg het om enige tyd te weier dat my gedoneerde kornea vir hierdie navorsingsprojek gebruik word. Ek is meegedeel dat hierdie navorsing

vrywillig is en dat ek my toestemming enige tyd mag onttrek sou dit my wens wees, sonder enige vooroordele teenoor my.

Ek is ook volledig in kennis gestel dat my kornea en stamselle vir geen finansiële doel gebruik sal word nie.

Ek verleen hiermee ook toestemming dat navorsing ingewin deur die gebruik van my kornea en stamselle, gepubliseer mag word sonder die gebruik van my naam.

Indien u enige navrae het in verband met die beoogde navorsing, kan u vir Dr. Leonard Heydenrych, die navorsingsleier, kontak by spoed bel 76622, of telefoonnommer 021 404 3525 in saal D4.

Incwadi Yesifungo

Isifungo sithathwe ngu gqirha:

………………………………………………………………….. (Igama)

………………………………………………………………….. (Tyikitya)

………………………………………………………………….. (Umhla)

Isifungo sithathwe ngu gqirha:

………………………………………………………………….. (Igama)

………………………………………………………………….. (Tyikitya)

………………………………………………………………….. (Umhla)

Isifungo sinikwe sisiguli kunye nomnikeli:

Umhla: ……………………………………………………

Mna, ………………………………………………………. (Igama elipheleleyo)

……………………………………………………………….. (Tyikitya)

Hospital Sticker

okanye

……………………………………………………………….. (Umhla wokuzalwa)

……………………………………………………………….. (Inombolo yesibhedlele)

Oku kukuvuma kokukhutshwa kwe mehlo lam (cornea) ukwenzela uphando lwakwa stem cell research.

Nanini na ndinalo ilungelo lokungavumi okuba imehlo endinikele ngalo lungasetyenziselwa oluphando.

Ndichazelwe ngabo phando ukuba imehlo (cornea and stem cells) aluzusetyenziselwa ingeniso nayaluphina uhlobo.

Ndiya vuma ukuba kolulwazi loluphando inkcukaca zam azizu chazwa okanye zipapashwe eluphandleni.

Koluphando uyakwazi ukunikezela okanye unganikezeli ngeliso lakho kuxhomekeke kuwe mguli ngayo, yonke into ezakwenziwa.

Okwesibini unalo ilungelo lokuthi hayi xa ungafuni igama likhutshewe kuxelelwe abanye abantu okanye koma bona kude.

Tsalela lo mnxeba umphathi, Dr Leonard Heydenrych kweli candela labaguli ward D4

(021) 404 - 3225 okanye (Speed dial) 76622

xa ufuna ukuqonda.

Ethics approval letter from the Faculty Research Ethics Committee

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
e-mail: sumayah.ariefdien@uct.ac.za

09 February 2010

REC REF: 067/2010

Dr L Heydenrych
Ophthalmology

Dear Dr Heydenrych

PROJECT TITLE: REVIEWING THE EX VIVO GROWTH PATTERN AND CHARACTERISTICS OF CULTURED CORNEAL AND LIMBAL STEM CELLS ON AMNIOTIC MEMBRANE AND APPROPRIATE SUBSTRATES

Thank you for submitting your study to the Research Ethics Committee for review

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 10th February 2011.

Please submit an annual progress report (FHS016) if the research continues beyond the expiry date. Alternatively please submit a study closure report (FHS 010) if the study is completed within one year so that we can close our file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

sAriefdien

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Human Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6626 • **Facsimile** [021] 406 6411
e-mail: lamees.emjedi@uct.ac.za

25 February 2011

HREC REF: 067/2010

Dr L Heydenrych
Opthalmology

Dear Dr Heydenrych

PROJECT TITLE: REVIEWING THE EX VIVO GROWTH PATTERN AND CHARACTERISTICS OF CULTURED CORNEAL AND LIMBAL STEM CELLS ON AMNIOTIC MEMBRANE AND APPROPRIATE SUBSTRATES

Thank you for submitting your progress report to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the FHS HREC has granted **annual approval** for the above-mentioned study.

Approval is granted for one year until 28 February 2012.

Please send us an annual progress report (website form FHS 016) if your research continues beyond the approval period. Alternatively, please send us a brief summary of your findings so that we can close the research file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

lemjedi

Supervisor Request Letter

UNIVERSITY OF CAPE TOWN

Division of Ophthalmology

Faculty of Health Sciences
H53 Old Main Building Groote Schuur Hospital
Observatory 7925 Cape Town South Africa
Tel: +27 21 406-6215/6
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Professor C.D. Cook MBChB DO MPH FCS(Ophth)SA FRCOphth
Email: colin.cook@uct.ac.za
24 April 2010

Professor DF Du Toit
Anatomy Department
Stellenbosch University

Dear Professor Du Toit

Re : Dr Leonard Heydenrych – MMed Research Project

Thank you for agreeing to co-supervise Dr Heydenrych's MMed research project. Dr Cockburn will be his supervisor here at UCT.

Dr Heydenrych, in discussion with Dr Cockburn, has prepared the protocol for this study. Ethical approval for the study has been obtained from the ethics committee of the University of Cape Town.

We are very grateful for the opportunity to collaborate with the anatomy department at Stellenbosch University, and we are very grateful for your supervision of Dr Heydenrych's study. As we understand, the research will be headed and supervised by you and will deal with the growth of cultured corneal stem cells. It will take place at the laboratories of the anatomy department at Stellenbosch University, where you have the infrastructure for this. The cells will be cultured from eviscerated corneas obtained from the division of ophthalmology at Groote Schuur Hospital.

With best wishes.

Yours sincerely

Colin Cook

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