Development of decellularised conjunctiva as a substrate for the ex-vivo expansion of conjunctival epithelium

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Abstract

Purpose

To develop a method to decellularise human conjunctiva. To characterise the tissue in terms of its DNA content, tensile strength, collagen denaturation, basement membrane, extracellular matrix components and its potential to support conjunctival epithelial growth.

Methods

Human conjunctival tissues were subjected to a decellularisation process involving hypotonic detergent and nuclease buffers. Variations in sodium dodecyl sulphate (SDS) concentration (0.05 – 0.5% (w/v)) were tested to determine the appropriate concentration of detergent buffer. DNA quantification, collagen denaturation, cytotoxicity and tensile strength were investigated. Human conjunctival cell growth by explant culture on the decellularised tissue substrate was assessed after 28 days in culture. Samples were fixed and paraffin embedded for immunohistochemistry including conjunctival epithelial cell markers and extracellular matrix proteins.

Results

Conjunctival tissue from 20 eyes of 10 donors (range 65-92 years) was used. Decellularisation of human conjunctiva was achieved to 99% or greater DNA removal (p<0.001) with absence of nuclear staining. This was reproducible at the lowest concentration of 0.05% SDS (w/v). No collagen denaturation (p=0.74) and no difference in tensile strength parameters was demonstrated following decellularisation. No significant difference was noted in the immuno-localisation of

collagen IV, laminin and fibronectin, or in the appearance of PAS-stained basement membranes following decellularisation. The decellularised tissue did not exhibit any cytotoxicity and explant culture resulted in the growth of stratified conjunctival epithelium.

Conclusion

Allogeneic decellularised human conjunctiva can be successfully decellularised using the described protocol. It represents a novel substrate to support the expansion of conjunctival epithelium for ocular surface cellular replacement therapies.

1. Introduction

The conjunctiva is a thin vascularised membrane that extends from the corneal limbus to the mucocutaneous junction of the eyelids. Homeostasis of the ocular surface is dependent upon normal conjunctival function including mucin production (Dartt 2004). The conjunctival epithelium is a stratified layer with a subpopulation of goblet cells (Forrester *et al.*, 2008). It may become irreversibly damaged for example by infective, inflammatory or neoplastic conditions and chemical trauma. Subsequent keratinisation, cicatrisation, forniceal contraction and corneal ulceration may result in painful loss of vision. Loss of conjunctival function also contributes to failure of limbal stem cell transplantation and poor visual outcomes (Ilari and Daya 2002). Non expanded conjunctival autografts are insufficient in size to reconstruct greater areas of loss or to reconstruct the fornices (Ang *et al.*, 2005). Human amniotic membrane has been used in attempts to reconstruct the ocular surface

following chemical burns and pterygium surgery, in mucous membrane pemphigoid and Stevens-Johnson syndrome (Honavar *et al.*, 2000; Tseng *et al.*, 2001; Barabino *et al.*, 2003; Ang *et al.*, 2005; Fernandes *et al.*, 2005). <u>It is, however, quickly degraded and prone to recurrent scarring and cicatrisation</u> (Barabino *et al.*, 2003; Zhao *et al.*, 2014). There is therefore a need to develop novel tissue engineered conjunctiva to enable ocular surface reconstruction.

Biological scaffolds derived from decellularised tissues have been successfully transplanted in humans for skin, heart valves and trachea (Crapo *et al.*, 2011, Elliott *et al.*, 2012). Animal studies using allogeneic and xenogeneic decellularised cornea have also proved successful (Zhang *et al.*, 2007). It is hypothesised that decellularised human conjunctiva is a good substrate for the *ex-vivo* expansion of conjunctival epithelium. Extracellular matrices are relatively conserved across species and confer the optimal three-dimensional environment providing a platform for recellularisation with the patient's own cells, demonstrated through a variety of organ models (Bolland *et al.*, 2007; Mirsadraee *et al.*, 2007). The decellularisation process renders tissue immunologically inert thereby minimising rejection risk and enabling the use of a large donor pool. Furthermore, with developments in stem cell biology and technologies directed at cellular expansion, a novel conjunctival construct developed on a decellularised conjunctival scaffold could be developed in vitro for the ocular surface reconstruction of individual patients.

A method for tissue decellularisation has been developed and patented by the University of Leeds in collaboration with NHS Blood and Transplant (NHSBT), UK to enable the decellularisation of heart valves, blood vessels and amnion (GB2375771, Fisher, Ingham and Booth: Wilshaw *et al.*, 2008). In this report, we describe an adaptation of the protocol described for the decellularisation of human dermis (Hogg *et al.*, 2013) to enable decellularisation of human conjunctiva. The aims of this study are to develop a decellularisation process for conjunctival tissue, characterise its properties and demonstrate the potential for the ex vivo expansion of conjunctival epithelium on its surface.

2. Materials and Methods

2.1.1 Tissues

Bulbar conjunctival specimens were obtained from human cadaveric donors within 24 hours of death. Donors were identified by the Merseyside Eye Retrieval Consortium or NHSBT National Referral Centre whose next-of-kin had consented for their ocular tissues to be used for research purposes. Ethical approval was obtained (11/NW/0766, National Health Service Health Research Authority). Tissue retrieval, storage and handling practices were in keeping with the Human Tissue Act and Data Protection Act. Tissue samples were prepared using 5 mm diameter trephines except for explant cultures in which 1mm x 1mm sections were dissected and for biomechanical testing in which approximately 15mm x 3mm sections were dissected with a scalpel (Swann-Morton no.10) and Westcott scissors. The basement membrane was identified under a microscope when freshly dissected and sutures

placed to mark the basement membrane side.

2.1.2 Decellularisation of tissues

Triplicate samples were treated in the same experiment in which all experimental reagents and steps were constant other than the concentration of SDS (percentage w/v). Reagents used in this study were produced to British/European Pharmacopoea standards by pharmaceutical suppliers (Nordic Pharma/Source BioScience) or were high-grade research reagents.

Tissue samples were first washed three times in Tris(hydroxymethyl)aminomethane (TRIS) buffered isotonic saline (Source Bioscience) for 20 minutes at 200rpm in an orbital incubator at 25°C. Cells were subsequently lysed using a hypotonic buffer comprising 10mM TRIS (VWR) and 0.1 % ethylenediaminetetraacetic acid (EDTA supplied by VWR) at pH 8 for 18 hours at 4°C agitated at 200rpm in an orbital incubator. Following this step, samples were treated in a detergent buffer by agitation at 200 rpm, 25°C for 24 hours in an orbital incubator to remove cellular fragments. The detergent buffer comprising 10mM TRIS (VWR) and 0.1% EDTA (VWR) was prepared with variation in sodium dodecyl sulphate (SDS) concentration (0.05%, 0.1% and 0.5% (w/v)). Samples were subsequently washed three times in TRIS buffered isotonic saline (Source Bioscience) for 20 minutes. This was followed by treatment in a nuclease buffer; 50mM TRIS (VWR), 10mM $MgCl_2$ (VWR), Benzonase 1U/ml (Novogen) for 3 hours with agitation at 150rpm at 37°C before washing twice in phosphate buffered saline (PBS) 0.1% (w/v) EDTA (VWR) and finally in PBS with 1% (w/v) Penicillin/Streptomycin (Sigma). The protocol for the

decellularisation of amniotic membrane was as described above other than the detergent buffer in which only 0.1%(w/v) SDS was used.

2.2 Isolation and quantification of DNA

DNA isolation was undertaken using a commercially available kit (DNAeasy, Invitrogen). Tissue samples were placed on tissue paper, removed and weighed individually and processed according to manufacturer's protocols. This has also been described for the decellularisation of human dermis (Hogg *et al.*, 2013). The extracted DNA was quantified using a PicoGreen assay (Invitrogen) by fluorometric analysis. A microplate fluorescent plate reader (FLX800, Biotek) was used for DNA quantification, determined after plotting a standard curve from known concentrations of calf thymus DNA control standards (Invitrogen).

2.3 Collagen denaturation

Samples were treated with either 0.1N sodium hydroxide for 16 hours at 25°C (positive control to unravel collagen fibrils), 50mM gluteraldehyde (Sigma) at 25°C (negative control for hydroxyproline estimation) or 10mg/ml alpha-chymotrypsin (Sigma-Aldrich) for 24 hours at 37°C. Denatured collagen results in the release of hydroxyproline into the supernatant along with a corresponding colour change detected using Ehlrich's reagent (Sigma) (Hogg *et al.*, 2013). Both the controls with pre-determined hydroxyproline concentrations and test samples were similarly treated as follows: 12N Hydrochloric acid (VWR) was added to a sample of each supernatant and autoclaved for 60 minutes at 121°C, 18 psi. Chloramine T reagent (Sigma) was then added to each sample at 25°C for 25 minutes. Ehlrich's reagent

was subsequently added at 65°C for 20 minutes and the resulting colormetric change measured using a microplate reader (ELX 808, Biotek).

2.4 In vitro contact cytotoxicity testing

A standard method for determining contact cytotoxicity was employed as outlined by the Biological evaluation of medical devices- part 5 (2009); ISO 10993-5 (BSI, 2009). Sections of decellularised tissue were attached to 24-well plates (Greiner) with Steristips™(3M). Cyanoacrylate glue (RS components) and Steristips™ alone were used as positive and negative controls respectively. All samples were tested in triplicate and against two cell types; primary human skin fibroblasts (passage 4) and an immortalized human conjunctival cell line (HCjE-Gi cells, passage 10). $1x10^5$ cells were seeded in each well and incubated for 48 hours in a 5% CO_2/air incubator at 37°C. HCjE-Gi cell media consisted of K-SFM (Invitrogen), 0.2ng/ml EGF (Invitrogen), 25μg/ml BPE (Invitrogen), 0.4mM $CaCl_2$ (Sigma), 1% (w/v) penicillin/streptomycin (Sigma). Primary human skin fibroblasts were cultured in DMEM (Sigma), 10% (w/v) fetal calf serum (Sigma), 1% (w/v) penicillin/streptomycin (Sigma). After 48 hours in culture, the samples were fixed in 70% ethanol and stained with Giemsa (VWR). Cells were visualized and photographed on an inverted microscope (Leica 090-135-002).

2.5 Explant culture

Bulbar conjunctival tissue was dissected from its underlying tenon's layer and divided into approximately 1x1mm tissue sections for use as explants. Five explants were seeded on decellularised conjunctival tissue sections mounted within tissue cell crowns (Scaffdex) orientated such that the basement membrane side of both the decellularised tissue and explants were opposed and the epithelial side faced upwards. The tissue explants were cultured for the first 24 hours in DMEM/F12 media (Invitrogen), 10% (w/v) fetal calf serum (Sigma) and penicillin/streptomycin 1% (w/v) (Sigma), and the subsequent 11 days in K-SFM media supplemented as above. After 12 days in culture, the tissues were airlifted and maintained in conjunctival epithelial stratification media to day 28: K-SFM (Invitrogen), 10ng/ml EGF (Invitrogen), 12mM $CaCl_2$ (Sigma), 1% (w/v) penicillin/streptomycin (Sigma), transferrin 5µg/ml (Sigma), insulin 5µg/ml (Sigma), triiodothyronine 1.4ng/ml (Sigma), adenine 12µg/ml (Sigma), hydrocortisone 0.4µg/ml (Sigma), epidermal growth factor 10ng/ml (Sigma). Media levels were monitored up to twice daily and media replenished as required to supply the air-liquid interface.

2.6 Histology

Samples including cellular and decellularised conjunctiva were prepared for paraffin wax histology by completing a standard cycle in a tissue processor (Citadel 2000, Thermo Shandon, UK). Following wax embedding, 5-10µm sections were cut using a microtome (Finesse 325, Thermo Shandon). Samples were subsequently stained with Haematoxillin and Eosin (H&E supplied by Surgipath), periodic acid schiff stain (PAS supplied by Sigma) and also deparaffinised sections were stained with DAPI (4',6-diamidino-2-phenylindole supplied by Sigma). Triplicate samples were taken

from at least three separate areas of the tissue sections to ensure that the samples were representative of the entire tissue section.

2.7 Immunohistochemistry

Deparaffinised sections on silanised slides (Dako) were submerged for 20 minutes at 95°C in Target Retrieval solution (Dako). Slides were washed in a buffer comprising 0.05% (w/v) Tween-20 (Sigma), 8.76 % (w/v) NaCL (Sigma), 6.05% (w/v) TRIS (VWR), pH 7.6. Immunohistochemical staining was undertaken using the Envivsion[TM] kit HRP anti-mouse/rabbit (Dako) according to the manufacturer's recommended standard protocol but included 20% goat serum block for 30 minutes (Dako) prior to incubation with primary antibody (antibody diluent, Dako) for 3 hours at room temperature. Slides were mounted in DPX mounting media (Sigma) and imaged on an Olympus BX60 microscope. A representative photograph was taken from the centre of each stained tissue section. Tissue sections were taken from at least 3 different areas from the paraffin embedded tissue block.

2.8 Biomechanical testing

The Lloyd Instruments Universal Testing machine (LRX plus, Lloyds Instruments) was used to determine the uniaxial tensile strength of tissue samples. Conjunctival sections were divided into approximately 15mm x 3mm sections and held within clamps. The thickness, length and width of each sample were individually measured with Vernier calipers (Digimatic CD-6"C, Mitutoyo UK; resolution 0.01mm). Three measurements of width and thickness were taken from each sample and the average thickness calculated. Each sample was attached to a 5N load cell and data including stress (MPa), strain and elastic modulus (MPa) were generated by

Nexygen software. For each of the cellular and decellularised tissue test groups, a minimum of 4 samples were tested from 3 different donors (n=15 and n=14 respectively).

2.9 Statistical analysis

IBM SPSS 22 was used for all statistical analysis and included repeated measures ANOVA and the paired t-test. Data were normalised by log transformation to enable parametric statistical testing. Tests of homogeneity were undertaken and satisfied for the ANOVA model using Box's test of equality of covariance matrices and Levene's test of equality of error variances.

3.0 Results

Conjunctival tissue from 20 eyes of 10 donors was used in this study; 5 male donors and 5 female donors, mean age 83.5 (range 65 to 92 years).

3.1 DNA detection and quantification

Effective decellularisation was demonstrated in the treatment groups subjected to the decellularisation protocol (p<0.001; Table 1). Post-hoc ANOVA Bonferroni analysis found no significant differences between the treatment groups defined by % SDS concentration (w/v); p≥0.1. In all treatment groups, less than 1% residual DNA (with the cellular control as the comparator) could be detected.

The lowest concentration of SDS (0.05%) tested using three different donors demonstrated that decellularisation was reproducible with no significant differences between donors (p≥0.1; Bonferroni post-hoc tests) and less than 1% residual DNA compared with cellular tissue (Table 2). Decellularisation of tissue was also confirmed by the absence of nuclear staining demonstrated using the nuclear DAPI stain (photomicrographs not included).

3.2 Tensile strength

The average width and thickness of amniotic membrane in mm (SD) were 5.9 (1.1) and 0.05 (0.01) respectively. The average width and thickness of conjunctiva in mm (SD) were 5.3 (1.5) and 1.05 (0.64) respectively. Amniotic membrane had the greatest elastic modulus (12.0 MPa) and was more than 3-fold higher in comparison to conjunctiva indicating greater stiffness (p<0.0001; Table 3). No significant difference in ultimate tensile stress (p=0.354) or elastic modulus (p=0.561) was found between cellular and decellularised conjunctiva or amniotic membrane suggesting no change in stiffness or biomechanical strength occurs with decellularisation in either tissue.

3.3 Collagen denaturation

Three samples were taken from each of the three donors with experimental repeats taken in triplicate for each (9 samples analysed per donor). Cellular and decellularised tissues prepared from three donors showed no significant difference in collagen denaturation (p=0.74). This was determined by the lack of significant difference in hydroxyproline concentration detectable in the experimental assays

prepared from cellular and decellularised tissue samples between three different donors (Figure 1).

3.4 Contact cytoxicity

In vitro cytotoxicity was undertaken using primary human fibroblasts and a human conjunctival cell line. Representative images of human fibroblasts are shown in Figure 2. Giemsa stained samples demonstrate cells in close proximity to and within the decellularised tissue, suggesting that the decellularised tissue is not cytotoxic to either fibroblasts or conjunctival epithelial cells. In contrast, a zone of inhibition and cellular debris can be visualised around the cyanoacrylate glue indicating non-viable cells and cytotoxicity.

3.5 Histology and immunohistochemistry

H&E staining (Figure 3) demonstrated an eosinophillic band at the surface of the tissue in keeping with the structure of the basement membrane. Qualitatively the cellular and decellularised tissues appeared to have a similar eosinophillic staining pattern, however, absence of basophilic Haematoxillin staining of nuclei in the decellularised sections were apparent. PAS staining of cellular and decellularised tissues showed the detected glycoprotein to be qualitatively similar before and after decellularisation, suggesting that the basement membrane was not disrupted by the treatment protocol. Qualitative analysis of the extracellular matrix components laminin, fibronectin and collagen IV before and after decellularisation were also in keeping with this observation (Figure 4).

H&E sections were taken of tissues in which primary conjunctival explants had been expanded show the formation of a stratified epithelium 2-4 cell layers thick (Figure 5). Figure 6 shows the abundant expression of CK19 throughout the tissue sample (the same tissue block was used as in Figure 5a). In contrast, CK7 and CK4 were expressed in a qualitatively lower proportion of cells. Although MUC5AC expression was qualitatively sparse, it was however, detectable within the tissue sections examined. Markers of progenitor cells ΔNp63-α and ABCG2 were also present in lesser frequency, with ABGC2 expression less abundant than ΔNp63-α. Levels of PCNA expression appear similar to that of ΔNp63-α. Caspase 3, a marker of apoptotic cells is also present and appears to be expressed in cells that are apical rather than basal in comparison to the other markers studied by qualitative observation of representative tissue sections.

4.0 Discussion

The overall aim of this study was to develop and characterise decellularised conjunctiva as a substrate for the *ex vivo* expansion of conjunctival epithelium.

The decellularisation protocol using an SDS concentration of 0.05% (w/v) was able to remove 99% or greater DNA content with reproducibility across donors. Although these data are supported by the number of experimental repeats (3 samples per donor from which 3 experimental repeats were drawn from each) it would also be of interest to investigate inter-donor variability using concentrations of SDS other than 0.05%. The tests for the tensile strength, quantitative collagen denaturation, histology and immunochemistry for extracellular matrix proteins found no evidence of degradation of the tissue or its extracellular matrix components following the

decellularisation process. Finally, preliminary experiments demonstrated the potential of decellularised conjunctiva to support *ex vivo* expansion of conjunctival epithelial cells forming a stratified epithelium expressing markers of differentiated conjunctival epithelial cells.

Decellularised tissue has not been strictly defined. Results from *in vivo* studies, in which tissue remodelling has been demonstrated in the absence of an adverse host immunological response, suggest 90-95% decellularisation is adequate (Crapo *et al.*, 2011; Hogg *et al.*, 2013). It follows therefore, that the protocol that we used to decellularise conjunctival tissue exceeds the previously defined standards of 50ng DNA/mg dry weight or tissue (Crapo *et al.*, 2011). Indeed, given our finding of cellular conjunctiva with 54ng/mg DNA, the standard of 50ng/mg DNA may not be appropriate when determining whether an adverse host response would be incited. Other criteria suggested by Crapo and colleagues include <200bp length DNA fragments. This parameter would be useful to investigate in future work.

Zhao and colleagues describe a decellularisation process used for porcine conjunctiva in which it is stated that tissues were left to dry by ventilation. In our study, tissue samples were placed on tissue paper, removed and weighed individually and processed according to manufacturer's protocols. DNA isolation was then undertaken using a commercially available kit (DNAeasy, Invitrogen). This has also been described for the decellularisation of human dermis (Hogg *et al.*, 2013). It seems likely therefore, that the discrepancy in results may be due to differences in the levels of tissue hydration when weighed and the assays used for DNA

quantification (Darzynkiewicz *et al.*, 2011). This makes it difficult to directly compare the amount of DNA between studies.

Hypotonic and hypertonic reagents are known to be effective in lysing cells but may leave cellular residues (Crapo *et al.*, 2011). This is counteracted by observations that SDS effectively removes nuclear and cytoplasmic remnants from even dense tissues, but may damage collagen matrices (Woods and Gratzer 2005; Crapo *et al.* 2011). We found, however, that there was no increase in hydroxyproline detectable following decellularisation thereby suggesting an SDS concentration of 0.05% represents an optimal balance between decellularisation and the lack of tissue disruption in human conjunctival tissue.

Tensile strength testing results found no significant difference in the stress at maximum load (MPa), strain at maximum load (%) or elastic modulus (MPa) between cellular and decellularised tissue suggesting that biomechanical strength is not altered by the decellularisation treatment. This is in keeping with other tissue models comparing cellular and decellularised tissue (Stapleton *et al.*, 2008; Campbell *et al.*, 2012; Hogg *et al.*, 2013). The tensile strength measurements are low for both conjunctiva and amniotic membrane in terms of their absolute values in comparison to other characterised tissues such as dermis and cartilage (Stapleton *et al.*, 2008; Campbell *et al.*, 2012, Hogg *et al.*, 2013). The tensile strength of conjunctiva and amniotic membrane differ in this study in comparison to that by Dreschler and colleagues. This may be due to methodological differences such as equipment used including the clamps used to secure tissues, measurement methods

used to assess tissue thickness and whether dumbbell shaped tissue sections were cut prior to use. Delicate tissues such as conjunctiva and amniotic membrane may become easily damaged whilst being handled, or if tissues are placed on adherent surfaces where they may be inadvertently stretched or become dehydrated. Measurement of their tensile strength is therefore fraught with difficultly and consistency in method is critical to ensuring reliability of any observed differences. Comparison of results between studies however may not be appropriate. Tensile strength characterisation is of particular importance given the current scientific climate in which synthetic materials are also under investigation as conjunctival substrates (Ang *et al.*, 2006).

The stiffness of a material itself has been recognised to influence the cellular phenotype (Petroll and Lakshman 2015). It is of interest to note the 3-fold greater elastic modulus of amniotic membrane in comparison to conjunctiva indicating greater stiffness. Indeed it has been recognised that amniotic membrane has a thick basement membrane and may account for these observations (Schrader *et al.*, 2009).

The extracellular matrix components laminin, fibronectin and collagen IV do not appear to be disrupted following decellularisation. This is apparent qualitatively in terms of the immuno-localisation of laminin, fibronectin and collagen IV, but also quantitatively in terms of denatured collagen whereby no difference between cellular and decellularised tissue could be demonstrated. Laminin, fibronectin and collagen IV appear to be present in both the basement membrane zone and epithelium in conjunctival tissues, a staining pattern that has been previously characterised

(Messmer *et al.*, 2012). The PAS (not shown) and H&E staining also confirm that the cellular and decellularised tissues appear to have very similar staining and the basement membrane layer appears unchanged. The structure of the conjunctiva is peculiar to its location and function. Apart from the removal of the cellular component, the extracellular matrix components and stiffness of the conjunctiva was unchanged by the decellularisation process. It might be expected therefore, that decellularised conjunctiva would possess the greatest potential to support a conjunctival epithelium with a subpopulation of progenitor and goblet cells by providing the most appropriate niche for these cells.

Preliminary primary cell culture experiments (not shown) found that seeding decellularised tissue with conjunctival explants was more effective in forming confluent cell layers than seeding the decellularised tissue with a cell suspension that had been initially expanded on tissue culture plates from explants. The orientation of tissue was crucial such that the greatest cellular expansion occurred when cells were cultured in direct contact with the basement membrane. Immunohistochemistry confirmed that the resulting stratified epithelium was of a conjunctival phenotype given the expression of CK19, CK7 and CK4 (Moll *et al.*, 1982; Kasper *et al.*, 1988; Fostad *et al.*, 2012). CK7, regarded as a goblet cell marker was found throughout the tissue sections suggesting these cells had the potential to produce mucin (Shatos *et al.*, 2003; Fostad *et al.*, 2012). MUC5AC is a gel forming mucin found exclusively in mature goblet cells. Its expression was sparse but detectable suggesting the presence of immature goblet cells given the degree of CK7 staining (Eidet *et al.*, 2012). To the best of our knowledge, this is the first conjunctival substrate-culture study in which CK7 positive conjunctival cells have

been demonstrated. The expression of progenitor cell markers ABCG2 and ΔNp63 was also apparent indicating the existence of progenitor cells in culture that may have the potential to replicate. This evidence, together with the observation of a proportion of cells expressing PCNA would suggest that the conjunctival construct has potential for self renewal.

Other biodegradable matrices have been considered to support *in vivo* conjunctival growth. A porous glycosaminoglycan co-polymer matrix has been described for the repair of full thickness conjunctival defects in rabbits and the forniceal depth compared with an un-grafted wound in the fellow eye (Hsu *et al.*,2000). A similar study describes the use of porous poly(lactide co-glycolide) (PGLA) scaffold modified by hyaluronic acid and collagen (Lee *et al.*, 2003). In these studies, forniceal shortening was significantly greater in the un-grafted eyes. In both studies, the un-grafted eyes had irregularly arranged collagen in keeping with scar tissue in comparison to a more regular collagen deposition demonstrated in the grafted eye (Hsu *et al.*,2000). Both substrates are examples degradable matrices. It should be noted however that both substrates were inelastic and lacked an epithelium (Elliot *et al.*, 2012). In contrast, conjunctival epithelium cultured on collagen matrices developed with an appropriate cell polarity and a basement membrane, but was limited to an organised monolayer (Tsai *et al.*, 1988).

A more promising elastic substrate for conjunctival regeneration is the use of plastic compressed collagen (Drechsler *et al.*, 2015). Confluent growth of conjunctival epithelium has been demonstrated *ex vivo* including a subpopulation of progenitor cells. The authors of the study report the ultimate tensile strength of the substrates and handing was similar to amniotic membrane. Another elastic resorbable polymer demonstrated to support the growth of conjunctival epithelium including goblet cells is ultrathin Poly (ε-Caprolactone) (Ang *et al.*, 2006). Like decellularised conjunctiva, both these substrates are biodegradable. Of the described substrates, decellularised conjunctiva may provide the most appropriate cues for cell proliferation and differentiation given that it presents the 3-dimentional microenvironment of native conjunctival tissue itself, a matrix that would be difficult to replicate in a synthetic substrate.

Given that an ideal conjunctival substrate would support mucin producing goblet cells, further optimisation of media constituents including growth factors and perhaps a lengthened time in culture could upregulate MUC5AC expression (Dartt 2004). Alternative methods to potentiate the culture of goblet cells could include the use of bronchial epithelial growth medium, epidermal growth factor mediated signalling or γ-secretase as studied by other research groups (Chung *et al.*, 2007; Hodges *et al.*, 2012; Tian *et al.*, 2014). Furthermore, seeding explants from conjunctival sites known to be rich in stem cells and goblet cells or developing methods to enhance cultures rich in progenitor/stem cells would result in optimised, self-renewing cultures (Stewart *et al.*, 2015). Decellularised trachea, a successful model of tissue engineering in patients, crucially required an *ex vivo* epithelial culture rich in stem cells with the appropriate angiogenesis and differentiation promoting growth factors

(Elliott *et al.*, 2012). To this end, techniques to expand and support cultures rich in conjunctival stem cells are acutely warranted and currently lacking. The optimal cell culture environment in which to produce conjunctival cultures with a functional population of stem and goblet cells are yet to be determined.

A soft, pliable graft derived from decellularised conjunctiva, with or without *ex vivo* expanded conjunctival epithelium, may suit a range of indications including glaucoma surgery, conjunctival replacement following resection of conjunctival tumours and conjunctival chemical burns. The role of decellularised human conjunctiva may be extended for other ocular cellular replacement therapies such as limbal stem cell transplantation. Indeed, a xenogenic conjunctival matrix has been demonstrated as an effective scaffold for corneal epithelium in rabbit model of limbal stem cell disease (Zhao *et al.*, 2014). This therefore demonstrates the range of cellular replacement therapies in which decellularised matrices provide major advancements in the field of tissue engineering.

5.0 Conclusion

This study has demonstrated the successful decellularisation of human conjunctiva without degradation of its mechanical properties or basement membrane structure. Following decellularisation the tissue can support the growth of a stratified conjunctival epithelium containing differentiated epithelial cells and goblet cells along with progenitor cells demonstrating its potential for clinical applications in the treatment of ocular surface disease.

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Table 1 DNA content of cellular tissues and decellularised tissues using SDS of varying concentration. Overall ANOVA model significant p<0.001. Data log transformed to enable parametric data analysis and ANOVA model satisfied following Levene's test of equality of variance (p=0.1). Bonferroni post hoc tests between 0.05%/0.1/0.5% SDS (w/v) treatment groups; p≥0.1

Treatment group	DNA ng/mg (SD)	Percentage DNA removal
Cellular control	54.5 (10.4)	-
0.05%	0.4 (0.2)	99.3
0.1%	0.2 (0.1)	99.7
0.5% SDS	0.3 (0.3)	99.4

Table 2 DNA content of cellular tissues and donor tissues decellularised with 0.05% SDS (w/v).

Tissue	DNA ng/mg (SD)	Percentage DNA removal
Cellular control	42.3 (7.4)	-
Donor a	0.4 (0.2)	98.9
Donor b	0.3 (0.1)	99.2
Donor c	0.3 (0.1)	99.3

Table 3 Tensile stress of cellular and decellularised conjunctiva and amniotic membrane. Data shown in this table was normalised by log transformation prior to ANOVA analysis. Overall ANOVA model for both ultimate tensile strength and elastic modulus p<0.0001 significant difference between tissues. No significant differences were found between cellular and decellularised tissues: p=0.354 ultimate tensile strength; p=0.561 elastic modulus.

Tissue	Ultimate tensile stress MPa (SD)	Elastic modulus MPa (SD)
Cellular conjunctiva	0.7 (0.5)	3.9 (5.6)
Decellularised conjunctiva	0.5 (0.9)	3.0 (3.6)
Cellular amnion	1.7 (1.1)	11.5 (6.1)
Decellularised amnion	1.8 (0.7)	12.0 (5.1)
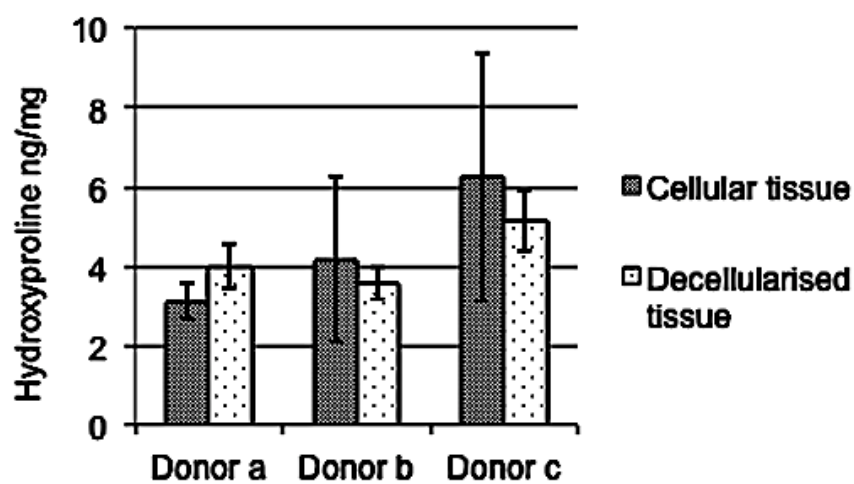
Figure 1 Hydroxyproline found in assays of paired samples of cellular and decellularised tissue (n=3 in each group, error bars +/-SD). Positive control 196.7 (+/- SD 31.9). Negative control 2.82 (+/-SD 0.9).
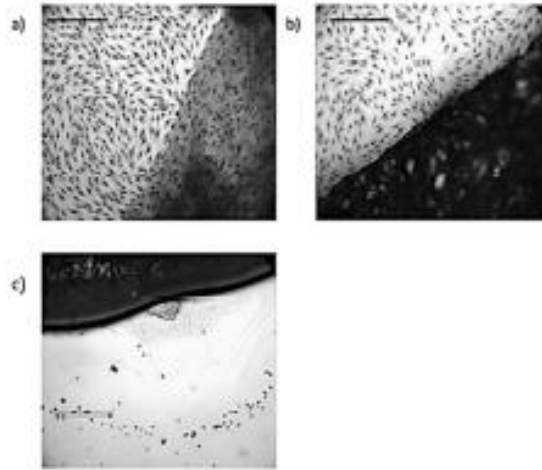
Figure 2 Representative photomicrographs of fixed Giemsa stained human conjunctiva cell line (HCjE-Gi cells in culture with materials fixed to tissue culture plates after 48 hours. a) decellularised conjunctiva with cells visualised in contact with tissue b) SteristripsTM, the experimental negative control known not to exhibit cytotoxicity, visualised with cells in close contact c) cyanoacrylate glue, the experimental positive control exhibiting cytotoxicity with surrounding HCjE-Gi and primary human skin fibroblast cellular debris and a large zone of inhibition. Scale bars 200µm.
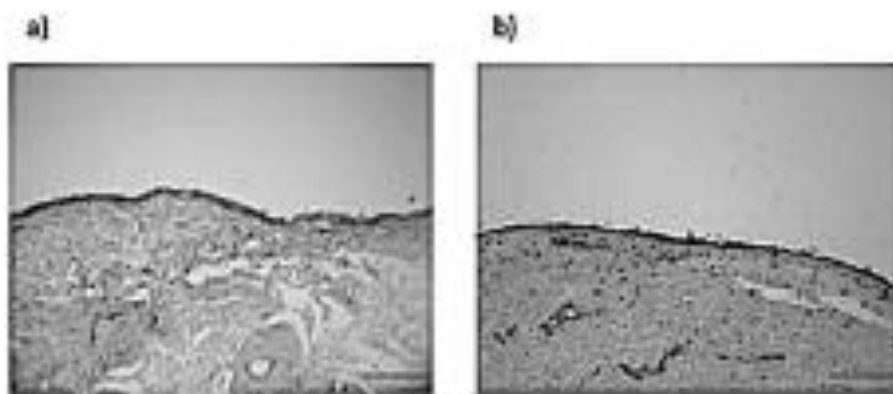
Figure 3 Representative photomicrographs of H&E stained sections. a) decellularised conjunctiva b) cellular conjunctiva. Scale bars 200 μm.
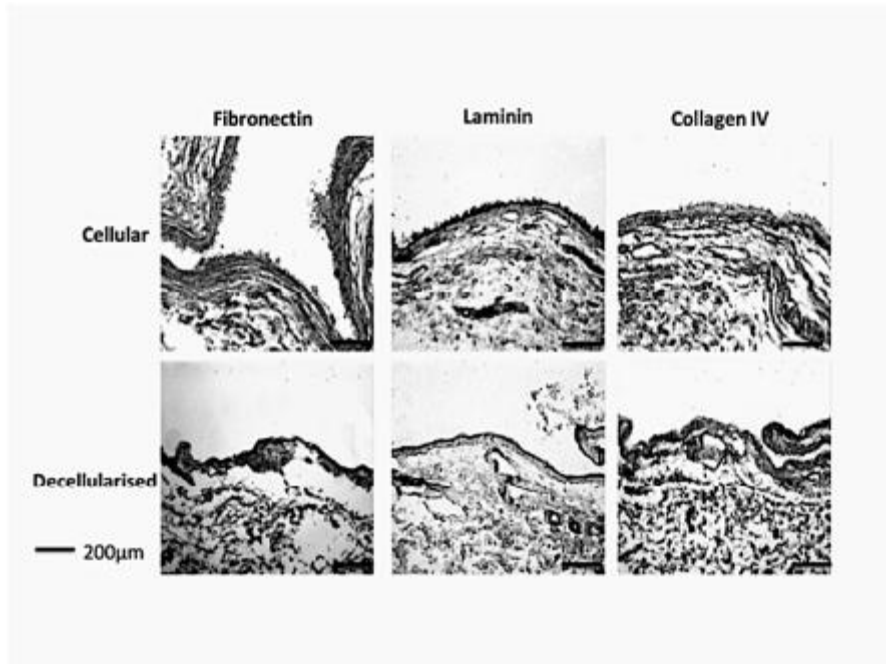
Figure 4 Representative photomicrographs of fixed and paraffin embedded matched cellular and decellularised tissues have stained with laminin, collagen IV and fibronectin. Black staining represents positive immunolocalisation of the extracellular matrix protein of interest.
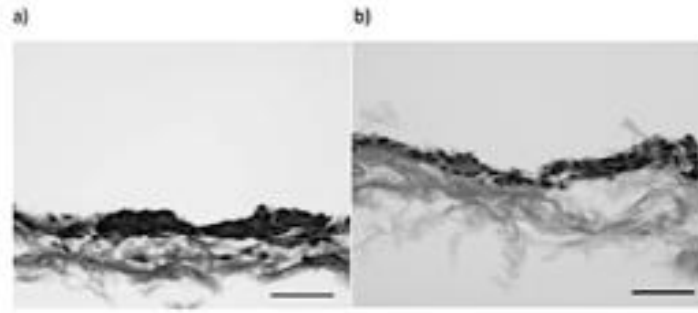
Figure 5 Representative photomicrographs of H&E stained sections. Allogeneic decellularised tissues have been recellularised with conjunctival epithelium a) and b). Scale bars 100 μm.
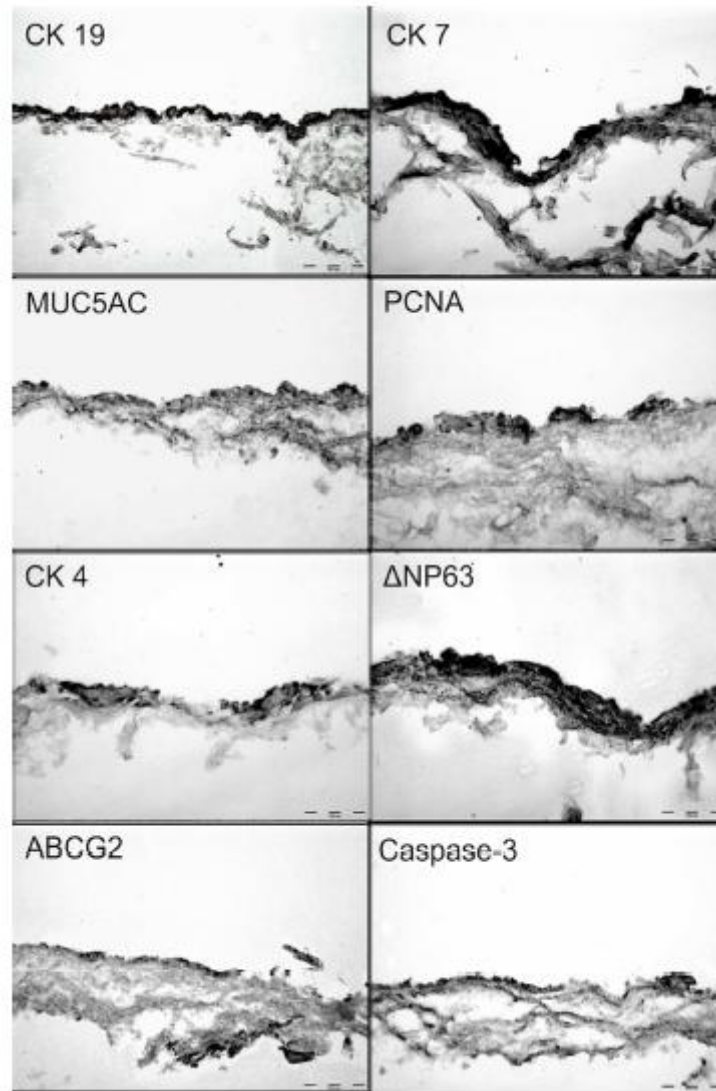
Figure 6 Representative photomicrographs of tissue sections of decellularised conjunctiva j recellularised using explants from donor k for immunohistochemistry. Black staining represents positive immunolocalisation of the respective marker studied. Scale bars 100μm.