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Molecular Solutions to Low Vision Resulting from Battlefield Injuries

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Molecular Solutions to Low Vision Resulting from Battlefield Injuries

We hypothesize that targeted molecular intervention can preserve vision threatened by battlefield trauma-induced corneal and retinal inflammation, corneal and retina/optic nerve apoptosis, ocular surface dry eye after refractive surgery, and retinal degeneration. We are studying the consequences of trauma-induced (1) corneal inflammation using a gene therapy approach of providing soluble Fas ligand to the cornea to determine if this ligand can suppress corneal inflammation in mice; (2) retinal inflammation by examining if transforming growth factor-beta, thrombospondin, and somatostatin, in subretinal space, can suppress inflammation within retina secondary to autoimmune uveoretinitis and light-induced damage in mice; (3) corneal cell death by apoptosis and promote regeneration by identifying the anti-apoptotic gene with the greatest capacity to suppress corneal cell apoptosis using mice; (4) retinal cell death and regeneration by using mice to determine if systemic treatment with lithium chloride can prevent collateral damage to retinal neurons and promote optic nerve regeneration; (5) dry eye by determining how to minimize dry eye after LASIK refractive surgery by developing new tests to predict pre-disposition to refractive surgery induced dry eye; and (6) retinal injury by generating stem cell polymer composites.
# TABLE OF CONTENTS

- INTRODUCTION .................................................................1
- BODY ..............................................................................1
- KEY RESEARCH ACCOMPLISHMENTS .........................5
- REPORTABLE OUTCOMES ...............................................6
- CONCLUSION .................................................................6
- APPENDIX .......................................................................7
Introduction

Body

An increasing percentage of battlefield injuries occur to the eye in modern warfare. Even treatable battlefield injuries to the eye can lead to blindness because of collateral damage to adjacent tissues. This blindness results from injury-induced inflammation, cell death, failure to regenerate and repair, and development of scar tissue. Task #5 is one portion of a multidisciplinary project that addressed corneal blindness resulting from abrasions, burns, and penetrating wounds acting on normal corneas or exaggerated in corneas that have undergone refractive surgery, as well as retinal blindness resulting from physical trauma, infection, or laser-induced injury that destroy retinal nerve cells. In task 5 our goal is to prevent the consequences of trauma to the cornea after refractive surgery by developing strategies to diagnose dry eye syndromes. Our specific objective was to determine if there are individuals in whom the goblet cells of the conjunctiva do not respond normally to neural and growth factor stimulation and if this abnormal response predisposes these individuals to developing chronic dry eye after laser refractive surgery. Our three subtasks were to 1: Determine if the response of conjunctival goblet cells to nerves and growth factors is altered by loss of corneal nerves (induced by a corneal wound). The loss of corneal nerves by a corneal wound mimics the loss of nerves induced in laser refractive surgery. 2: Determine if human goblet cells from normal human controls respond to the growth factor EGF, the b-adrenergic agonist isoproterenol, and the cholinergic agonist carbachol. 3: Determine if patients with a change in the goblet cell population that contains mucin (filled) or is empty of mucin (unfilled) will have an increased rate of dry eye symptoms and traumatic complications after laser refractive surgery. We will additionally determine if there are any low abundance inflammatory proteins in tears that predict development of post-surgical dry eye or epithelial complications.

I. Research accomplishments for Subtask 1: This task was completed in 2008 and was reported on the interim report submitted on June 30, 2009.

II. Research accomplishments for Subtask 2: This task was completed in 2008 and was reported on the interim report submitted on June 30, 2009.
III. Research accomplishments for Subtask 3: As indicated in our 2008 interim report, Dr. Dimitri Azar our initial collaborator moved from Massachusetts Eye and Ear Infirmary and we have continued the study with COL Kraig S. Bower, LTC Charles Coe, and Ms. Denise Sediq from Walter Reed Army Medical Center. As discussed in our 2008 report with the failure of Subtask 2, we changed portions of the clinical study. We decided to continue collecting impression cytology specimens, but instead of stimulating them, we analyzed them by quantifying the number of filled goblet cells per total number of goblet cells using immunofluorescence microscopy. In addition, we began a collaboration with Dr. Robert Sack of SUNY State College of Optometry, New York, NY. An initial subset of Schirmer strips already being used to determine tear volume were sent to Dr. Sack for analysis. He analyzed the tears absorbed on the strips by microarray analysis for inflammatory mediators. A protocol modification was approved to incorporate these changes. After the initial subset presented in our 2009 report no further analysis has been completed due to microarray analysis funding shortages. Additional funds are necessary to continue Schirmer strip microarray analysis.

As of 22 January 2010 we have enrolled 145 out of a total of 146 subjects. The 146th subject will be enrolled in the first week of February 2010. Three subjects were withdrawn prior to treatment leaving 142 subjects enrolled and treated- (73 PRK and 69 LASIK). One subject is scheduled for treatment in February 2010. Two patients were disenrolled from the study after treatment (1 LASIK and 1 PRK) leaving 140 subjects for follow up. Follow up rates have been outstanding with 140/140 (100%) at 1 month, 135/140 (96.4%) at 3 months, 77/83 121/128 (94.5%) at 6 months, and 77/90 (85.6%) at 12 months. Follow-up care and testing is ongoing and we anticipate a February 2011 completion date. Tests to analyze the tear film of subjects before and after surgery are being completed as scheduled. These tests will be analyzed for statistical differences and correlated with dry eye in individual subjects once the study has been completed.

There was one adverse event in a subject who experienced acute, non-granulomatous anterior uveitis OS 2 weeks following uncomplicated LASIK. The subject was treated with Prednisolone Acetate 1.0% ophthalmic suspension q2hrs and cyclopentolate 1% ophthalmic solution TID, which resolved the uveitis. The PI felt this was unrelated to the
surgery or the study participation. This was reported in the July 2009 report. There have been no additional adverse events.

Impression cytology was used to determine what percentage of conjunctival goblet cells are filled (contained mucin) compared to those that had already secreted their mucin and not refilled (empty). Conjunctival impression cytology for goblet cell analysis was performed on 24 patients who did not develop postoperative dry eye (non-dry eye patients). Of these 24 patients 12 patients (8 females, 4 males mean age 31.4 years) underwent LASIK and 12 patients (8 females, 4 males, mean age 29.5 years) underwent PRK. In addition impression cytology was performed 10 patients who developed dry eye after refractive surgery (dry eye patients). Of these 10 patients, 5 patients (2 females, 3 males mean age 28.4 years) underwent LASIK and 5 patients (2 females and 3 males mean age 28.4 years) had PRK. Normal (non-dry eye) patients were defined as those who had no symptoms, no superficial punctual keratitis (SPK), no punctual plugs, and two of the following: McMonnies questionnaire <10, TBUT >10 sec, or Schirmer test > 10 mm. Dry eye patients were defined as those who had symptoms, SPK, punctal plugs, and two of the following: McMonnies questionnaire >20, TBUT <5 sec, or Schirmer test < 5 mm. Patients were assessed for dry eye at 9 mo after surgery. These two groups were normal and moderate to severe dry eye and represented two widely separated groups. No mild or suspect dry eye patients were included.

Samples taken preoperatively and then 1 week, 1 month and 3 months after surgery were analyzed. For impression cytology (IC) samples, a drop of anesthetic was placed in the left eye of each patient. After 5 minutes a second drop of anesthetic was placed in the left eye. A piece of nitrocellulose membrane was then placed on the superior conjunctiva and even pressure was applied to the membrane. This process was repeated for the temporal conjunctivae. Membranes were placed cell side down on a slide. Each membrane was gently pressed uni-directionally onto a slide with a glass ball. The slides were fixed in 100% methanol. IC slides were rinsed in 1X PBS, blocked for 30 min in PBS containing 2% BSA and 2% triton X-100. Slides were incubated with cytokeratin 7 (K7, a specific marker for conjunctival goblet cells) in PBS containing 2% BSA for 1.5 hours at room temperature or overnight at 4° C. Slides were rinsed 3 times in PBS after which time the secondary antibody Cy 2 diluted 1:100 and Helix pomatia agglutinin lectin (HPA) indicative for the presence of glycoconjugates diluted 1:100 in
PBS with 2% BSA were added to the slides. Coverslips were mounted with PVA/DABCO containing DAPI that labels cell nuclei for counting the total number of cells. Cells from five fields on each slide were counted. The cells were analyzed for DAPI alone, K7 alone, HPA alone, and both K7 and HPA. DAPI staining indicated the total number of cells in each field, cells positive for K7 indicated goblet cells and K7 and HPA positive cells indicated filled goblet cells.

Data was presented as mean ± SEM. Values for superior and temporal IC were combined. Data was analyzed using Student’s t-test with p<0.05 considered statistically significant.

We found that in normal patients the percent of total conjunctival cells collected that were goblet cells decreased at 1 wk and 1 mo after LASIK, but not PRK, compared to the value preoperatively. The goblet cell percentage returned to preoperative levels by 3 mo after surgery. In patients who developed dry eye, compared to preoperative levels, the percentage of goblet cells transiently decreased at 1 wk in individuals with PRK and decreased at 1 wk and 1 mo in those with LASIK. The goblet cell percentage returned to preoperative levels by 3 mo after surgery.

The number of cells that were goblet cells (filled plus empty) was set to 100%. The percentage of filled and empty goblet cells was then calculated. These two values when summed added up to 100%. In normal patients the percentage of goblet cells that were filled did not change after PRK or LASIK surgery. In contrast in patients who developed dry eye the percentage of filled goblet cells increased at 1 mo postoperatively compared to preoperative levels, but returned to preoperative levels at 3 mo. In those with LASIK the percentage of filled goblet cells increased at 1 wk, 1 mo and 3 mo after surgery compared to before surgery. When the preoperative values were compared between normal and dry eye patients, in non-dry eye there was a greater percentage of filled than empty goblet cells. In contrast, in dry eye there was a greater number of empty than filled goblet cells.

We conclude that: 1) LASIK is damaging to conjunctival goblet cells in both normal and dry eye patients, but the cells recover by 3 mo, 2) Refractive surgery blocks neural stimulation of goblet cells thus increasing the number of filled goblet cells in the dry eye
patients, 3) The preoperative secretory status of the conjunctival goblet cells in dry eye patients could predispose these individuals to dry eye after refractive surgery, and 4) LASIK induced changes in goblet cell secretion do not recover in dry eye patients and could be the cause of chronic dry eye. An oral presentation based on this work was presented at the Fourth Annual Military Refractive Surgery Meeting in January 2010. Three abstracts based on this work have been accepted for presentation at the Association for Research in Vision and Ophthalmology 2010 Annual Meeting.

No further analysis of the tears collected onto Schirmer strips for analysis of inflammatory mediators was performed during the past year.

**Key Research Outcomes**

- Enrolled 145 subjects for LASIK or PRK with excellent rate of follow-up visits.
- Performed analysis of tear film before and after surgery using standardized tests described in our protocol to determine individuals who develop chronic dry eye after refractive surgery.
- Analyzed goblet cell population in a subset of subjects for number of filled, empty, and total conjunctival goblet cells.
- Found that LASIK is damaging to conjunctival goblet cells in both normal and dry eye patients, but the cells recover by 3 mo.
- Demonstrated that refractive surgery appears to reduce neural stimulation of goblet cells thus increasing the number of filled goblet cells in the dry eye patients.
- Showed that the secretory status of the conjunctival goblet cells in dry eye patients could predispose these individuals to dry eye after refractive surgery.
- Demonstrated that LASIK induced changes in goblet cell secretion do not recover in dry eye patients and could be the cause of chronic dry eye.
- Found that low abundance tear proteins can be successfully collected in a busy clinical setting using Schirmer test strips and subsequently analyzed in University molecular biology lab.
- Found that changes in specific inflammatory proteins in tears can be predictive of dry eye and epitheliopathy after refractive surgery.
- Suggested that in the immediate post-operative period, there are changes in both the magnitude of tear proteins and goblet cells that contribute to dry eye, but
beginning at 1 month, the clinical exam and patient demographics are the best predictors of post-operative dry eye.

Reportable Outcomes


Conclusions

We conclude that: 1)LASIK is damaging to conjunctival goblet cells in both normal and dry eye patients, but the cells recover by 3 mo, 2) Refractive surgery appears to reduce neural stimulation of goblet cells thus increasing the number of filled goblet cells in the dry eye patients, 3) The secretory status of the conjunctival goblet cells in dry eye patients could predispose these individuals to dry eye after refractive surgery, 4) LASIK induced changes in goblet cell secretion do not recover in dry eye patients and could be the cause of chronic dry eye, 5) Changes in specific inflammatory proteins in tears can be predictive of dry eye and epitheliopathy after refractive surgery, 6) In the immediate post-operative period, there are changes in both the magnitude of tear proteins and goblet cells that contribute to dry eye, but
beginning at 1 month, the clinical exam and patient demographics are the best predictors of post-operative dry eye.

**Appendix**


Predicting Post-Operative Dry Eye: Multivariate Analysis of Clinical Findings, Tear Proteins, and Goblet Cells

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Abstract:

Purpose: To examine the significance of subjective symptoms, the clinical examination, conjunctival goblet cells, and inflammatory tear proteins and cytokines in predicting post-operative dry eye in refractive surgery patients.

Methods: A prospective, non-randomized, multicenter study comparing the effect of LASIK and PRK on the clinical findings of cochet-bonnet aesthesiometry, tear break up time (TBUT), rose Bengal staining (RB), videokeratoscopy surface indices, percent of filled goblet cells (GC), 16 cytokines, 7 matrix metalloproteinases, 2 matrix metalloproteinases inhibitors, and subjective sx of dry eye quantified using the McMonnies questionnaire. Discriminant analysis was conducted at 1 week (1W), 1 month (1M), and 3 months (3M) to determine if significant differences existed across the predictor variables of two groups: subjects with and without postoperative dry eye.

Results: Seventy-two eyes underwent either PRK(n=39) or intralase LASIK (n=33). At one week post-op, only MMP10 (Wilks’ lambda=0.461) and IL1α(Wilks’ lambda=0.302) were significant predictor variables. At one month, percent filled of goblet cells (WL=.791), Rose Bengal Staining (WL=0.617), and age (WL=0.533) were different across the two groups. At 3M, McMonnies questionnaire (WL=0.724), Rose Bengal staining (WL=0.565), Schirmer’s with anesthesia (WL=0.478), and age (WL=0.413) were significantly different across the two groups. The level of tear proteins were not significantly different across the two groups at either 1M or 3M and at no time point did videokeratoscopy surface indices distinguish between the dry eye group and non-dry eye group.

Conclusions: In the immediate post-operative period, there are changes in both the magnitude of tear proteins and goblet cells that contribute to dry eye. Beginning at 1M, the clinical exam and patient demographics are the best predictors of post-operative dry eye.

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Normal Goblet Cell (GC) Response after Photorefractive Keratectomy (PRK) and Laser assisted in situ keratomileusis (LASIK)

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Abstract:

Purpose: To examine the post-op GC response after uncomplicated PRK and LASIK.

Methods: Dry eye (DE) questionnaire, Schirmer test, tear breakup time, and Rose Bengal staining were tested pre- and post-op. Patients whose post-op course was complicated by DE were excluded. Impression cytology samples (ICS) were taken from the superior and temporal conjunctivae of 12 PRK (8F, 4M) and 8 LASIK patients (3F, 5M) pre-op and at 1W, and 1 and 3M post-op. ICS were fixed on glass slides and stained with three indicators: DAPI to mark cell nuclei to determine the total number of cells (GC and squamous epithelial cells); with anti-keratin 7 (K7) to mark GC bodies to determine the percentage of cells which were GC (% GC); and with K7 and helix pomatia agglutinin to mark GC secretory product to determine the percentage of GC which were filled (% Filled GC). (Figure) Five random areas were selected as representative fields of the total slide. Results were compared using RM-ANOVA with p<0.05 considered significant.

Results: Mean age±SD of the PRK group was 28.5±5.1 and LASIK was 32.8±5.1 years (p=0.18). Mean ablation depth of PRK was 55.40±18.10µm and LASIK was 56.01±17.85µm (p=0.91). The % GC did not change significantly over time in either the PRK group [46.2 ±27.6% pre, 34.8±22.7% 1W, 41.8±25.9% 1M, 45.5±21.9% 3M (p=0.43)] or the LASIK group [35.6 ±20.6% pre, 22.8±19.8% 1W, 17.4±14.9% 1M, 36.5±22.8% 3M (p=0.18)]. The % Filled GC did not change significantly over time in either the PRK group:64.7±35.4% pre, 80.1±17.9% 1W, 76.6±20.9% 1M, 68.0±33.2% 3M (p=0.29) or the LASIK group: 55.3 ±36.9% pre, 74.4±22.1% 1W, 70.3±27.6% 1M, 64.0±27.5% 3M (p=0.24).

Conclusions: Preliminary results indicate that neither the percentage of GC nor the proportion of GC which are filled changes significantly over time after either PRK or LASIK in patients uncomplicated by post-op DE. GC response in post-surgical DE patients is currently under investigation.
Author Disclosure Information:  D.S. Ryan, None; M.A. Shatos, None; K.S. Bower, None; R.K. Sia, None; L. Peppers, None; C.D. Coe, None; E. Guilbert, None; R.S. Howard, None; D.A. Dartt, None.

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Refractive Surgery Alters the Conjunctival Goblet Cell Population in Individuals Who Develop Dry Eye

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Abstract: **Purpose:** To determine if the population of goblet cells (GC) and filled goblet cells changes in the conjunctiva of individuals who develop dry eye after laser-assisted in situ keratomileusis (LASIK) or photorefractive keratectomy (PRK).

**Methods:** Pre- and post-op tear film status was evaluated by slit lamp biomicroscopy, Schirmer test, tear breakup time and McMonnies DE questionnaire. Patients who developed clinically significant DE were included for this analysis. Impression cytology samples (ICS) were taken from the superior and temporal conjunctivae pre- and at 1W, 1M and 3M post-op. ICS on membranes were transferred to glass slides and stained with anti cytokeratin-7 (K7) to identify GC bodies; Helix pomatia agglutinin (HPA) for GC secretory product, and DAPI for cell nuclei. Five random fields were counted for each sample. Total cell number was determined by counting DAPI stained nuclei; unfilled GC number by K7 positive cells (no HPA), and filled GC by K7 cells with HPA. Statistical analyses were performed using student`s t-test.
**Results:** 10 post-op DE patients were studied [5 LASIK, (3M, 2 F, average age 28.4y); 5 PRK (3M, 2F, average age 28.4 y)]. The average number of GC per patient was significantly different between PRK (72.5±11.8 cells) and LASIK (169.5±36 cells). The number of cells significantly declined at 1W in both PRK (47.3±17.9 cells) and LASIK (63.8±17.9 cells). At 3M, the number of cells had returned to pre-op levels. The percentage of total GC (filled plus unfilled) significantly decreased at 1W in both PRK (21.2±6.7%) and LASIK (24.3±6.7%) when compared to their respective pre-op levels of 31.1±6.5% and 50.6±8.5%, but recovered at 3M. For PRK, when the total number of GC was set to 100%, the percentage of filled GC significantly increased at 1W (59±13%) and 1M (76.9±7.4) post-compared to pre-op (42.6±11.9%) and began to recover at 3M. LASIK induced a significant increase in filled GC at 1W (55±12%) and 1M (60.5±13%) post-op compared to pre-op (23.8±8.5%) and began to return to the pre-op levels.

**Conclusions:** We conclude that in individuals who develop dry eye after refractive surgery, goblet cells are sensitive to stress and are easily lost. The remaining goblet cells are predominately filled goblet cells indicating that neural regulation of goblet cell secretion is deregulated but begins to recover at 3 mo.

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