Cryopreserved Amniotic Membrane as Transplant Allograft: Viability and Post-transplant Outcome

Natasha M. J. Perepelkin,[2] Kirsten Hayward,[2] Tumelo Mokoena,[1] Michael J. Bentley,[1]

Lisa U. Ross-Rodriguez,[1,2,3] Leah Marquez-Curtis,[2,3]

Locksley E. McGann,[2] Jelena L. Holovati[1,2] and Janet A. W. Elliott[2,3]

[1]Comprehensive Tissue Centre, Alberta Health Services, Edmonton, Alberta

[2]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta

[3]Department of Chemical and Materials Engineering, University of Alberta, Edmonton, Alberta

Corresponding Author:

Janet A. W. Elliott

Department of Chemical and Materials Engineering

University of Alberta, Edmonton, AB Canada T6G 1H9

Email: janet.elliott@ualberta.ca

Phone: (780)-492-7963

Fax: (780)-492-2881

Abstract

Amniotic membrane (AM) transplantation is increasingly used in ophthalmological and dermatological surgeries to promote re-epithelialization and wound healing. Biologically active cells in the epithelial and stromal layers deliver growth factors and cytokines with anti-inflammatory, anti-bacterial, anti-immunogenic and anti-fibrotic properties. In this work, confocal microscopy was used to show that our cryopreservation protocol for AM yielded viable cells in both the stromal and epithelial layers with favorable post-transplant outcome. AM was obtained from Caesarean-section placenta, processed into allograft pieces of different sizes (3 cm × 3 cm, 5 cm × 5 cm, and 10 cm × 10 cm) and cryopreserved in 10% dimethyl sulfoxide using non-linear controlled rate freezing. Post-thaw cell viability in the entire piece of AM and in the stromal and epithelial cell layers was assessed using a dual fluorescent nuclear dye and compared to hypothermically stored AM, while surveys from surgical end-users provided information on post-transplant patient outcomes. There was no significant statistical difference in the cell viability in the entire piece, epithelial and stromal layers regardless of the size of allograft piece ($p = 0.092$, 0.188 and 0.581, respectively), and in the entire piece and stromal layer of hypothermically stored versus cryopreserved AM ($p = 0.054$ and 0.646, respectively). Surgical end-user feedback ($n = 49$) indicated that 16.3% of AM allografts were excellent and 61.2 % were satisfactory. These results support the expanded clinical use of different sizes of cryopreserved AM allografts and address the issue of orientation of the AM during transplant for the treatment of dermatological defects and ocular surface disorders.

Keywords: amniotic membrane, stroma, epithelium, cryobiology, cryopreservation, ocular surgery, dermal surgery, tissue transplantation

Introduction

Since the first documented report of the use of fetal membrane in skin transplantation over a century ago (Davis 1910), human amniotic membrane (AM) has been widely applied in the management of burns, dermatological defects and ocular surface reconstruction (Lo and Pope 2009; Liu et al. 2010; Meller et al. 2011; Mamede et al. 2012; Fairbairn et al. 2014; Malhotra and Jain 2014; Zelen et al. 2015). The AM is thin (0.02 to 0.05 mm), lightweight, elastic and almost transparent rendering it suitable for these therapeutic applications (Lo and Pope 2009; Malhotra and Jain 2014). It constitutes the innermost layer of the amniotic cavity and consists of a single layer of cuboidal epithelial cells firmly adhered to a thick basement membrane, which is in turn attached to an avascular stromal layer, also known as the amniotic mesenchyme (Fairbairn et al. 2014). This stromal layer is further composed of a compact sheet of reticular fibers, a fibroblast layer and a spongy layer covered in mucin, a glycoprotein lubricant, which allows the AM to be easily separated from the placenta by means of blunt dissection (Niknejad et al. 2008; Riau et al. 2010; Malhotra and Jain 2014). The cells in the epithelial and stromal layers produce biological factors and mediators that contribute to the therapeutic benefits of AM including anti-bacterial, anti-viral, anti-inflammatory, anti-fibrotic, anti-angiogenic, pro-apoptotic, pro-epithelialization and analgesic properties (Wolbank et al. 2009; Mamede et al. 2012; Werber and Martin 2013; Ricci et al. 2013; Fairbairn et al. 2014). Aside from being a source of non-immunogenic multipotent cells, AM can also serve as a scaffold and substrate for the growth, migration and adhesion of resident cells and thus find widespread applications in regenerative medicine, cellular therapy and tissue engineering (Niknejad et al. 2008; Shortt et al. 2009; Niknejad et al. 2011; Mamede et al. 2012; Kim et al. 2014). In addition to its favourable features, AM is readily available because placentas are traditionally considered as medical waste after childbirth. Over 100 allograft pieces can be obtained from one placenta for multiple transplant applications; hence AM banking provides an excellent opportunity for readily available allografts. Despite rigorous donor selection criteria, the time frame between infection and seroconversion may mask a donor's seropositivity; therefore banking the AM allows a window not only for serological re-testing, but also sterility assessment and functional evaluation.

There have been several approaches to preserve AM including hypothermic storage, lyophilisation, chemical preservation and cryopreservation, all of which can cause varying severity of cellular damage (Hennerbichler et al. 2007; Lo and Pope 2009; Riau et al. 2010; Allen et al. 2013; Tehrani et al. 2013). Lyophilisation is a means of

physically removing water from the tissue through drying while chemical preservation utilizes a high salt or glycerol concentration to sequester and reduce free water. The most common cryopreservation protocol reported in the literature involves the use of 50% glycerol and storage at –80 °C (Adds et al. 2001; Shortt et al. 2009; Hermans 2011; Schulze et al. 2012; Cooke et al. 2014). This procedure decellularizes the AM and results in low viability although the tissue morphology and structure are maintained (Kruse et al. 2000; Shortt et al. 2009; Cooke et al. 2014). Storage of AM in 50% glycerol at –80 °C for up to two years was shown not to impair its sterility and expression of extracellular matrix proteins (Thomasen et al. 2011; Cooke et al. 2014), leading to the conclusion that AM grafts seem to function primarily as a scaffold rather than as a source of fully functional cells. On the other hand, 463 thawed AM samples previously cryopreserved in 10% DMSO at –80 °C and stored in the vapor phase of liquid nitrogen at –140 °C, have been successfully transplanted in patients for corneal reconstruction; their efficacy has been attributed to the viability of cells (40% by trypan blue exclusion) which purportedly contributed growth factors for epithelialisation (Rama et al. 2001). Moreover, cryopreserved AM and fluid allograft containing live cells, proteins, cytokines and growth factors healed 90% (18 out of 20) of patients with foot and ankle wounds (Werber and Martin 2013), suggesting that AM cell viability is an important quality indicator of transplant allografts. However, it has recently been realized that protocols for evaluation of cell viability in AM are tedious and do not yield reliable results (Laurent et al. 2014).

The methods employed for the processing and preservation of AMs can drastically modify their structural and biochemical composition and potentially impair important signalling pathways that are crucial for their intended therapeutic use. Because AM transplantation is becoming more widespread, it is important to establish protocols adaptable for the clinical banking of AM that include verification of the graft quality and viability before its release for transplantation. Cryopreservation is the banking method currently used by the Comprehensive Tissue Centre (CTC) in Edmonton, Alberta, Canada for preserving AMs for regional and national ophthalmological and dermatological transplant applications. The CTC is one of only three full-service tissue banks in Canada, with accreditation and licences from Health Canada and the American Association of Tissue Banks, and is responsible for the recovery, processing, storage and distribution of tissues including ocular, musculoskeletal, cardiovascular, skin, surgical bone, and AM allografts for transplantation.

To the best of our knowledge, this paper is the first to investigate cell viability throughout the different layers, i.e. the epithelial and stromal layers, of the AM. Layer-specific viability may be important when deciding the best orientation that AMs should be applied on a patient during a transplant. For example, one recommended technique described for ophthalmological indications involves suturing the AM to the ocular surface with the epithelial side up and the stromal surface in contact with the eye in order to facilitate adherence of the membrane and migration of resident epithelial cells (Dua and Azuara-Blanco 1999; Rama et al. 2001). On the other hand, the orientation of the AM plays only a minor role in the "onlay" technique where the AM is temporarily placed on the cornea as a patch and then detached after one to two weeks (Meller et al. 2011). In any case, it is important to determine whether cryopreservation affects the viability of the cells in the different layers of the AM in order to address the issue of which side should be in contact with the damaged tissue when the AM is transplanted. Moreover, because the application of AM allografts also depends on the size of the area requiring tissue reconstruction (e.g., smaller for cornea versus bigger for skin burn wound), we investigated whether the size of the allograft affects the viability of the cell layers. CTC uses the same cryopreservation protocol for different AM sizes, but different packaging; therefore, we investigated whether post-thaw viability is affected by the different sizes of the AM allograft. We also compared the viability of cells in hypothermically stored and cryopreserved AMs to validate the acceptability of cryopreserved AMs for transplant applications. Finally, we report surgical feedback on the post-transplant AM allografts provided by CTC for corneal surgeries using the Cells, Tissues and Organs Surveillance System (CTOSS).

Materials and Methods

Selection of donors of AM

AM donor selection for this study was based on Health Canada, Canadian Standards Association (CSA) and American Association of Tissue Banks (AATB) standards. Informed consent was obtained from mothers who were scheduled for a Caesarian section (gestation week 39). A questionnaire on the donor's medical and social history was completed and additional transmissible disease tests were performed, including a physical examination and serology testing for Human Immunodeficiency Virus Type 1 and Type 2 (HIV-l/ll), Herpes Simplex Virus, Hepatitis B and C, Syphilis, Human T-lymphocytic Virus Type 1 and 2 (HTLV l/ll), Cytomegalovirus, West Nile Virus and Toxoplasma. Any positive serology result or high-risk behaviour from the social/medical questionnaire was a basis

for exclusion from this study. Samples used for preliminary experiments were procured following the procedures approved by the Human Research Ethics Board of the University of Alberta. All other samples were obtained following the quality management protocols of the CTC and approved by the provincial health authority board, Alberta Health Services.

Processing and cryopreservation of AM

Shortly after delivery in an operating room, the placenta was packaged under sterile conditions and transported on wet ice (2 to 10 °C) to the CTC in two sterile bags filled with Ringer's lactate solution (Baxter Corp., Mississauga, ON, Canada). Following aseptic techniques, the placenta was placed under a Class 100 Level II biosafety cabinet where the AM was separated from the placenta by means of blunt finger dissection (Fig. 1a). Once separated, the AM was washed twice in Ringer's lactate solution and gently massaged to remove all blood clots (Fig. 1b). The AM was then immersed for a minimum of two hours in X-VIVO 10 media (Lonza, Walkersville, MD, USA) containing 10,000 units/mL penicillin and 10,000 µg/mL streptomycin (GIBCO, Life Technologies, Grand Island, NY, USA). Sterility testing on a sample was carried out before and after the decontamination step. Within three hours after decontamination the AM was placed on nitrocellulose filter paper (Pall Corporation, Port Washington, NY, USA), cut into 3 cm × 3 cm, 5 cm × 5 cm, and 10 cm × 10 cm pieces and stored in individual sterile pouches with cryopreservation solution containing X-VIVO 10 media and 10% dimethyl sulfoxide (DMSO, Origen BioMedicals Inc., Austin, TX, USA) (Fig. 1c). The pouches were heat-sealed and cooled in a CryoMed controlled-rate freezer (Thermo Scientific, Waltham, MA, USA). Non-linear controlled-rate freezing was used to cryopreserve the AMs. The samples were initially cooled to the same temperature as the chamber (4 °C) and further down to –5 °C at 1 °C/min. To induce ice nucleation the chamber was cooled at 30 °C/min to –55 °C then warmed at 10°C/min to –15 °C. Ice nucleation was recorded to take place at –10.9 ± 0.45 °C and was accompanied by the release of the latent heat of fusion. The sample was further cooled at 1 °C/min to –50 °C and then at 5 °C/min to –70 °C and finally transferred to liquid nitrogen vapour phase for storage (≤ –130 °C).

Preliminary experiments to optimize cell viability assessment

Cryopreserved AMs (3 cm × 3 cm pieces) were thawed in a room temperature water bath (21 to 29 °C) for 5 min, removed from the plastic packaging using sterile forceps and incubated in Ringer's lactate solution for 2 min. The

tissues were transferred into phosphate buffered saline (PBS, Life Technologies) on ice and cut into approximately 1 cm × 1 cm pieces. An individual piece was placed on a slide and dual SYTO® 13 (Molecular Probes, Eugene, OR) and ethidium bromide (EB) stain (Sigma-Aldrich, St. Louis, MO) (9 μM SYTO® 13 and 5.7 μM EB in PBS) was added drop-wise until the tissue was covered. The tissue was incubated on ice for 2 to 10 min, cover-slipped using the stain as mounting media and sealed.

Slides were examined using the Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems Inc., Concord, ON, Canada) with an HCX PLAPO CSx63 water immersion lens (NA=1.2). The fluorescence of SYTO® 13 (509 nm, green) and EB (605 nm, red) were simultaneously detected under the 488 nm excitation wavelength of the argon beam. The argon beam was set at 30% power, with bandwidths set between 492 nm and 541 nm for SYTO® 13, and 613 nm and 735 nm for EB. The Leica Application Suite Advanced Fluorescence (LAS AF, Leica Microsystems) acquisition software was used to image the tissues. Images (1024 × 1024) were acquired at 100 Hz speed, with each piece of tissue imaged at the epithelial level and in 5 μm increments throughout the stromal layer (from 15.5 μm to 95.5 μm). The numbers of red and green cells were counted manually in the preliminary experiments, and the percent viability was calculated from the ratio of the number of green cells and the total number (green + red) of cells.

Prospective analysis of AM viability

Prospective experiments examined how cryopreservation and processing different sizes of AM sections affect the quality of the final allograft for transplant issued by the CTC, as well as the potential differences in the AM cell viability between the stromal and epithelial AM layer. “Fresh” hypothermically stored AM pieces (after AM processing, but before cryopreservation step) 10 cm × 10 cm (n = 9), cryopreserved 3 cm × 3 cm pieces (n = 9), cryopreserved 5 cm × 5 cm pieces (n = 9) and cryopreserved 10 cm × 10 cm pieces (n = 10) were stained with 9 μM SYTO® 13 and 5.7 μM EB, visualised using the Plan-Apochromat 20×/0.8 M27 objective on a Zeiss Observer LSM 710 confocal microscope and analyzed using 2011 ZEN software (Carl Zeiss, Oberkochen, Germany). Live cells that stained green with SYTO® 13, dead cells that stained red with EB, and cells with double staining (yellow) were quantitated using IMARIS x64 7.6.5 software (Bitplane Scientific, South Windsor, CT, USA). The percent viability was calculated from the ratio of the number of green cells to the total number (green + red + yellow) of cells.

One-way ANOVA statistical analysis was used to compare percent viability among the three different sizes of cryopreserved AMs (3 cm × 3 cm, 5 cm × 5 cm and 10 cm × 10 cm) in the entire piece, the epithelial layer and the stromal layer using the SPSS version 22.0 software (IBM, Armonk, NY). Paired t-tests were used to determine statistical significance of differences in the percent viability in the entire piece, epithelial and stromal layers of "fresh" hypothermic versus cryopreserved 10 cm × 10 cm AM pieces. A p-value < 0.05 was deemed statistically significant.

Controls used in the prospective study included a "fresh" hypothermically stored AM, and a positive control, a negative control, and an auto-fluorescence control. The "fresh" AM was processed the same way as the cryopreserved AMs but instead of being placed in individual sterile pouches containing cryopreservation solution, it was placed in X-VIVO 10 media and stored at 4 °C for a total of 6 hours after processing. The positive control was cryopreserved AM that was placed in 70% ethanol, and the negative control consisted of a slide with no tissue and with EB and SYTO® 13 stain. To check for auto-fluorescing cells, PBS was added to cryopreserved AM instead of EB/SYTO® 13 stain.

Retrospective analysis: Cells, Tissues and Organs Surveillance System (CTOSS) reports

The Cells, Tissues and Organs Surveillance System (CTOSS) is a Canadian national adverse event surveillance system related to organ and tissue transplantation with the goals of monitoring and analysis of the incidence and prevalence of adverse events in the transplant recipient populations and facilitating the reporting of specific adverse events to the Public Health Agency of Canada. As part of this surveillance program, CTC requests surgical end-users to provide follow-up information on patient diagnosis associated with the tissue transplant and tissue transplant recipients' outcome and presence of any tissue-related adverse reaction or event 90 days after the transplant. CTOSS responses from CTC AM transplants were collected and summarized from the submissions dating January 1st to December 31st, 2013. The transplant outcomes were classified under four categories; no reply, unsatisfactory, satisfactory, and excellent. If the response was unsatisfactory or in case of an identified adverse event, the transplant surgeon was asked to elaborate with additional information.

Results

Preliminary assessment of cell viability by confocal microscopy

The membrane-impermeant EB dye binds very strongly with nucleic acids of membrane-compromised cells and emits a red fluorescence, while the membrane-permeant SYTO® 13 is a lower affinity nucleic acid stain that permeates all cells and emits a green fluorescence. The viability of cells in the epithelial and stromal layers was determined by counting green and red cells from five 3 cm × 3 cm cryopreserved AM tissues that had been cut into 1 cm × 1 cm pieces each. Figure 2a is a snapshot taken from a 3D movie constructed from confocal microscope images showing that cells are denser in the epithelial layer and are more scarce in the stromal layer. In the preliminary experiments, the overall viability of AM following cryopreservation was 62.3 ± 39.4% (mean ± SD). When examined separately, the stromal layer cell viability was 63.3 ± 40.3% and the epithelial layer cell viability was 57.0 ± 32.5%. Figure 2b shows the cell viability in the stromal layer of AM tissues (n = 5) as a function of distance from the epithelium (5 μm increments from 15.5 μm to 95.5 μm). It is important to point out that the number of cells detected in the stromal layer was very low (0 to 40 cells per measurement at one depth), resulting in very high standard deviations in the presence of outliers. Nevertheless, these preliminary results indicate that there does not appear to be a correlation between tissue depth and percent viability.

Analysis of AM viability controls

Focused on the epithelium, the "fresh" hypothermic control AM stained with SYTO® 13/EB clearly showed both live (green) and dead (red) cells (Figure 3a). Figure 3b shows a positive control where cells were chemically killed, and confirms that dead cells took up EB and fluoresced red even in the presence of the SYTO® 13 stain. The negative control (no tissue) confirmed the absence of cells (Figure 3c) while the auto-fluorescence control slide demonstrated the absence of background signal (Figure 3d).

Viability of cells in the epithelial and stromal layer

The difference in cell density between the epithelial and stromal layers is clearly seen in Figure 4. In the epithelial layer, all three sizes of cryopreserved AMs (3 cm × 3 cm, 5 cm × 5 cm, and 10 cm × 10 cm) and "fresh" hypothermically stored AMs demonstrated similar percent viability, where approximately 40% of cells remained viable after cryopreservation (Figure 4, top). Figure 4 (bottom) shows the variability in the number of red and green cells in the stromal layer for all sizes of cryopreserved AMs and "fresh" hypothermically stored AMs. Figure 5 shows the percentage viability in the entire tissue, and in the epithelial and stromal layers of different sizes of

cryopreserved and "fresh" (hypothermically stored) AMs. There was no significant statistical difference in the percent viability of cells in the entire piece, the epithelial layer and the stromal layer regardless of the size of cryopreserved AM tissues (p values = 0.092, 0.188 and 0.581, respectively). The percent viability in the entire tissue and in the stromal layer of the "fresh" hypothermically stored AMs was also not statistically different from that of their cryopreserved counterparts (p values = 0.054 and 0.646 respectively). The only statistically significant difference was in the % viability in the epithelial layer of the fresh versus cryopreserved AM pieces (p = 0.028).

Cells, Tissues and Organs Surveillance System (CTOSS) Reports

From January 1st to December 31st, 2013, CTC received 49 CTOSS post-operative transplant reports on AMs issued by the CTC. The compliance response rate from transplant surgeons was 80%. The clinical diagnoses associated with CTC AM transplant are summarized in Figure 6. Of the 49 CTOSS post-transplant responses, 16% of AM allografts (8/49) were rated excellent and described as a good promoter of healing with no inflammation, infection or scarring on follow-up appointments with transplant recipients. The majority of surgeons assessed the AM quality as satisfactory/fair/good which caused minimal scarring and promoted a good healing response 82% (40/49). The remaining 2% (*i.e.*, 1 report) deemed the AM as unsatisfactory, but the reason indicated ("patient presented new recurrence of pterygium") was neither specific to the AM allograft nor classified as an allograft-associated adverse event.

Discussion

This paper is the first to report a comparison in cell viability across the layers of cryopreserved AM and the findings have implications in the use of AM for transplant. Cell viability in different layers of the AM was assessed using fluorescent nuclear dyes detectable by confocal microscopy, a technique not routinely performed by clinical tissue banks. Others have shown that trypan blue exclusion and Giemsa staining are sufficient to provide an indication of tissue integrity (Kruse et al. 2000; Rama et al. 2001; Hennerbichler et al. 2007; Niknejad et al. 2011; Laurent et al. 2014). However, confocal microscopy offers distinct advantages over conventional fluorescent microscopy including the capability to control the depth of field, eliminate or reduce background noise from the focal plane, collect serial optical sections, and obtain extremely high-quality images from thick specimens. In fact, laser confocal microscopy has been employed to resolve the four distinct layers of the AM (Kobayashi et al 2008).

First, in this work, it was found that the cell viability in the entire tissue (overall) and in the stromal layer of "fresh" hypothermically stored AMs were not statistically different from those in the cryopreserved AMs. There was a slight statistical difference in the viability of cells in the epithelial layer between the fresh and cryopreserved AMs, but overall the CTC cryopreservation protocol did not compromise the quality of the AM allografts. Cryopreservation using 10% DMSO has also been employed by others (Rama et al. 2001; Hennerbichler et al. 2007; Ricci et al. 2013; Laurent et al. 2014), with viability ranging from 15–40%, corroborating the findings here. Interestingly, the studies that reported the upper limit (40%) made use of trypan blue in the post-cryopreservation assessment of cell viability. Trypan blue is a membrane-impermeant stain that binds to intracellular proteins in membrane-damaged cells rendering them a bluish color while leaving membrane-intact cells unstained. It has been shown that trypan blue viability measurements in samples with <70% viability show higher measured cell viability when compared to fluorescent-based detection methods (Mascotti et al. 2000). This work employed SYTO® 13, a DNA/RNA binding stain that permeates all cells and fluoresces green, along with ethidium bromide, a high affinity nuclear stain that permeates cells with damaged plasma membranes and stains them red. Cells with partially damaged membranes can take in a small amount of EB that is not enough to fully block the SYTO® 13 fluorescence and fluoresce yellow. In this work a more stringent calculation was applied by counting purely green cells as live and doubly-stained cells as dead.

Furthermore, the results presented herein on the quality of DMSO-cryopreserved AM support previous findings which showed that membrane integrity (by acridine orange/propidium iodide staining) and mitochondrial activity (by MTT assay) of glycerol-preserved and "fresh" hypothermically stored AM allografts are no different (Adds et al. 2001). Moreover, the post-transplant surveillance data reported here suggest effectiveness and safety of AM allografts used for treatment of various clinical disorders. Similarly, it was previously reported that both fresh and frozen AM tissues resulted in an improvement in visual acuity, when transplanted to a patient with total corneal epithelial failure (Adds et al. 2001). In rats with induced liver fibrosis, both freshly prepared and AM cryopreserved in 10% DMSO elicited the same anti-scarring effects (Ricci et al. 2013). Also, there was no difference detected in fresh, cryopreserved or lyophilized AM in terms of antibacterial property (Tehrani et al. 2013), expression of toll-like receptors (Micera et al. 2014), expression of complement inhibitory protein CD59 (Füst et al. 2012), and expression of trefoil factor family peptide (TFFP)-3, which is involved in ocular surface restitution after corneal injury (Schulze et al. 2012).

Next, in this work no statistical difference was found in the overall percent viability of cells and the cell viability in the epithelial layer and the stromal layer; although the number of cells in the epithelial layer is significantly more than in the stromal layer. Questions still remain as to whether the intact or decellularized AM is better for ocular surface reconstruction and dermatological applications. Glycerol-preserved AM has been shown to produce a number of growth factors including epidermal growth factor, keratinocyte growth factor, hepatocyte growth factor and basic fibroblast growth factor that can contribute to epithelialization after AM transplantation (Koizumi et al. 2000). Higher levels of these growth factors were detected in AM with intact epithelium; moreover, the epithelial layer secreted more of them compared to the stromal layer, suggesting that AM layers might exert different effects on corneal reconstruction and may influence the use of AM depending on the nature of injury (Koizumi et al. 2000). For example, when used as a matrix to support the growth of chondrocytes for cartilage repair, it was shown that the less cell-populated stromal layer but not the epithelial layer supported growth and adhesion (Díaz-Prado et al. 2010). There is also an ongoing debate with regards to the orientation (epithelial-side or stromal-side) of the AM when transplanted to damaged tissue. In one study, amniotic membrane that was sutured to damaged cornea with the epithelial side up promoted rapid healing in four of five patients with persistent epithelial defects (Azuara-Blanco et al 1999). The findings presented in this paper could suggest that for AM allografts cryopreserved by CTC, the orientation may not matter if it is strictly percent cell viability that is desired because the cell viability is not significantly different between the two layers; however, there is a difference in cell numbers and biological action between the two layers that may be important in specific clinical applications.

In this work it was also found that the overall percent viability of cells and the cell viability in the epithelial layer and the stromal layer of cryopreserved AM was almost unaffected by the size of the allograft. The application of AM allografts depends on the size of the area requiring tissue repair, e.g. smaller for ocular reconstruction and bigger for a skin burn wound. Previously, CTC cryopreserved only 1.5 cm × 1.5 cm, 3 cm × 3 cm, and 5 cm × 5 cm AM allograft pieces, but recently the CTC has implemented the 10 cm × 10 cm size after appropriate validation. Our results show that the current cryopreservation protocol does not affect the percent viability of the stromal and epithelial layers of different sizes of AM allografts, thus expanding the possible use of larger AM allograft pieces as a therapeutic option for patients with burn wounds, ulcers, epidermolysis bullosa and Stevens-Johnson syndrome without compromising quality and efficacy. The CTOSS reports collected for this study suggested that the majority of surgeons viewed the AM allografts from CTC as being of satisfactory quality for corneal surgeries. Although it is

desirable to garner 100% feedback, for the 80% of transplant tissues that received feedback, the current high level of approval from transplant specialists (98% of tissues rated satisfactory or better) can propel an increase in the transplant of AM allografts as primary therapy instead of just an alternative option, and broaden its usefulness in tissue repair other than ocular.

Although the percentage of viable cells in either hypothermically stored or cryopreserved AM is less than 40%, the cells exist in an environment composed of extracellular matrix-associated and cell structural proteins (Hopkinson et al. 2006). This becomes relevant in situations when AM allografts are primarily used as a matrix or scaffold. For example, lyophilized AM is a suitable matrix for *ex vivo* culture of rat endothelial cells because lyophilisation exposed the extracellular components of the basement membrane which induced the adhesion and proliferation of the endothelial cells (Niknejad et al. 2011). Decellularized or denuded AM grafts are also preferred for tissue engineering applications (Niknejad et al. 2008; Hopkinson et al. 2008). In fact, it has been reported that denuded AM can promote better cell proliferation, differentiation and adhesion, as well as more uniform cell outgrowth compared to intact AM (Koizumi et al. 2006). Moreover, the presence of an amniotic epithelium has been shown to hinder the uniform expansion of explants cultured on the membrane and delay the formation of strong hemidesmosomal attachment necessary for faster wound healing (Burman et al. 2004).

In conclusion, we showed that there are no statistical differences in cell viability between the epithelial and stromal layers of AMs, between cryopreserved and hypothermically stored AM in the entire piece and stromal layer, and between different sizes of AM allograft pieces (3 cm × 3 cm versus 5 cm × 5 cm and 5 cm × 5 cm versus 10 cm × 10 cm). Our results support the expanded use of AM allografts in the management and treatment of chronic wounds, dermatological defects and ocular surface disorders.

Acknowledgements

This work was partially funded by the Canadian Institutes of Health Research (CIHR MOP 86492, OGBF INO 126778, INO 131572, and MOP 133684). J. A. W. Elliott holds a Canada Research Chair in Thermodynamics. The authors would like to thank Luciana Da Silveira Cavalcante for her assistance with statistical analysis.

REFERENCES

Adds PJ, Hunt CJ, Dart JK (2001) Amniotic membrane grafts, “fresh” or frozen? A clinical and in vitro comparison. Br J Ophthalmol 85:905–7.

Allen CL, Clare G, Stewart EA, et al (2013) Augmented dried versus cryopreserved amniotic membrane as an ocular surface dressing. PLoS One 8:e78441. doi: 10.1371/journal.pone.0078441

Azuara-Blanco A, Pillai CT, Dua HS (1999) Amniotic membrane transplantation for ocular surface reconstruction. Br J Ophthalmol 83:399–402. doi: 10.1111/j.1442-9071.2006.01408.x

Burman S, Tejwani S, Vemuganti GK, et al (2004) Ophthalmic applications of preserved human amniotic membrane: A review of current indications. Cell Tissue Bank 5:161–175. doi: 10.1023/B:CATB.0000046067.25057.0a

Cooke M, Tan E, Mandrycky C, et al (2014) Comparison of cryopreserved amniotic membrane and umbilical cord tissue with dehydrated amniotic membrane/ chorion tissue. J Wound Care 23:465–476.

Davis JS (1910) Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. Johns Hopkins Hosp Reports 15:307–396.

Díaz-Prado S, Rendal-Vázquez ME, Muiños-López E, et al (2010) Potential use of the human amniotic membrane as a scaffold in human articular cartilage repair. Cell Tissue Bank 11:183–95. doi: 10.1007/s10561-009-9144-1

Dua HS, Azuara-Blanco A (1999) Amniotic membrane transplantation. Br J Ophthalmol 83:748–752.

Fairbairn NG, Randolph MA, Redmond RW (2014) The clinical applications of human amnion in plastic surgery. J Plast Reconstr Aesthetic Surg 67:662–75. doi: 10.1016/j.bjps.2014.01.031

Füst A, Pállinger E, Stündl A, et al (2012) Both freshly prepared and frozen-stored amniotic membrane cells express the complement inhibitor CD59. Sci World J 2012:815615. doi: 10.1100/2012/815615

Hennerbichler S, Reichl B, Pleiner D, et al (2007) The influence of various storage conditions on cell viability in amniotic membrane. Cell Tissue Bank 8:1–8. doi: 10.1007/s10561-006-9002-3

Hermans MHE (2011) Preservation methods of allografts and their (lack of) influence on clinical results in partial thickness burns. Burns 37:873–81. doi: 10.1016/j.burns.2011.01.007

Hopkinson A, McIntosh RS, Shanmuganathan V, et al (2006) Proteomic analysis of amniotic membrane prepared for human transplantation: characterization of proteins and clinical implications. J Proteome Res 5:2226–35. doi: 10.1021/pr050425q

Hopkinson A, Shanmuganathan VA, Gray T, et al (2008) Optimization of amniotic membrane (AM) denuding for tissue engineering. Tissue Eng Part C Methods 14:371–81. doi: 10.1089/ten.tec.2008.0315

Kim EY, Lee K-B, Kim MK (2014) The potential of mesenchymal stem cells derived from amniotic membrane and amniotic fluid for neuronal regenerative therapy. Biochem Mol Biol Reports 47:135–40.

Kobayashi A, Sugiyama K, Li W, Tseng S (2008) In vivo laser confocal microscopy findings of cryopreserved and fresh human amniotic membrane. Ophthalmic Surg Lasers Imaging 39:312–318.

Koizumi N, Inatomi T, Sotozono C, et al (2000) Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 20:173–177.

Koizumi N, Rigby H, Fullwood N, et al (2006) Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells. Graefe's Arch Clin Exp Ophthalmol 245:123–134.

Kruse FE, Joussen AM, Rohrschneider K, et al (2000) Cryopreserved human amniotic membrane for ocular surface reconstruction. Graefe's Arch Clin Exp Ophthalmol 238:68–75.

Laurent R, Nallet A, Obert L, et al (2014) Storage and qualification of viable intact human amniotic graft and technology transfer to a tissue bank. Cell Tissue Bank 15:267–75. doi: 10.1007/s10561-014-9437-x

Liu J, Sheha H, Fu Y, et al (2010) Update on amniotic membrane transplantation. Expert Rev Ophthalmol 5:645–661. doi: 10.1586/eop.10.63.Update

Lo V, Pope E (2009) Amniotic membrane use in dermatology. Int J Dermatol 48:935–40. doi: 10.1111/j.1365-4632.2009.04173.x

Malhotra C, Jain AK (2014) Human amniotic membrane transplantation: Different modalities of its use in ophthalmology. World J Transplant 4:111–21. doi: 10.5500/wjt.v4.i2.111

Mamede AC, Carvalho MJ, Abrantes AM, et al (2012) Amniotic membrane: from structure and functions to clinical applications. Cell Tissue Res 349:447–58. doi: 10.1007/s00441-012-1424-6

Mascotti K, McCullough J, Burger SR (2000) HPC viability measurement: Trypan blue versus acridine orange and propidium iodide. Transfusion 40:693–696. doi: 10.1046/j.1537-2995.2000.40060693.x

Meller D, Pauklin M, Thomasen H, et al (2011) Amniotic membrane transplantation in the human eye. Dtsch Arztebl Int 108:243–8. doi: 10.3238/arztebl.2011.0243

Micera A, Jirsova K, Normando EM, et al (2014) Molecular and biochemical expression of TLRs in human amniotic membrane: a comparative study of fresh and cryopreserved specimens. Graefe's Arch Clin Exp Ophthalmol 252:267–74. doi: 10.1007/s00417-013-2540-z

Niknejad H, Deihim T, Solati-Hashjin M, Peirovi H (2011) The effects of preservation procedures on amniotic membrane's ability to serve as a substrate for cultivation of endothelial cells. Cryobiology 63:145–51. doi: 10.1016/j.cryobiol.2011.08.003

Niknejad H, Peirovi H, Jorjani M, et al (2008) Properties of the amniotic membrane for potential use in tissue engineering. Eur Cell Mater 15:88–99.

Rama P, Giannini R, Bruni A, et al (2001) Further evaluation of amniotic membrane banking for transplantation in ocular surface diseases. Cell Tissue Bank 2:155–63. doi: 10.1023/A:1020158206073

Riau AK, Beuerman RW, Lim LS, Mehta JS (2010) Preservation, sterilization and de-epithelialization of human amniotic membrane for use in ocular surface reconstruction. Biomaterials 31:216–25. doi: 10.1016/j.biomaterials.2009.09.034

Ricci E, Vanosi G, Lindenmair A, et al (2013) Anti-fibrotic effects of fresh and cryopreserved human amniotic membrane in a rat liver fibrosis model. Cell Tissue Bank 14:475–88. doi: 10.1007/s10561-012-9337-x

Schulze U, Hampel U, Sel S, et al (2012) Fresh and cryopreserved amniotic membrane secrete the trefoil factor family peptide 3 that is well known to promote wound healing. Histochem Cell Biol 138:243–50. doi: 10.1007/s00418-012-0943-2

Shortt AJ, Secker GA, Lomas RJ, et al (2009) The effect of amniotic membrane preparation method on its ability to serve as a substrate for the ex-vivo expansion of limbal epithelial cells. Biomaterials 30:1056–65. doi: 10.1016/j.biomaterials.2008.10.048

Tehrani FA, Ahmadiani A, Niknejad H (2013) The effects of preservation procedures on antibacterial property of amniotic membrane. Cryobiology 67:293–8. doi: 10.1016/j.cryobiol.2013.08.010

Thomasen H, Pauklin M, Noelle B, et al (2011) The effect of long-term storage on the biological and histological properties of cryopreserved amniotic membrane. Curr Eye Res 36:247–55. doi: 10.3109/02713683.2010.542267

Werber B, Martin E (2013) A prospective study of 20 foot and ankle wounds treated with cryopreserved amniotic membrane and fluid allograft. J Foot Ankle Surg 52:615–21. doi: 10.1053/j.jfas.2013.03.024

Wolbank S, Hildner F, Redl H, et al (2009) Impact of human amniotic membrane preparation on release of angiogenic factors. J Tissue Eng Regen Med 3:651–654. doi: 10.1002/term.207

Zelen CM, Snyder RJ, Serena TE, Li WW (2015) The use of human amnion/chorion membrane in the clinical setting for lower extremity repair. Clin Podiatr Med Surg 32:135–146. doi: 10.1016/j.cpm.2014.09.002

Figure captions:

Fig. 1 (a) Separation of AM from placenta by means of blunt finger dissection; (b) AM washed in Ringer's lactate solution to remove all blood clots; (c) AM placed on nitrocelluose filter paper and kept in cryopreservation solution (X-VIVO 10 media and 10% DMSO)

Fig. 2a Cross-sectional view taken from a 3D movie constructed from confocal microscope images of AM with cell-packed epithelial layer and less dense stromal layer

Fig. 2b Cell viability in the stromal layer of AM as a function of depth from the epithelium. Results from Tissue #1 were excluded because of a rip in the tissue during slide preparation. Cells were visualised in a single field under 630× magnification with the Leica SP5 confocal microscope. The results represent mean ± SD from 1 cm × 1 cm pieces cut out from each 3 cm × 3 cm cryopreserved AM tissue (n = 5).

Fig. 3 SYTO® 13/EB staining of (a) fresh hypothermically stored AM tissue; (b) positive control for dead cells; (c) negative (no tissue) control; (d) auto-fluorescence control

Fig. 4 Representative staining with SYTO® 13/EB of the epithelial layer (top) and stromal layer (bottom) of different sizes of cryopreserved and "fresh" hypothermically stored AM

Fig. 5 Comparison of percent viability in the entire tissue and in the epithelial and stromal layers of different sizes of cryopreserved AM pieces (3 cm × 3 cm (n = 9), 5 cm × 5 cm (n = 9) and 10 cm × 10 cm (n = 10)), and hypothermically stored (fresh) AM (n = 9). Statistically significant difference was found in the percent viability of cells in the epithelial layer of fresh versus cryopreserved 10 cm × 10 cm AM pieces.

Fig. 6 CTC AM transplant recipient diagnoses (CTOSS recipient forms January – December 2013, n = 49, 80% response compliance)

Fig. 1

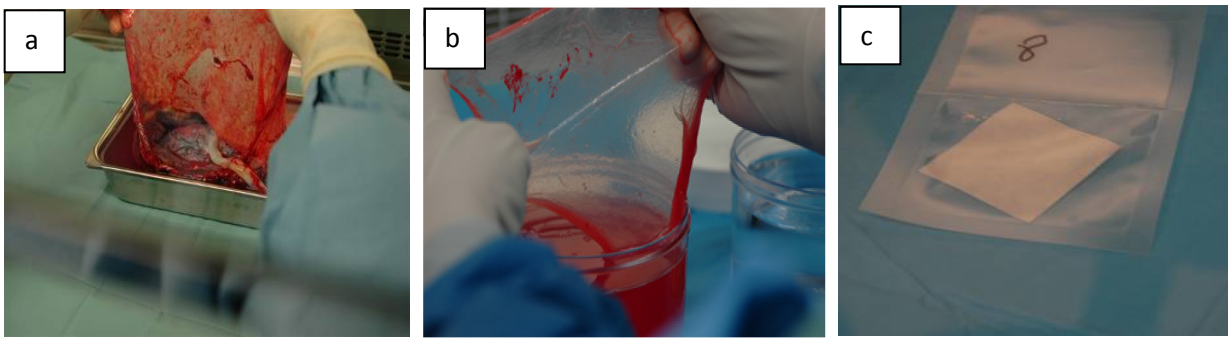


Fig. 2a

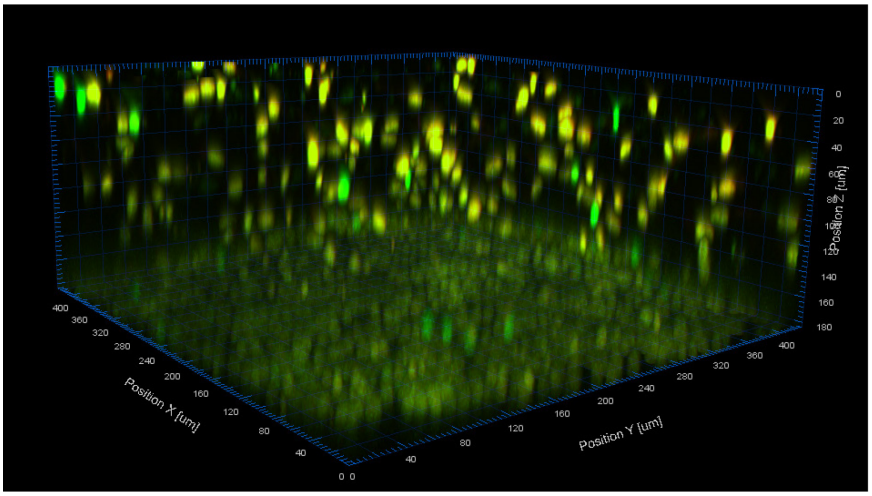

Position X [um]
Position Y [um]
Position Z [um]
Stromal layer
Epithelial layer

Fig. 2b

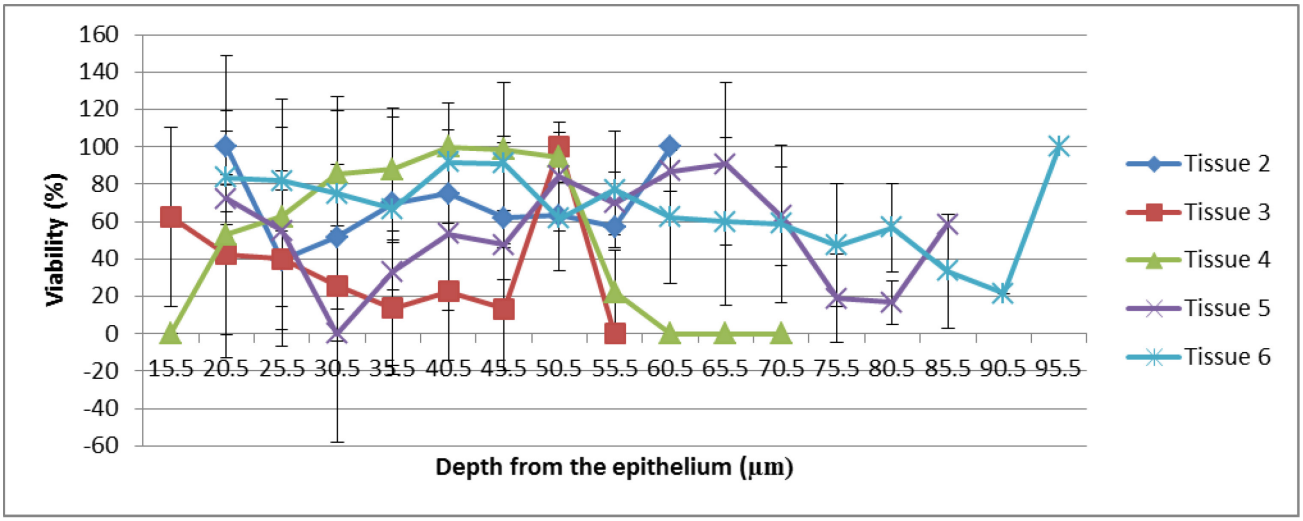

Viability (%)
160
140
120
100
80
60
40
20
0
-20
-40
-60
15.5 20.5 25.5 30.5 35.5 40.5 45.5 50.5 55.5 60.5 65.5 70.5 75.5 80.5 85.5 90.5 95.5
Depth from the epithelium (µm)
Tissue 2
Tissue 3
Tissue 4
Tissue 5
Tissue 6

Fig. 3

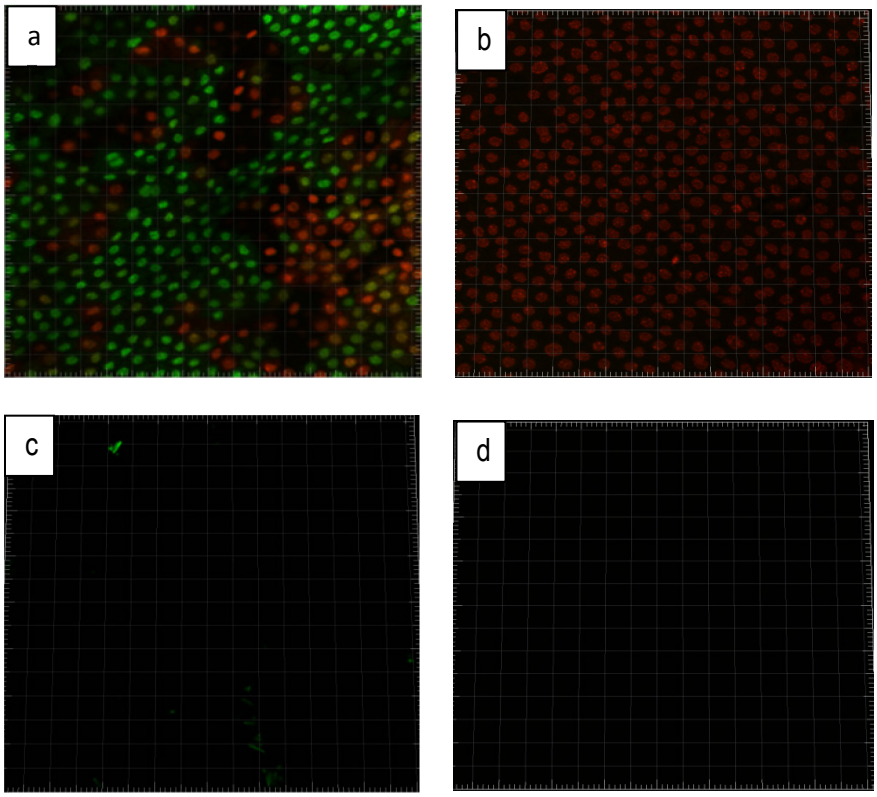


Fig. 4

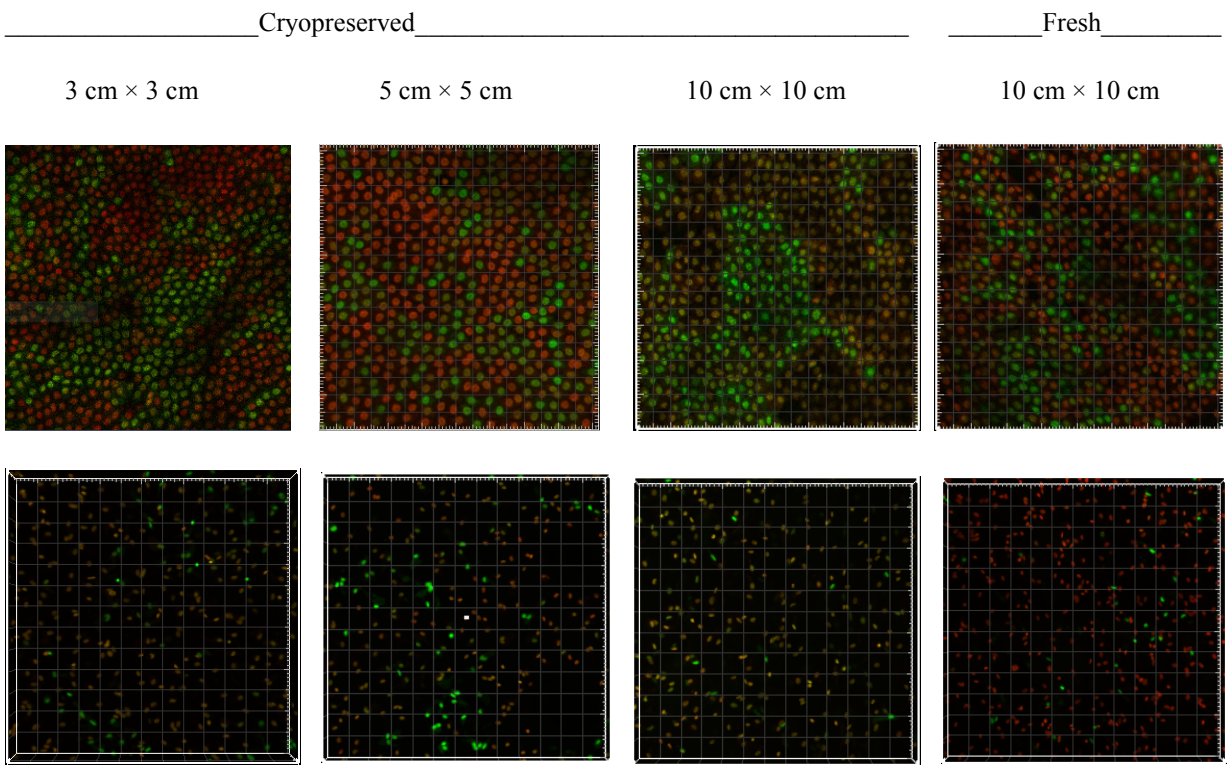


Fig. 5

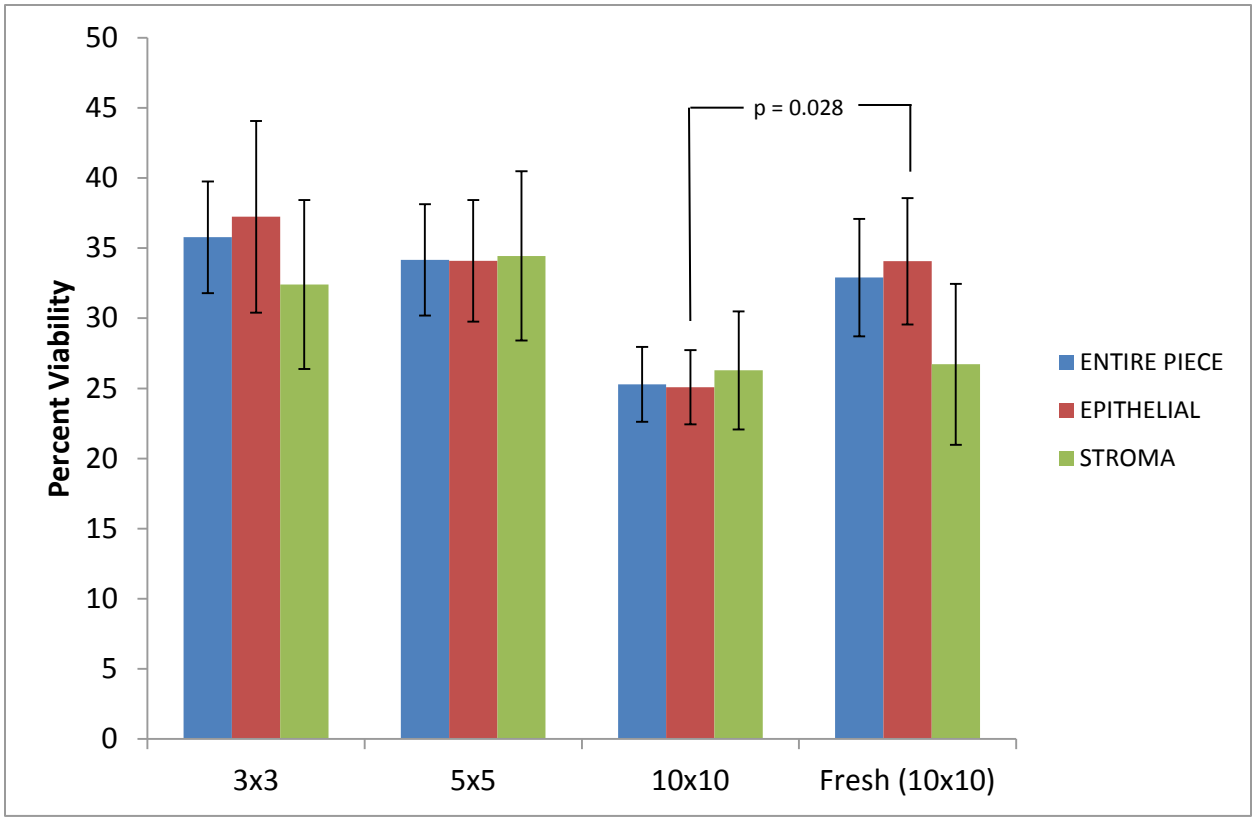
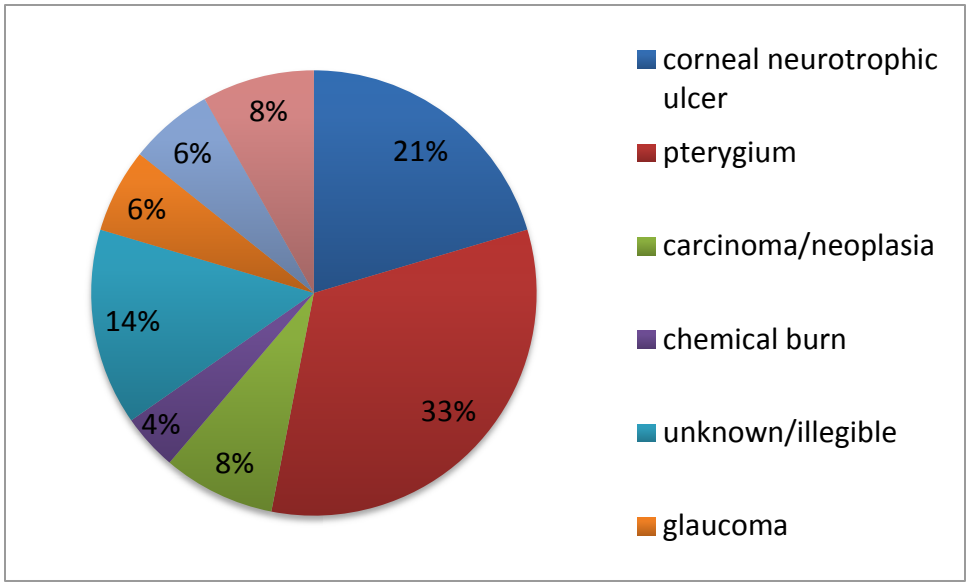

50
45
40
35
30
25
20
15
10
5
0
Percent Viability
p = 0.028
3x3
5x5
10x10
Fresh (10x10)
ENTIRE PIECE
EPITHELIAL
STROMA

Fig. 6


21%
33%
8%
4%
14%
6%
6%
8%
corneal neurotrophic ulcer
pterygium
carcinoma/neoplasia
chemical burn
unknown/illegible
glaucoma