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14. ABSTRACT Purpose: The overall goal of this research is to preserve vision of patients recovering from severe facial burns by providing an improved method to reduce development of corneal defects, inflammation, infection and opacification. Scope: To improve and understand the properties of the degradation-resistant crosslinked amniotic membranes for treating cornea of burn patients, Major findings: Identified three crosslinking methods that produced amnion with maximum protection against enzymatic degradation while remaining flexible enough to conform to cornea shape. Determined that protein crosslinking greatly reduces availability of beneficial factors in native amnion. Selected two crosslinking methods for in vivo evaluation. Demonstrated that with moderate crosslinking (chemical or photo crosslinking), one could improve with performance of the membrane (reduce degradation) without compromising its anti-inflammatory ability.				
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Crosslinked Amniotic Membrane vs Cryopreserved Amniotic Membrane for the Treatment of Severe Exposure Keratopathy in the New Zealand White Rabbit

Introduction:

Patients with severe facial burns often suffer indirect damage to their eyes as a sequela of ocular surface exposure keratopathy. Burn wound contracture of the periocular skin causes cicatricial ectropion resulting in ocular exposure. With loss of the blink reflex the patient quickly develops exposure keratitis. Due to its anti-inflammatory properties, amniotic membrane has developed into a mainstay of treatment to help maintain the ocular surface when skin grafting is delayed. However due to a host of factors, to include elevated room temperature, desiccation, and inflammatory cytokines in the tear film, the amniotic membrane breaks down rapidly, lasting only 1-3 days. Some patients have near total body surface area involvement of their burns, resulting in delays of weeks or months before skin is available for grafting to the eyelids. Replacing amniotic membrane every 1-3 days for such a prolonged time, results in significant expense and utilization of scarce resources. Utilizing our exposure keratopathy model developed for this experiment, we therefore sought to evaluate the efficacy and longevity of two different cross-linked amniotic membrane methods, diimide covalently crosslinked and Rose Bengal photo crosslinked vs. commercially available cryopreserved amniotic membrane, to determine if we could improve the longevity of the amniotic membrane without losing its anti-inflammatory properties.

BODY

This research project is a translational research project involving collaboration between myself and Dr. Irene Kochevar from the Wellman Group, Department of Dermatology, Massachusetts General Hospital, Boston. In this project, through telephone consultation with me, Dr. Kochevar developed the methodology to crosslink the amniotic membranes. Additionally, Dr. Kochevar developed, designed, and constructed the argon green laser delivery hand piece, the laser parameters, and treatment thresholds used to crosslink the rose Bengal impregnated amniotic membrane. After the parameters were determined, I then performed experimentation in-vivo using the New Zealand White Rabbit.

Methods:

Thirty-six New Zealand White Rabbits were included in this study. The IACUC committee at our institution approved the study and all housing, care and procedures were performed by appropriately trained personnel under the guidance of a veterinarian. Utilizing our previously reported model of exposure keratopathy, the right upper and lower eyelids were subjected to blepharoplasty and the nictitating membrane was removed. The left eyes remained undisturbed to serve as controls.

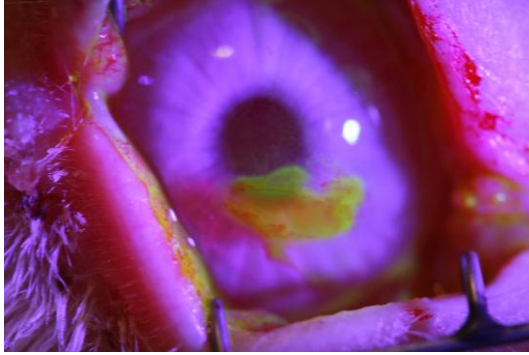


Fig. 1: Exposure keratopathy model. The upper and lower eyelids have undergone blepharoplasty and the nictitating membrane removed.

Application of amniotic membrane: One week following initial injury the rabbit was returned to the operating room where cryopreserved amniotic membrane (AM), diimide covalently crosslinked amniotic membrane (D-AM) was draped over the cornea and sutured to the limbal conjunctiva (n=12 for each arm, fig. 2).



Fig 2: Application of amniotic membrane in the New Zealand white rabbit exposure keratopathy model.

Clinical examination with fluorescein staining and slit lamp photography was performed on days 3, 5, 7, 14, 21 and 28. The rabbits were returned to the operating room to re-suture, patch (fig. 3) or replace the amniotic membrane whenever exposure of the ocular surface occurred.

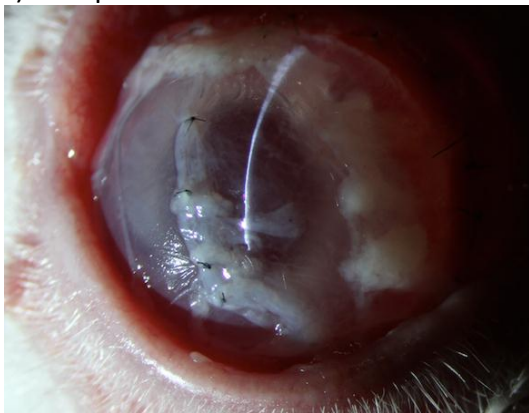


Fig. 3: Patched amniotic membrane after partial breakdown.

The number and type of procedure was recorded for each rabbit. Histopathological analysis was performed by the veterinary pathologist. The groups were compared utilizing an ANOVA with Tukey adjustment.

Tear Film Analysis: the rabbits from each treatment group underwent tear analysis of inflammatory chemokines/cytokines and matrix metalloproteinases commercially available multiplex immunoassays utilizing the Luminex bead array were procured. Tested chemokines/cytokines include EGF, FGF-2, Eotaxin, IFN γ , GRO, MDC, PDGF-BB, IL-17A, IL-1RA, IL-3, IL-6, IL-8, MP-1a and VEGF. A separate assay was used to test for matrix metalloproteinase 9 (MMP 9) given its known up-regulation in dry eye models and clinical scenarios. The tested cytokines were chosen based on their reported prevalence in dry eye states and the manufacturer recommendation of the ability of the human test to cross-react with our rabbit species. Samples were collected at baseline (prior to initial injury), at day 6 or 7 (prior to placement of amniotic membrane), day 13 or 14 and day 20 or 21. The tears were collected and analyzed using the method described by VanDerMeid, et al (1). The investigator, with gloves, placed a Schirmer strip over the lid margin at the junction of the lateral and middle third of the lower lid and kept in place for 5 minutes with the eyes closed in the absence of topical anesthetic. The strips were removed with gloves and tear volume in millimeters was recorded. Each strip was placed in a sterile 2-ml centrifuge tube, stored on ice for 20 minutes to 1 hour, and then stored at -20⁰ C until processed.

To determine the feasibility and sensitivity of the kits for recovering cytokines and matrix metalloproteinases (MMPs) from the Schirmer strips, the low internal quality controls (QC-1) from each cytokine and MMP kit were prepared as described in the kit assay protocol. Twenty μ l of each QC-1 was aliquotted to a Schirmer strip, allowed to flow for 1 minute and the strip was transferred to a 2 ml eppendorf tube and frozen at -20⁰ C for 24 hours. For percent recovery, 20 μ l of each QC was simultaneously aliquotted into a separate eppendorf tube and frozen at -20⁰ C for 24 hours. Each experiment for the strips and diluted samples were prepared in triplicate.

To extract the cytokines and MMPs from the Schirmer strips, 200 μ l of the analyte kit assay buffer (1% BSA in phosphate buffered saline with sodium azide) or extraction buffer (0.5 M NaCl and 0.5% Tween-20) was added to each 2 ml centrifuge tube and incubated for 3 hours at ambient temperature on a rocker, and then stored in ice. The strip was transferred to a new 2 ml tube and residual liquid was removed by pinching the strip at the 25mm mark in the sealed tube cap. The sample was then centrifuged at 100g for 10 seconds. The liquid was combined with stored extraction buffer. The Schirmer strips were discarded. Each 20 μ l frozen sample was diluted in 180 μ l of Assay or extraction buffer and treated to the same extraction regimen as described for the strips. Results were compared across groups using a repeated measures ANOVA analysis with a tukey adjustment for multiple comparisons.

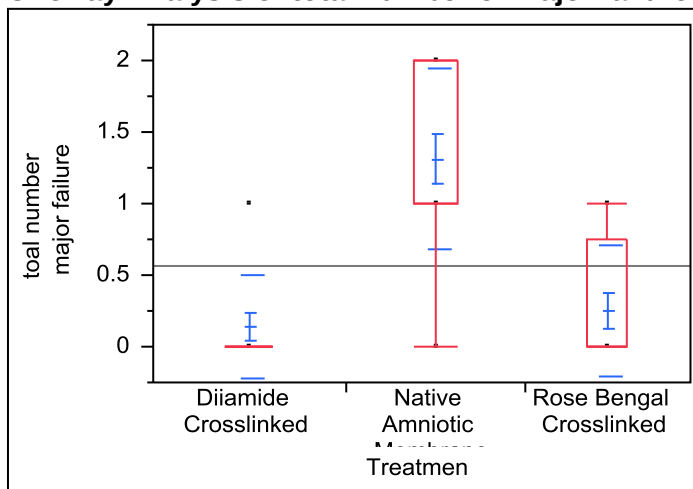
Histopathologic Analysis: Following euthanasia on day 28, treated globes were enucleated and the amniotic membrane was removed. The globes were fixed in Modified Davidson's solution for 24-48 hours and then transferred to 10% buffered neutral formalin. After fixation, the globe was transected sagittally and both hemispheres were embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin. Corneal sections were analyzed for mononuclear and

polymorphonuclear inflammation and vascularization of the corneal stroma, stromal edema, endothelial necrosis, and regeneration of the corneal epithelium. Each category of interest was graded (0=none, normal; 1=minimal, less than 25% affected; 2=mild, less than 50% affected; 3=moderate, less than 75% affected; and 4=severe, greater than 75% affected). The scores were compared across groups utilizing an ANOVA.

Findings:

Surgical Experience: The cryopreserved amniotic membrane required 12 replacement procedures, 6 reattachments, and 8 patch grafts (3 <50%, 5 =>50%). The Diimide group required 2 replacements, 1 patch (<50%) and 24 reattachment procedures. The Rose Bengal Group required (3 replacement procedures, 0 patches and 16 reattachment procedures). It was evident that the crosslinked groups required fewer major interventions (patches and replacements) and were thus more resistant to degradation, however, the stiffness from the crosslinking led to more failures at the suture-tissue interface. This accounted for the extra reattachment procedures.

Oneway Analysis of total number of major failures By Treatment



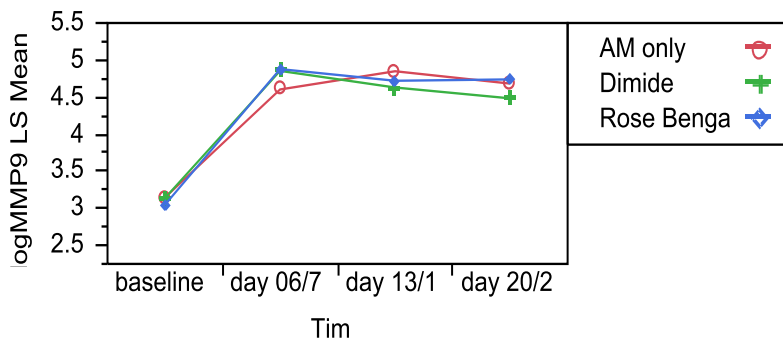
The crosslinked membranes had few major failures (Diimide vs AMG. $P < 0.0001$), and the (Rose Bengal vs. AMG $p = 0.0005$). There were no difference in the total number of failures. Using a nonparametric comparison using the Wilcoxon method, there was a statistically significant difference in the cost comparison of rose Bengal vs. AMG, $p = 0.0159$

Tear Analysis: EGF, FGF-2, IL-17A, IL-3, IL-6, IL-8 and MP-1a were very rarely found at detectable levels across treatment groups and time periods. As such, these chemokines/cytokines were not included in the statistical analysis. Eotaxin, IFN γ , GRO, MDC, PDGF-BB, IL-1RA and VEGF were

generally found in reportable levels, although there was great fluctuation throughout the time points and across treatment groups. There was a single time-point (day 6/7) where the level of VEGF was significantly higher in the AM group (ANOVA, $p=0.0024$). The Eotaxin (eosinophil chemokine) plot was noteworthy for a statistically significant difference at baseline with the Diimide amnion starting at lower levels than the untreated and Rose Bengal group ($p=0.0220$), however by day 6 onward there were no statistically significant differences between the groups. On the IL-1RA plot the diimide was statistically lower than the rose Bengal level at days 13/14 and 20/21 ($p=0.0210$, and $p=0.047$) however neither were statistically lower than the amniotic membrane.

On many of the plots the Diimide did trend lower than untreated and Rose Bengal however there was no statistical significance.

MMP 9 was recovered in significantly elevated quantities in both groups following the initial injury. There was no significant difference between groups at any time point during the study (ANOVA, $p=0.7958$).



Histopathologic Analysis: The major histologic change in all corneas is epithelial regeneration, characterized by increased mitotic figures in the basal cell layer of the corneal epithelium, occasionally accompanied by individual necrotic epithelial cells and mild epithelial hyperplasia. Most corneas have one or more bullae with occasional fibrosis in the superficial corneal stroma at the level of Bowman's layer. The corneal stroma reveal minimal infiltration by mononuclear and polymorphonuclear cells and rarely contain reactive blood vessels in any of the groups. ANOVA with a tukey adjustments did not find any statistically significant factors between the three groups

Discussion:

The prevention of vision threatening complications from exposure keratopathy is a difficult and often expensive undertaking in any critically ill patient. Those patients on the burn ward exponentially increase the cost and the difficulty given the comorbid conditions of the patient and the prolonged need for alternative means of ocular surface protection. Our study has shown that cross-linking of amniotic membrane could potentially save thousands in materials and facility utilization by decreasing procedures three fold.

A previous case series claimed that a glutaraldehyde cross-linked amniotic membrane was able to last up to 90 days in a human patient (2). This is much longer than we saw in our study, although we did have 12 of 17 cross-linked amniotic membranes that required no patching or replacement for the full 28 days of the study. We did not place a cone on any of the rabbits, so it is possible that many of the procedures were secondary to self-injury as opposed to natural degradation of the amniotic membrane. We also used carbodiimide cross-linking, which may be better tolerated by corneal epithelial cells at the theoretical expense of the longevity seen with glutaraldehyde cross-linking (3).

Our study did not find any significant difference between the levels of inflammatory mediators in the tear film. There are several limitations to this analysis. First, there are no commercially available rabbit multiplex arrays to test for these chemokines/cytokines. We used the recommendations from the manufacturer (EMD Millipore Corporation, Billerica, MA) to test those factors that cross react with the human. To more accurately reflect the inflammatory milieu in the rabbit, further studies of normal levels and disease model levels (exposure without amniotic membrane) would be needed to accurately develop positive and negative controls.

We were able to confirm the results published by VanDerMeid, et al (1) by showing that all of the tested cytokines/chemokines/MMPs could be quantitatively analyzed after extraction from Schirmer strips.

Like the tear film analysis, the histopathologic analysis revealed no difference in the ability of the carbodiimide cross-linked amniotic membrane, or rose Bengal photocrosslinked amniotic membrane to suppress inflammation. This is in accordance with recently published results from Lai, et al stating that carbodiimide cross-linked amniotic membrane retains anti-inflammatory activity (4).

There are also limitations to our model for exposure keratopathy. The burn ward in our institution routinely keeps room temperature at 100+°F, contributing to the harsh environment for the ocular surface. This could not be recreated in our study. Also, there were limitations in how quickly we could return to the operating room, resulting in delays before the corneas could be re-covered. This likely resulted in artificially elevated levels of inflammatory mediators.

Future areas of study should focus on further establishing the baseline characteristics of this model of exposure keratopathy, namely reference values of the chemokines, cytokines and MMPs shown to be mediators in exposure keratopathy. Alternative methods of cross-linked amniotic membrane should also be explored.

Depending on the results of these further studies, human safety testing should be undertaken so that cross-linked amniotic membrane can be added to our armamentarium to combat severe exposure keratopathy.

Conclusions:

Cross-linked amniotic membrane may be a viable alternative to cryopreserved amniotic membrane in the treatment of severe exposure keratopathy with the potential to reduce treatment time and cost with preserved efficacy.

Key Research Accomplishments (Johnson and Kochevar)

- Determined that amniotic membrane can safely undergo carbodiimide crosslinking and rose Bengal crosslinking. The resultant tissue can protect the cornea as well as the gold standard, cryopreserved amniotic membrane, as determined by histopathological analysis
- Determined that by crosslinking amniotic membrane moderately, you can reduce the number of replacements and patches to amniotic membrane grafts. Given the cost of the amniotic membrane, this has the potential to save thousands of dollars annually in our burn center alone.
- Determine that moderate amounts of crosslinking will result in more returns to the OR for reattachments. (This might be mitigated by reducing the amount of crosslinking, or directly photo-crosslinking the amnion directly to the cornea.- possible future experiment)
- Determined that the crosslinking procedure does not reduce the tissues anti-inflammatory properties, as measured by our cytokine/chemokine profile

Reportable Outcomes

I.E. Kochevar, E. Verter, T. Gisel, R.W. Redmond, A.J. Johnson, Corneal Protection for Burn Patients, Medical Health Services Research Symposium, Aug 12-16, 2012, Fort Lauderdale, FL, Poster 12-015. A video presentation of this poster was featured as a video highlight on the CDMRP home page (<http://cdmrp.army.mil>).

A.J. Johnson, P. Buttke, I. Kochevar, H-C.Wang, S. Cora, S. DeMartelaere, Surgical Model for Evaporative Loss Dry Eye Model in the New Zealand White Rabbit, Association for Research in vision and Ophthalmology, June 10-16, Seattle WA Poster 6046-A0109.

*poster currently being submitted to ARVO Denver 2015.

Resources:

1. VanDerMeid K, Su S, Krenzer K, et al. *A method to extract cytokines and matrix metalloproteinases from Schirmer strips and analyze using Luminex*. Molecular Vision 2011; 17:1056-63.
2. Spoerl E, Wollensak G, Reber F, Pillunat L. *Cross-linking of human amniotic membrane by gluteraldehyde*. Ophthalmic Res. 2004 Mar-Apr; 36(2):71-7.
3. Lai JY, Ma DH. *Gluteraldehyde cross-linking of amniotic membranes affects their nanofibrous structures and limbal epithelial cell culture characteristics*. Int J Nanomedicine. 2013; 8: 4157-68.
4. Lai JY, Lue SJ, Cheng HY, Ma DH. *Effect of matrix nanstructure on the functionality of carbodiimide cross-linked amniotic membranes as limbal epithelial cell scaffolds*. J Biomed Nanotechnol. 2013 Dec; 9(12):2048-62.
5. Verter EE, Gisel TE, Yang P, et al. *Light-initiated bonding of amniotic membrane to cornea*. Invest Ophthalmol Vis Sci. 2011 Dec 9;52(13):9470-7.