AWARD NUMBER: W81XWH-14-1-0495

TITLE: Inflammation Modulatory Protein TSG-6 for Chemical Injuries to the Cornea

PRINCIPAL INVESTIGATOR: Samuel Fulcher, MD

CONTRACTING ORGANIZATION:CENTRAL TEXAS VETERANS RESEARCH FOUNDATION TEMPLE TX 76504

TYPE OF REPORT: Final

REPORT DATE: SEPTEMBER 2018

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
SEPTEMBER 2018	Final	30SEP2014 - 30JUN2018

4. TITLE AND SUBTITLE Inflammation Modulatory Protein TSG-6 for Chemical Injuries to the Cornea

5a. CONTRACT NUMBER
W81XWH-14-1-0495

5b. GRANT NUMBER
MR130174

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Samuel Fulcher, MD

Email: samuel.fulcher@va.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
TEXAS VETERANS RESEARCH FOUNDATION
TEMPLE TX 76504

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project tested the efficacy of the anti-inflammatory protein TSG-6 for the treatment of alkali injury of the cornea in a rat model. We established the time sequence of inflammation, and the cellular and molecular changes which occur after exposure of rat corneas to different concentrations of alkali. These data set the conditions to test the efficacy of topical, intraocular, and intravenous TSG-6, in the promotion of corneal healing and restoration after alkali injury. We found that TSG-6 applied with these methods did not provide a statistically significant benefit to the cornea after alkali injury as evaluated by corneal clarity, although biochemical markers of inflammation suggested limited benefit with intraocular and intravenous application. Further experiments studied the effectiveness of extracellular vesicles, which contain TSG-6, in the treatment of corneal alkali and nitrogen mustard injury, however no significant benefit was observed in these preliminary studies.

15. SUBJECT TERMS

cornea, alkali injury, rat model, inflammation, chemical injury, TSG-6, regeneration

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	166	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. INTRODUCTION:

This project aims to study the treatment of chemical injury of the cornea with a natural anti-inflammatory protein, TSG-6, which has a novel mechanism of action. Chemical injuries of the eye are difficult to treat, and may lead to severe debilitation or blindness with few patient treatment options. The wartime threat for chemical injury to the eye is evidenced by the thousands of soldiers injured with mustard gas in the Iran-Iraq war. The mechanism of injury after chemical ocular injury includes inflammation secondary to trauma, and may be alleviated with anti-inflammatory agents like TSG-6. TSG-6 works by inhibiting inflammation at the earliest stage, and is effective in the treatment of mild chemical injuries caused by alcohol and mechanical scraping in a mouse model. This study is designed to determine if TSG-6 will be effective with more severe chemical alkali injuries of the cornea, and which would more closely mimic injuries from mustard gas or other severe caustic agents. We will treat rat corneas injured with different concentrations of alkali using topical, anterior chamber, and IV TSG-6 to determine the efficacy and time course of treatment as evaluated by clinical improvement and corneal clearing, and biochemical markers of inflammation.

2. KEYWORDS:

cornea, alkali injury, rat model, inflammation, chemical injury, TSG-6, regeneration

3. ACCOMPLISHMENTS:

a. Project Goals

The specific goals for this project as pertaining to the SOW and amendments included:

1) Specific Aim 1 Subtask 1: Obtain ACURO approval for study initiation
2) Specific Aim 1 Subtask 2: Determine the acute phase timing and patterns of inflammatory cytokines and chemokines in response to the severity of alkali injury for 0.1N, 0.5N, and 1N injury to the rat cornea
3) Specific Aim 1 Subtask 3: Determine the timing and patterns of cellular changes in response to the severity of alkali injury (histopathology)
4) Specific Aim 2 Subtask 1: Determine the efficacy for topical and intraocular TSG-6 after corneal alkali injury to the rat cornea for varying concentrations of alkali
5) Specific Aim 2 Subtask 2: Determine the therapeutic window for topical administration of TSG-6
6) Specific Aim 3 Subtask 1: Test for efficacy and dose response of intravenous administration of TSG-6

7) Specific Aim 3 Subtask 2: Determine the therapeutic window for IV TSG-6 administration
8) Specific Aim 3 Subtask 3: Test for synergistic effect of topical and intravenous administration of TSG-6
9) Specific Aim 3 Subtask 4: Determination of maximal rescued
10) Specific Aim 4 (per no cost extension) Subtask 1: Determine the for therapeutic effect of AC MSC exosome on corneal alkali injury
11) Specific Aim 5 (per no cost extension) Subtask 1: Determine the acute phase timing and patterns of inflammatory cytokines and chemokines in response to the severity of vesicant injury
12) Specific Aim 5 Subtask 2: Determine the for therapeutic effect of topical MitoQ on vesicant injury (nitrogen mustard)
13) Specific Aim 5 Subtask 3: Determine the for therapeutic effect of topical TSG-6 on vesicant injury (nitrogen mustard)
14) Specific Aim 5 Subtask 4: Determine the for therapeutic effect of AC MSC exosome on vesicant injury (nitrogen mustard)

b. Goals Achieved

1) Specific Aim 1 Subtasks 1-3: ACURO approval was achieved 24 Sept 2014. Subtasks 2 and 3 were completed by 4/1/2015, and the results were presented in part at the World Ophthalmology Congress 5-9 February 2016 **(Appendix 1)**, and published in total in the journal Current Eye Research **(Appendix 2)** Choi H, Phillips C, Oh JY, Stock EM, Kim DK, Won JK, **Fulcher S:** Comprehensive Modeling of Corneal Alkali Injury in the Rat Eye. Curr Eye Res. 2017 Oct; 42(10):1348-1357.

Rat corneas were exposed to varying concentrations of NaOH for 30 seconds using filter paper soaked with NaOH, and then the corneas were thoroughly rinsed with 40cc of BSS. The corneas were photographed for clinical scoring at varying time points, and the corneas were collected for cytokine PCR assay for RNA at varying intervals after injury at 2 hr, 4 hr, and 1,2,3 and 5 days for a total of 6 time points.

Initial experiments at the 0.1N injury level resulted in an injury that was insufficient to distinguish a possible treatment benefit of TSG-6, and an amended SOW was submitted and approved to focus on the 0.5N and 1N injury levels.

The peak of inflammation for the 1N injury levels from chemokine studies indicated that the peak of inflammation occurred by day 1, and then subsided. The inflammatory cytokine peaks at day 1 for IL1β and IL6 were shown to be dependent on level of alkali injury which increased in a linear manner for 0.5N and 1N injuries. The level of corneal opacity peaked by day 3 and continued to remain high without improvement through day 21. These results demonstrated that

early treatment is indicated for TSG-6, and that the efficacy of TSG-6 would be readily established by comparing outcomes with the untreated controls **(Appendix 3, slides 2-4).**

The time course of injury data for the 1N injury level was extended to day 21 for histological analysis, and for further delineation of the time course of inflammation using thrombomodulin as a marker for neovascularization, and collagen I alpha 1 as a marker for fibrosis. These markers indicated that corneal neovascularization and fibrosis increase through day 21 post injury, as corroborated on histological examination which demonstrates endothelial lined vascular channels in the corneal stroma with dense corneal stromal fibrosis by day 21 **(Appendix 3, slide 5)**.

Neutrophil infiltration and myeloperoxidase expression peaked at day 1, while macrophage infiltration in the central cornea peaked from day 7 to day 14. Markers of acute inflammation IL-1B peaked at day one, followed by a decline and secondary peak at day 5, and declined thereafter to day 21, although was upregulated from baseline through the 21 day course. IL-6 peaked early at 4 hours and returned to baseline by day 2 **(Appendix 2, pgs 11-13).**

Markers of corneal neovascularization VEGF and fibrosis TGF-β peaked at 4 hours and one day respectively, and then returned to normal by day 21. Corneal neovascularization was observed to increase until day 15 when it plateaued, while corneal fibrosis as measured by type 1 collagen staining peaked at day 7 and remained elevated through day 21 **(Appendix 2, pg 14).**

The combined results of Specific Aim 1 Subtasks 1-3 established the proper testing conditions required to test the efficacy of TSG-6 for subsequent treatment studies.

2) Specific Aim 2 Subtasks1-2: Determine the efficacy for topical and intraocular TSG-6 after exposure to 0.5N and 1N alkali injuries, completed by end September 2015. These experiments did not demonstrate any statistically significant benefit after 0.5N or 1N injuries for TSG-6 with regard to inflammatory cytokine message or protein at day 7, or with regard to corneal opacity for either topical application, or anterior chamber injection into the eye **(Appendix 4, slides 2-15).** As no benefit was discovered for topical TSG-6, a therapeutic window for application (Specific Aim 2, Subtask2) could not be established, and further testing with topical TSG-6 alone was discontinued.

3) Specific Aim 3 Subtasks 1-2: Determine the efficacy, dose response, and therapeutic window of IV TSG-6 administration. These data were reported at the Annual Symposium for the American Society of Cataract and Refractive Surgery 5-9 May 2017, Los Angeles, CA **(Appendix 5).** Initial experiments completed by end of July 2015 with IV TSG-6 did not demonstrate statistically significant benefit for corneal clarity or inflammatory markers **(Appendix 4, slides 16-21).** Further testing demonstrated benefit on day 1 for inflammatory markers and the 0.5N injury with IV TSG-6, but not by day 7, and no benefit was observed with respect to corneal clarity **(Appendix 5, slides 11-12)**. Similarly, further tests of anterior chamber

TSG-6 improved inflammatory markers on day 1 for the 0.5N injury but did not improve corneal clarity or later inflammatory markers by day 7 **(Appendix 5, slides 15-16)**.

We investigated the time window of application for IV TSG-6 and found that while IV TSG-6 given at the time of injury results in a modest reduction in inflammatory markers, TSG-6 given 4 hours after injury does not result in the same reduction. Our results confirm the work of others which indicates that TSG-6 must be given as close to the time of injury as possible to be effective **(Appendix 6, slides 1-4)**.

4) Specific Aim 3, Subtasks 3-4: Test for synergistic effect of topical and intravenous administration of TSG-6 and determine maximal rescued. We investigated for synergistic effect of IV and topical TSG-6 applied at the time of injury and found no synergistic effect with respect to inflammatory markers or corneal clarity, which further confirms the lack of efficacy of topical TSG-6 after chemical injury to the eye. **(Appendix 7, slides 1-3).** As there was no benefit to combined topical and IV TSG-6, maximal rescued by treatment was not determinable.

5) These data for Specific Aims 1-3 were presented at the Annual Symposium of the American Society of Cataract and Refractive Surgery 13-17 April 2018, Washington DC **Appendix 8, slides 1-13),** and have been submitted to the journal, Ophthalmology, for publication **(Appendix 9, submitted manuscript). These data demonstrate that topical TSG-6 is ineffective for the treatment of corneal alkali injury, and that while AC and IV TSG-6 have some anti-inflammatory signaling at Day 1 post injury and treatment, the effect is gone by Day 7, and no therapeutic benefit to restore corneal clarity was observed.**

6) Specific Aim 4 (amended SOW and protocol) Subtask 1: Expose the corneas of rats to alkali and test the effectiveness of mesenchymal stem cell (MSC) exosomes through intraocular injection**.** MSC exosomes are known to contain TSG-6, and we tested the application of AC MSC exosomes after corneal alkali injury. These data show a trend for reduced inflammatory signaling at day 1 for IL1-β, IL6, and CCL2, but not for ELANE or VEGF however this did not reach statistical significance **(Appendix 10, slides 2-4)**. There was no trend noted at day 7 for inflammatory markers **(Appendix 10, slides 5-7). A potentially exciting finding was that corneal clarity at day 4 was significantly clearer, with a trend for better clarity through day 7 (Appendix 10, slide 8). This is the only experiment which was significant for improved corneal clarity after alkali injury and would indicate further investigation of MSC exosomes for chemical injury to the cornea is warranted.**

7) Specific Aim 5 (amended SOW and protocol) Subtask 1: Determine the acute phase timing and patterns of inflammatory cytokines and chemokines in response to the severity of vesicant injury. We tested the effect of nitrogen mustard on the rat cornea and established the inflammatory markers and acute phase timing of expression at day 2, as well as degree of corneal opacity **(Appendix 11, slides 2-4).**

8) Specific Aim 5 (amended SOW and protocol) Subtasks 2-4: Determine the for therapeutic effect of topical MitoQ, AC TSG-6, and AC MSC exosomes on vesicant injury (nitrogen mustard). We tested the efficacy of topical anti-apoptic protein MitoQ, AC anti-inflammatory protein TSG-6, and AC MSC exosomes for the treatment of nitrogen mustard injury to the rat cornea as part of the de novo protocol from 1 October 2017 through 30 June 2018. We found that there was no therapeutic benefit for corneal opacity or inflammatory markers at day 2 harvest for MitoQ or TSG-6 **(Appendix 11, slides 5-8).** There was no benefit observed for corneal clarity or inflammatory markers on days 1-2 post mustard injury treated with AC exosomes **(Appendix 12, slide 2-3).**

c. Training Opportunities and Professional Development

Nothing to report

d. Dissemination of Findings and Results

1) Paper presented 5-9 February 2016 at the World Ophthalmology Congress in Guadalajara Mexico titled: **Comprehensive Profiling of Alkali Injuries to the Cornea. (Appendix 1)**

2) Manuscript published in Current Eye Research Choi H, Phillips C, Oh JY, Stock EM, Kim DK, Won JK, **Fulcher S:** Comprehensive Modeling of Corneal Alkali Injury in the Rat Eye. Curr Eye Res. 2017 Oct; 42(10):1348-1357 **(Appendix 2).**

3) Paper presented at the Annual Symposium for the American Society of Cataract and Refractive Surgery 5-9 May 2017, Los Angeles, CA **(Appendix 5).**

4) Paper presented at the Annual Symposium of the American Society of Cataract and Refractive Surgery 13-17 April 2018, Washington DC **(Appendix 8).**

5) Manuscript submitted, notification pending to Ophthalmology titled **Efficacy of the Anti-inflammatory Protein TSG-6 for Corneal Alkali Injury in a Rat Model.** If this journal does not accept this paper, the manuscript will be submitted in order to additional journals for publication **(Appendix 9).**

e. Plans for Next Reporting Period

1) End of study. We have submitted a pre-application to CDMRP as a continuation study to further investigate the efficacy of MSC exosomes in the treatment of the vesicant nitrogen mustard injury to the cornea.

4. IMPACT

a. Principal Discipline

The results of this work for this report are the most encompassing and complete of any other reported study that we are aware of for chemical alkali injuries to the cornea and will serve

the ophthalmic community in the future as a benchmark model. The complete description of time course of injury with inflammatory cytokine and protein expression, clinical evaluation, and histopathology correlates to 21 days post injury is not duplicated in the literature. We have further demonstrated that TSG-6 alone is not effective in a corneal alkali injury model, although TSG-6 has been proven beneficial in several other models of inflammatory injury. Interestingly, we have also shown that MSC exosomes, which contain TSG-6, may be a better method to improve corneal clarity after chemical injury, and we are interested to investigate if this method would be beneficial for an injury that is more closely confined to the surface of the eye as seen with chemical warfare mustard agents. Therefore, we have laid the groundwork for further investigation into the application of techniques in Regenerative Medicine for the treatment of chemical injury of the cornea.

b. Other Disciplines

The results of this work, albeit with lack of demonstrated efficacy of TSG-6 to date with topical or anterior chamber application, will help investigators in the field of Regenerative Medicine focus on alternate methods of delivery, and show that while TSG-6 has been shown to be effective in a model of mild chemical corneal injury induced by ethanol, it is insufficient to benefit more severe ocular alkali chemical injuries when applied topically or in the anterior chamber.

c. Technology Transfer

Nothing to report

d. Impact on Society

Nothing to report

5. CHANGES/PROBLEMS

a. Changes

1) None to report, end of study.

b. Actual/Anticipated Problems

1) None to report, end of study.

c. Changes in Expenditures

1) None to report, end of study.

d. Changes in Animal Use

1) None to report, end of study.

6. PRODUCTS

a. Publications/papers/presentations

1) see 3.d. above under Accomplishments

b. Websites

1) Central Texas Veterans Research Foundation **http://www.ctvrf.org/research-programs**

c. Technologies/Techniques

1)Nothing to report

d. Inventions/Patents/Licenses

1)Nothing to report

7. PARTICIPANTS/COLLABORATING ORGANIZATIONS

a. Individuals

1) Name: Hosoon Choi, PhD

Project Role: Research Scientist

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 44

Contribution to Project: Dr. Choi has performed rat surgery to establish injury levels, performed chemokine and cytokine assays, and protein production and purification

2) Name: Casie Phillips

Project Role: Animal Technician, research assistant

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 42

Contribution to Project: Casie has assisted Dr. Choi in animal surgery, performs postoperative care, assists with laboratory techniques, prepares and stains histology specimens

3) Name: Samuel Fulcher, MD

Project Role: PI

Researcher Identifier (e.g. ORCID ID): NA

Nearest person month worked: 45

Contribution to Project: Dr. Fulcher performs the duties of the PI, and scores the clinical injuries by grading photographs of the corneal opacity

b. Collaborating Institutions

1) Texas A&M Institute for Regenerative Medicine, Temple, Texas. Dr. Darwin Prockop and his team have served as unpaid consultants, and shared bench laboratory space with our team.

2) Central Texas Veterans Research Foundation, Temple, Texas. CTVRF is the sponsoring agency

3) Central Texas Veterans Health Care System, Temple, Texas. CTVHCS is the employer of Dr. Fulcher, and has allotted time for research activities, and has supported the project with laboratory space and equipment

4) Baylor Scott and White Health Care, Temple, Texas. BSW is the sponsor of the IACUC and operates and maintains the vivarium where experiments were performed and employs the supervising veterinarian who oversees all animal research projects.

8. SPECIAL REPORTING REQUIREMENTS

a. No Collaborative Awards to report

b. Quad Chart, (appendix 13)

9. APPENDICES, SEE APPENDICES 1-13

Appendix 1 Final Report

Presented at the World Ophthalmology Congress

5-9 February 2016

Guadalajara, Mexico

Comprehensive Profiling of Alkali Injuries to the Cornea

Hosoon Choi[1,2], Casie Phillips[1,2], Darwin Prockop[2], Joo Youn Oh[4], Roxanne Reger[2], Eileen Stock[3], Samuel Fulcher[1]

[1]Central Texas Veterans Research Foundation Temple, TX
[2]Institute for Regenerative Medicine, Texas A&M Health Science Center College of Medicine at Scott & White, Temple, TX

[3]Baylor Scott and White Health Care Temple, TX

[4]Department of Ophthalmology, Seoul National University Hospital, Seoul, Korea

Objective/Purpose;
Corneal chemical injuries may cause extensive tissue damage which can result in permanent visual impairment. These injuries often result from accidents occurring in the home or work place, however no effective therapy exists for severe chemical injuries of the cornea. Numerous cellular interactions and alterations occur after corneal chemical injury which are mediated by leukocytes, fibroblasts and endothelial cells, and are influenced by the combined actions of proteinases, growth factors, and cytokines which are directed to corneal regeneration and healing. This study comprehensively examined the effect of alkali injury to the cornea over time in order to further research in the development of a novel therapy with the anti-inflammatory protein TNF-stimulating gene 6 (TSG-6). TSG-6 may modulate the excessive inflammatory response that exacerbates the injury to the cornea caused by chemical exposure to the eye.

Keywords; cornea, alkali injury, rat, inflammation, chemical burn

Materials and Methods;
A corneal alkali injury was produced with a 3-mm diameter circular piece of filter paper soaked in 1 N NaOH, and applied to the central cornea on the right eye for 30 seconds. Immediately after alkali exposure, the ocular surface was rinsed with 50 mL of PBS for 2 minutes. Gross examination for cornea opacity was performed under a dissecting microscope. The injured corneas were fixed in 10% buffered formalin and embedded in paraffin. Tissues were stained with hematoxylin and eosin (H&E) for histopathological examination, and were examined with immunohistochemistry. Neutrophil infiltration was examined by assays for myeloperoxidase (MPO) which is contained within neutrophil granules. Real-time PCR (RT-PCR) was used to evaluate mRNA expression levels of cytokines, chemokines, and genes involved in neovascularization, lymphangiogenesis and fibrosis.

Results and Conclusion
Corneal opacity rapidly developed after injury by day 1, and persisted throughout the study period (21 days). Corneal neovascularization developed as early as day 1, and increased over the entire study period. The inflammatory response as measured by biochemical markers correlated with concentration of NAOH applied and began within 2 hours of injury, and persisted throughout the study period. Neovascularization, lymphangiogenesis and fibrosis progressed throughout the time course of the study period.
Presentation Type; Free papers **Topic Categories;** Cornea, External Eye Diseases, Eye Trauma

Current Eye Research

Taylor & Francis
Taylor & Francis Group

Comprehensive Modeling of Corneal Alkali Injury in the Rat Eye

Journal:	*Current Eye Research*
Manuscript ID	NCER-2016-0690
Manuscript Type:	Original Articles
Date Submitted by the Author:	24-Dec-2016
Complete List of Authors:	Choi, Hosoon; Central Texas Veterans Research Foundation, Phillips, Casie; Central Texas Veterans Research Foundation Oh, Joo Youn; Seoul National University Hospital, Department of Ophthalmology Stock, Eileen; Perry Point VA Medical Center, Cooperative Studies Program Coordinating Center Kim, Dong-Ki ; Texas A and M University System, Institute for Regenerative Medicine Won, Jae-Kyung; Seoul National University Hospital, Department of Pathology Fulcher, Samuel ; Central Texas Veterans Healthcare System, Department of Surgery
Keywords:	corneal alkali injury, inflammation, corneal neovascularization, fibrosis, cytokine

Comprehensive Modeling of Corneal Alkali Injury in the Rat Eye

Hosoon Choi, PhD [*], Casie Phillips, Joo Youn Oh, MD, PhD [1], Eileen M. Stock, PhD [2], Dong-Ki Kim PhD [3], Jae-Kyung Won, MD, PhD [4], and Samuel Fulcher, MD [5]

Central Texas Veterans Research Foundation, Temple, Texas, United States of America

[1] Department of Ophthalmology, Seoul National University Hospital, Daehak-ro, Jongno-gu, Seoul, Republic of Korea

[2] Cooperative Studies Program Coordinating Center, VA Maryland Health Care System, Perry Point, Maryland, United States of America

[3] Institute for Regenerative Medicine, Texas A&M Health Science Center, College Station, Texas, United States of America

[4] Department of Pathology, Seoul National University Hospital, Daehak-ro, Jongno-gu, Seoul, Republic of Korea

[5.] Central Texas Veterans Health Care System, Temple, Texas, United States of America

*to whom correspondence may be addressed.

1901 S. 1st St. (Room 3R34)

Temple, TX 76504-7451
Phone: 254-743-1204
E-mail:hchoi@medicine.tamhsc.edu

Keywords: corneal alkali injury, inflammation, corneal neovascularization, fibrosis, cytokine

Running Title: Corneal Alkali Injury

Abbreviations

DAMPs: damage associated molecular patterns

CNV: corneal neovascularization

H&E: hematoxylin and eosin

MPO: myeloperoxidase

MSCs: Mesenchymal Stem cells

Conflict of interest statement

Authors have no financial disclosures that would be a potential conflict of interest with this manuscript.

Funding Support

This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0495.

Abstract

Aim: To examine the various effects of alkali injury to the cornea over a 21-day period.

Methods: Alkali injury was induced in one eye of male Lewis rats. Corneal opacity and corneal neovascularization were assessed daily. Real-time qRT-PCR analysis and immunohistochemical staining were conducted to examine inflammation, neovascularization, and fibrosis.

Results: We found that within 2 hours of chemical exposure, corneal opacification rapidly developed with an acute increase in various cytokine expressions while several cytokines demonstrated a secondary peak by day 7. Early neutrophil infiltration peaked at day 1 post-injury while macrophage infiltration peaked at day 7. Throughout the time course of the study, corneal opacity persisted and neovascularization, lymphangiogenesis, and fibrosis progressed.

Conclusions: This study highlighted the cellular, biochemical, clinical, and histopathological changes throughout the progression of a corneal alkali injury. These findings will help to better understand the pathogenesis of an ocular alkali injury and the development of therapy for this injury.

Introduction

Chemical trauma to the eye, including alkali injury, is among the most difficult of eye injuries to treat and manage. Despite therapeutic interventions, rehabilitation of vision takes months to years in many cases, commonly results in permanent, irreversible vision loss [1]. Alkali injury to the eye, in particular, has a grim prognosis due to the rapid and deep penetration of the alkali which results in massive tissue destruction of the eyelid and ocular surface including conjunctiva, limbus, and cornea, as well as intraocular structures to include the trabecular meshwork, lens, iris, and retina [2]. The rapid penetration of lipophilic alkali into the eye results in saponification of tissue with the subsequent release of proteolytic enzymes, which leads to further tissue destruction and inflammatory responses [3]. Destruction of the limbal epithelium results in chronic ocular surface disease, which portends a poor prognosis for corneal transplantation and future vision rehabilitation, while intractable glaucoma, cataract formation, and conjunctival and eyelid scarring may require multiple surgeries across subspecialties over months and years [4, 5]. Advancement in the understanding of the pathophysiology and natural history of ocular alkali injury will help facilitate the development of new treatment modalities for these devastating injuries.

Corneal trauma induces neutrophil infiltration into the cornea, facilitated with the release of secretoneurin within 15 minutes from the time of injury. Injured stromal keratocytes release damage associated molecular patterns (DAMPs), including heat shock protein (HSPB4). DAMPs in turn activate resident macrophages through the toll like receptor

(TLR)/NF kB axis, which results in neutrophil chemotaxis and infiltration peaking at 24-48 hours post injury [6]. Neutrophil degranulation leads to extensive further tissue damage, and in combination with loss of epithelium from the initial toxic insult, can lead to corneal opacification, stromal ulceration and lysis, and corneal perforation. Late phase inflammation contributes to continuing corneal opacification, stromal fibrosis, and neovascularization.

Activated stromal keratocytes proliferate and migrate into the damaged corneal stroma, differentiate into myofibroblasts, and synthesize types I and III collagen, α-smooth muscle actin, and extracellular matrix components such as fibronectin [1, 7]. These components of corneal wound healing disrupt the regular array of corneal collagen fibrils, which results in corneal opacification and scarring. Corneal neovascularization (CNV) contributes further to opacification by disruption of the regular collagen matrix, and edema through vascular extravasation [8].

The limited effectiveness of current treatment for corneal alkali burns underscores the need for more effective management options and the development of new modalities, which depend upon reliable and reproducible animal models. Animal models of corneal alkali burn, which use filter paper saturated with sodium hydroxide are widely used, but as yet have not fully characterized the molecular mechanisms of injury. Here we present the extensive molecular characterization of corneal alkali injury in a rat model with clinical and histopathologic correlation.

Materials and Methods

Animal model of a corneal alkali injury

This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal use protocol was approved and monitored by the Institutional Animal Care and Use Committee of Central Texas Veterans Health Care System and Baylor Scott & White Healthcare. In conducting research using animals, the investigators adheres to the laws of the United States and regulations of the Department of Agriculture. Eight-week-old male Lewis rats (LEW/Crl) purchased from Charles River Laboratories (Wilmington, MA) received a minimum of five days acclimation. Corneal alkali injuries were produced with a 4-mm diameter circular filter paper soaked in 1 N NaOH, then applied to the central cornea of the right eye for 30 seconds under general ketamine/xylazine anesthesia. Topical analgesia (0.5% proparacaine hydrochloride ophthalmic solution; Sandoz Inc. Princeton, NJ) was applied prior to injury. Immediately after alkali exposure, the ocular surface was flushed with 50 mL of PBS (pH 7.4) for 2 minutes. Antibiotic (Vigamox; 0.5% moxifloxacin hydrochloride ophthalmic solution; Alcon Laboratory, Inc. Fort Worth, TX) was applied to the cornea and the eyelid was sutured with size 7-0 silk suture (Ethicon, Inc. Cincinnati, OH). Tarsorrhaphy is performed to prevent scratching of the cornea and to reduce the risk of bedding, found on the floor of the enclosure, from entering the eye. The suture is removed 24 hours after the time of injury or at time of harvest for those involved in the two and four hour study groups.

The rats were euthanized with ketamine/xylazine at 2 hours, 4 hours and 1, 2, 3, 5, 7, 14 and 21 days after alkali injury. Corneal tissue was isolated and submitted for histological and immunohistochemical analysis, RNA expression, or protein isolation.

Biomicroscopic examination

Gross examination and photography of the corneas were performed daily under isoflurane anesthesia using a dissecting microscope for cornea opacity grading. Grading was performed independently by two experienced ophthalmologists blinded to the allocation of the animals in each group.

The cornea opacity scoring system followed that of Gupta el.al. [9] as outlined below:

0 = no opacity

1 = minimal superficial (non-stromal) slight opacity

2 = moderate stromal opacity; anterior chamber and iris both well visualized

3 = significant stromal opacity; pupil visible with haze

4 = intense stromal opacity; pupil visible and anterior chamber not visible

Corneal Neovascularization (CNV) was graded as follows:

0 = No Neovascularization

1 = Neovascularization in the peripheral cornea within one third of the corneal diameter as measured from the limbus

2 = Neovascularization within two thirds of the diameter from the limbus

3 = Neovascularization observed in the entire cornea

Histopathology

Rat corneas were excised after euthanasia, fixed in 10% buffered formalin, and embedded in paraffin. Specimens were cut into 4 μm sections and stained with hematoxylin and eosin (H&E) for histopathological examination. Antigens for immunohistochemical analyses were retrieved with a steamer in epitope retrieval solution (10 mM sodium citrate pH 6).

The samples were blocked in 3% goat serum (Vector Laboratories, Burlingame, CA) / 0.2% Triton X100 in PBS for 30 minutes at room temperature and subsequently incubated with primary antibody diluted in 3% goat serum in PBS overnight at 4ºC. Sections incubated with rabbit or mouse IgG served as negative controls. After washing with PBS, the samples were incubated with a biotinylated secondary IgG antibody for 1 hour at room temperature and washed again with PBS. Rabbit polyclonal anti-neutrophil elastase antibody (1:150) (ab21595, Abcam) was used for neutrophil infiltration detection, and mouse monoclonal anti-thrombomodulin (CD141) antibody (1:50) (ab33513, Abcam) was used for the detection of neovascular endothelial cells. Mouse monoclonal anti-CD68 antibody (ED1) (1:200) (sc59103, Santa Cruz Biotechnology) was used for macrophage infiltration detection. Rabbit anti-type I collagens (1:200) (ab34710, Abcam), rabbit anti-type III collagens (1μl/ml) (ab7778, Abcam) were used for fibrotic extracellular matrix secretion. Mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (1:200) (A5228, Sigma) was used to detect myofibroblasts. Anti-rabbit IgG (1:5000) (Abcam) or anti-mouse IgG (1:5000) (Abcam) were used as secondary antibodies. Avidin-biotin-peroxidase complex technique was used for immunostaining

according to the manufacturer's protocol with nickel enhancement (Vectastain ABC kit, DAB Peroxidase Substrate Kit, Vector Laboratories). The nuclei were counterstained with Hematoxylin QS (Vector Laboratories).

Enzyme-linked immunosorbent assay (ELISA)

Neutrophil infiltration was examined by ELISA for myeloperoxidase (MPO) contained within neutrophil granules. Corneal specimens were placed on ice and homogenized in 500 µL of tissue extraction buffer (Life Technologies, Carlsbad, CA) using a sonicator at 90% amplitude (Sonic Dismembrator model 120, Fisher Scientific, Waltham, MA), then centrifuged at 12,000 g for 15 minutes at 4 ºC. Supernatants were collected for ELISA. Measurements were performed with Rat MPO ELISA kit according to the manufacturer's specifications (Hycult Biotechnology, Uden, The Netherlands).

mRNA expression analysis

Real-time qRT-PCR was used to evaluate mRNA expression levels of cytokines, chemokines, and genes involved in neovascularization, lymphangiogenesis and fibrosis. Cornea total RNA was extracted with the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration and purity (A260/A280) were measured with a NanoDrop ND-1000 V 3.2.1 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Real time amplification was performed with QuantiTect Probe RT-PCR Kit (Qiagen) and analyzed on 7900HT Fast Real-Time PCR system (Applied Biosystems). The Taqman gene expression assays used are as follows: interleukin-1β (IL-1β; Rn00580432_m1), IL-1α (Rn00566700_m1), IL-6

(Rn01410330_m1), IL-10 (Rn01483988_g1), C-X-C Motif Ligand 1 (CXCL1; Rn00578225_m1), monocyte chemoattractant protein-1 (MCP-1/CCL2; Rn00580555_m1), CCL3 (Rn01464736_g1), tumor necrosis factor alpha (TNF-α; Rn99999017_m1), vascular endothelial growth factor A (VEGF-A; Rn01511601_m1), neutrophil elastase (ELANE; Rn01535456_g1), F4/80 (Emr1; Rn01527631_m1), CD68 (Rn01495634_g1), CD163 (Rn01492519_m1), Thrombomodulin (Thbd, CD141; Rn00582226_s1), Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE1; Rn01510421_m1), type I collagens (Col1a1; Rn01463848_m1), type III collagens (Col3a1; Rn01437650_m1) α-smooth muscle actin (α-SMA, ACTA2; Rn01759925_g1). For relative quantitation of gene expression, rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe (Rn01775763_g1) were used.

Statistical analysis.

Data are expressed as mean ± SEM. All analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). The MPO ELISA and real-time qRT-PCR data were evaluated by one-way ANOVA with Tukey's Multiple Comparison Test. The Kruskal-Wallis one-way ANOVA with Dunn's post-hoc tests was carried out for opacity grade and CNV grade. Opacity and CNV grades classify observation into discrete categories. The resulting ordinal scale data requires non-parametric analysis. The A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Biomicroscopic and Histopathological examination

Representative photos in Figure 1A depict the progression of 1N NaOH induced corneal injury over time. The initial alkali injury resulted in corneal epithelial destruction in all injured eyes. Significant corneal opacification developed immediately upon injury and progressed until day 9. Corneal opacity stabilized from days 9 to 14, and slowly improved thereafter until day 21 (Figure 1B). Corneal epithelial denudation was observed at the center and periphery of the cornea (Figure 1C, D). Inflammatory infiltrates and neovascularization of the corneal stroma were detected in the peripheral cornea on day 1 (Figure 1D). By day 21, Regeneration of corneal epithelium was completed and corneal epithelial thickness was normalized. Densely compacted opaque stroma was recognized by day 7 as (Figure 1C, D).

Inflammatory cell infiltration

Inflammatory cell infiltration, including neutrophils and macrophages, were observed in corneal specimens after alkali injury. Neutrophil infiltration was evaluated by the level of MPO protein, a major component of neutrophil primary granules in cornea lysate [10]. Neutrophil elastase mRNA transcription levels were detected by real-time qRT-PCR as an additional marker for neutrophil infiltration. MPO was detected as early as 4 hours after injury, peaked at days 1 and 3, then reduced by 17% of the peak level by day 5 (Figure 2A). Neutrophil elastase expression demonstrated a similar pattern, but peaked

at 4 hours after injury with reduction to 29% of the peak level by day 7 then returned to baseline levels by day 21 (Figure 2B). Neutrophil elastase positive neutrophils were detected in day 1 but rarely present in the center of the cornea thereafter (Figure 2F), however there was increased presence of neutrophils in the periphery of the cornea from day 1 to day 7 (Figure 2G). Macrophage infiltration was evaluated by CD68 and Emr1 (EGF-like module-containing mucin-like hormone receptor-like 1 also known as F4/80) expression, and was detected 2 hours after injury (Figure 2C, D). Macrophage infiltration peaked from days 7 to 14 post injury. Pan-macrophage marker CD68 staining demonstrated the macrophage infiltration in the corneal periphery as early as day 1, however macrophages were not observed centrally until day 7 (Figure 2H, I). M2 macrophage (CD163 expressing cells) infiltration peaked at day 1 and day 7 (Figure 2E).

Cytokine Expression

Real-time qRT-PCR analysis was performed to assess the expression of genes associated with pro-inflammatory cytokines (IL-1β, IL-1α, IL-6, and TNF-α), anti-inflammatory cytokines (IL-10), and chemotactic chemokines (CXCL1, CCL2, and CCL3) (Figure 3). Levels of IL-1β increased by 2 hours after injury and peaked at day 1, after which it reduced until day 5, and was followed by a secondary peak observed on day 7. IL-1β expression level remained upregulated through day 21. IL-1α expression increased early and peaked at 4 hours, followed by below baseline levels by days 1 and 2. A secondary increase of IL-1α expression was observed at day 3 and remained elevated through day 21. IL-6 demonstrated an early peak at 4 hours, followed by a

decrease to baseline levels by day 2. TNF-α expression was detected in normal cornea but post injury fell to subnormal levels, returning to baseline by day 7. Thereafter, TNF-α expression increased to day 14, and returned to normal by day 21. IL-10 peaked at 2 hours after injury, but quickly dropped by hour 4. Enhanced expression was observed at day 1 then gradually decreased to baseline by day 7. Chemokines CXCL1, CCL2 and CCL3 expression showed early increases and peaked at 4 hours or by day1. CCL3 demonstrated a robust second peak at day 3 as inflammation progressed.

Corneal neovascularization (CNV)

CNV progressively increased post injury until it plateaued at day 15 (Figure 4A). VEGF expression demonstrated an early steep increase at 2 and 4 hours post injury, returned to baseline by day 2, then increased to a second peak from days 5 to 14, returning to normal by day 21 (Figure 4B). The expression of endothelial marker thrombomodulin (CD141) showed a very similar expression pattern with that of VEGF, except CD141 remained elevated through day 21 (Figure 4C). CD141 positive vessels were found in the periphery of the cornea from day 1 but do not appear in the center of the cornea until day 7 (Figure 4F, G). The expression of lymphatic endothelial cell marker, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1), was enhanced at day 1, then gradually decreased by day 21 to near baseline levels (Figure 4D).

Fibrosis

Corneal fibrosis after injury may lead to scar formation. The expression level of collagen type I is increased during fibrosis and its expression is enhanced in pathological fibrotic processes influenced by TGF-β [11]. The expression of TGF-β peaked at day 1, fell to

baseline levels by days 2 and 3, increased from day 5 to a smaller secondary peak by day 7, and returned to baseline by day 21 (Figure 5A). Expression of collagen type I was transiently increased 2 and 4 hours after alkali injury and peaked by day 7 (Figure 5B). Increased collagen type I staining was observed from day 7 onward. The pattern of α-SMA expression mirrors that of collagen type I (Figure 5C). Increased collagen type I and α-SMA staining in corneal stroma was detected from day 1 in the peripheral cornea, and by day 7 in the center of the cornea (Figure 6 A, B, C, D). However, the increase of the collagen type III was not prominent. Histological imagery and gene expression values exhibit a slight increase of collage III in the corneal stroma for days 3 onward (Figure 6 E, F), though gene expression also indicates a significant increase at day 1(Figure 5D).

Discussion

Alkali injury of the cornea can be devastating and often leads to permanent vision loss and chronic pain. No effective therapeutic options currently exist for severe corneal chemical injury, and corneal transplantation to restore vision carries a grim prognosis. Animal models for corneal alkali injury have been developed and extensively used to study therapeutic alternatives, more recently showing a strong emphasis on inflammation and CNV. Previous studies have demonstrated a decrease of inflammatory response following ocular injury using cytokine inhibitors including TNFα inhibitor [12-14] and IL-6 inhibitor [15, 16], in addition to various anti-inflammatory molecules [17-20]. As for CNV research, the main focus is VEGF, as this is a prime source of

neovascularization [21-26]. Cell and-tissue therapies were investigated. Mesenchymal Stem cells (MSCs) [27-30] including limbal MSCs [31], omental cells [32], and amniotic fluid [17, 33-35] demonstrate improvement of the disease progress. There are few reports which extensively describe the elaboration of inflammatory markers, and which correlate clinical and histopathological findings for an extended period of time. We describe a rat model for corneal alkali injury which details the time course of inflammatory marker expression, as well as the clinical and histopathological findings over a 21 day period which could serve as a reproducible and noninvasive model to investigate the mechanisms of inflammation, fibrosis, and neovascularization of the alkali injured cornea. Similar to other published reports, this study works with 8 week old male Lewis rats. It is unknown at this time is gender or age affects injury progression. As more adults are treated for ocular chemical injuries than children, we intend to pursue future age-related studies for comparison of injury progression using rats six months of age or older.

The inflammatory cell response was influenced by cytokines and growth factors, elaborated by damaged tissue throughout the process of injury and subsequent repair. Alkali injury induced an acute early response of the cornea and immune system as shown by the rapid increase of the expressions of genes involved in inflammation, CNV, and fibrosis. Corneal opacification rapidly developed with all corneas scored as grade 2 within 2 hours of injury, while a significant number (5 out of 16) of corneas were scored as grade 3 within 4 hours. Neutrophil infiltration began by 4 hours post injury and remained elevated until day 3. Activated macrophage marker (EMR1) was significantly increased by 2 hours, but the increase of CD68 expression was minimal until 3 days

after injury. These findings indicate that during the acute phase, resident macrophages were activated, while macrophage infiltration was minimal. The observed high expression of CD163 between day 1 and day 7 indicated the presence of M2 macrophages which decrease inflammation and encourage tissue repair during the injury process [36]. Gene expression of cytokines IL-1β, IL-1α, IL-6, IL-10, and TNF-α, and chemokines CXCL1, CCL2, CCL3 were significantly increased within 2 hours. The rapid response implies the source of the cytokines was the cornea or resident macrophages rather than infiltrating leukocytes. Cytokine and chemokine expression remained elevated except for decreased expression of TNF-α, IL-10 and CCL2 by 4 hours after injury. Neovascularization related genes, VEGF, VEGFR1, and thrombomodulin were significantly increased within 2 hours, however expression of LYVE1, which is expressed in lymphangiogenesis, was shown to peak 1 day post-injury. Collagen type I and α-smooth muscle actin increased within 2 hours after injury, peaked at day 7 and remained elevated until day 14.

Our data are consistent with the observations of others which demonstrated an initial rapid increase in corneal neutrophil infiltration [37] though there are contradictory observations for macrophage infiltration. Uchiyama et. al.. [37] demonstrated a peak in macrophage infiltration at day 1, where we observed a peak between days 7 and 14, which is consistent with the remodeling and repair of tissue damage seen in chronic inflammatory responses to injury. The day 7 peak in macrophage infiltration that we observed was comparable with the reports of Han et.al. [37, 38].

As the initial acute inflammatory response subsided by day 7, evident by the decline in expression of IL-6, IL-10, CXCL1, and CCL2, the expression of cytokines increased related to a secondary delayed inflammatory response, evident by the late increase in IL-1α, IL-1β, TNF-α and CCL3 expressions. Furthermore, increases in corneal fibrosis related genes including TGF-β, α-SMA and collagen type I, were seen in conjunction with the increase in macrophage marker gene expression. There are few reports of long term time course studies which describe the secondary delayed inflammatory response in corneal alkali injury, however low level increases of IL-1β, TNF-α, CCL2, and TGF-β [37] have been observed at day 7.

The degree of corneal opacity increased continuously through day 14 followed by a slight improvement at day 21, which matches previously reported data [39]. Others have reported that corneal opacity does not significantly improve by 7 weeks after injury [40]. The normal cornea is avascular, which is instrumental in the maintenance of corneal clarity and function. CNV induced by injury leads to the irregular arrangement of collagen and aggravates corneal scarring. CNV has been reported to continuously increase within 1-2 weeks of chemical injury, [37-39, 41-43] and the CNV was persistent for up to 10 weeks after injury [40]. Stromal fibrosis which follows corneal injury limits corneal transparency due to the disorganization of the regular and uniform array of collagen fibrils in the normal cornea. This disruption of the regular array of collagen fibrils disrupts the transmittance of light through the cornea resulting in a loss of clarity [44]. Goldman and Benedem also recognized that light cannot resolve structures substantially smaller than its wavelength, therefore a larger distance of stromal fiber caused by edema can lead to loss of transparency [45].

Additionally, the expression of α-SMA is increased following alkali injury which results in further loss of corneal clarity as corneal fibrosis and scarring ensues. Our data demonstrate that fibrosis related genes were increased acutely by 2 hours, returned to normal levels at day 2, increased again to reach a peak at day 7, and then gradually declined until day 21. Collagen type III expression displayed an increasing trend at later time points, yet was less prominent when paralleled with previous reports [37, 46].

The extensive description that we have presented in this model highlights the cellular, biochemical, clinical, and histopathological changes that occur following a corneal alkali injury. Providing gene expression, histological findings, and clinical analysis, this report encompasses a more thorough understanding of disease progression. Our data illustrates histological and biological outcomes from multiple time points for a more extensive time interval which can provide a thorough reference for future research.

This data adds to the body of knowledge regarding chemical injury to the eye, and as such may serve to assist in the development of novel therapeutic treatment for the management of these devastating injuries.

Funding/support

Publication of this article was supported by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0495. Opinions, interpretations, conclusions and recommendations are those of the author and are not

necessarily endorsed by the Department of Defense. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office.

1. Wagoner MD. Chemical injuries of the eye: current concepts in pathophysiology and therapy. Surv Ophthalmol 1997;41:275-313.

2. Peate WF. Work-related eye injuries and illnesses. Am Fam Physician 2007;75:1017-1022.

3. Eslani M, Movahedan A, Afsharkhamseh N, et al. The role of toll-like receptor 4 in corneal epithelial wound healing. Invest Ophthalmol Vis Sci 2014;55:6108-6115.

4. Spector J, Fernandez WG. Chemical, thermal, and biological ocular exposures. Emerg Med Clin North Am 2008;26:125-36, vii.

5. Sharma N, Singh D, Sobti A, et al. Course and outcome of accidental sodium hydroxide ocular injury. Am J Ophthalmol 2012;154:740-749.e2.

6. Oh JY, Choi H, Lee RH, et al. Identification of the HSPB4/TLR2/NF-kappaB axis in macrophage as a therapeutic target for sterile inflammation of the cornea. EMBO Mol Med 2012;4:435-448.

7. Zieske JD, Guimaraes SR, Hutcheon AE. Kinetics of keratocyte proliferation in response to epithelial debridement. Exp Eye Res 2001;72:33-39.

8. Qazi Y, Wong G, Monson B, et al. Corneal transparency: genesis, maintenance and dysfunction. Brain Res Bull 2010;81:198-210.

9. Gupta N, Kalaivani M, Tandon R. Comparison of prognostic value of Roper Hall and Dua classification systems in acute ocular burns. Br J Ophthalmol 2011;95:194-198.

10. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997;89:3503-3521.

11. Fine A, Goldstein RH. The effect of transforming growth factor-beta on cell proliferation and collagen formation by lung fibroblasts. J Biol Chem 1987;262:3897-3902.

12. Cade F, Paschalis EI, Regatieri CV, et al. Alkali burn to the eye: protection using TNF-alpha inhibition. Cornea 2014;33:382-389.

13. Ferrari G, Bignami F, Giacomini C, et al. Safety and efficacy of topical infliximab in a mouse model of ocular surface scarring. Invest Ophthalmol Vis Sci 2013;54:1680-1688.

14. Lu P, Li L, Liu G, et al. Critical role of TNF-alpha-induced macrophage VEGF and iNOS production in the experimental corneal neovascularization. Invest Ophthalmol Vis Sci 2012;53:3516-3526.

15. Sari ES, Yazici A, Aksit H, et al. Inhibitory effect of sub-conjunctival tocilizumab on alkali burn induced corneal neovascularization in rats. Curr Eye Res 2015;40:48-55.

16. Sakimoto T. Potential Application of Biological Products for the Treatment of Ocular Surface Inflammation. Cornea 2015;34 Suppl 11:S153-7.

17. Saika S, Miyamoto T, Yamanaka O, et al. Therapeutic effect of topical administration of SN50, an inhibitor of nuclear factor-kappaB, in treatment of corneal alkali burns in mice. Am J Pathol 2005;166:1393-1403.

18. Bignami F, Giacomini C, Lorusso A, et al. NK1 receptor antagonists as a new treatment for corneal neovascularization. Invest Ophthalmol Vis Sci 2014;55:6783-6794.

19. Yamada J, Dana MR, Sotozono C, et al. Local suppression of IL-1 by receptor antagonist in the rat model of corneal alkali injury. Exp Eye Res 2003;76:161-167.

20. Kim DW, Lee SH, Shin MJ, et al. PEP-1-FK506BP inhibits alkali burn-induced corneal inflammation on the rat model of corneal alkali injury. BMB Rep 2015;48:618-623.

21. Liarakos VS, Papaconstantinou D, Vergados I, et al. The effect of subconjunctival ranibizumab on corneal and anterior segment neovascularization: study on an animal model. Eur J Ophthalmol 2014;24:299-308.

22. Chen WL, Lin CT, Lin NT, et al. Subconjunctival injection of bevacizumab (avastin) on corneal neovascularization in different rabbit models of corneal angiogenesis. Invest Ophthalmol Vis Sci 2009;50:1659-1665.

23. Su W, Li Z, Li Y, et al. Doxycycline enhances the inhibitory effects of bevacizumab on corneal neovascularization and prevents its side effects. Invest Ophthalmol Vis Sci 2011;52:9108-9115.

24. Hoffart L, Matonti F, Conrath J, et al. Inhibition of corneal neovascularization after alkali burn: comparison of different doses of bevacizumab in monotherapy or associated with dexamethasone. Clin Experiment Ophthalmol 2010;38:346-352.

25. Lee SH, Leem HS, Jeong SM, et al. Bevacizumab accelerates corneal wound healing by inhibiting TGF-beta2 expression in alkali-burned mouse cornea. BMB Rep 2009;42:800-805.

26. Lee KJ, Lee JY, Lee SH, et al. Accelerating repaired basement membrane after bevacizumab treatment on alkali-burned mouse cornea. BMB Rep 2013;46:195-200.

27. Almaliotis D, Koliakos G, Papakonstantinou E, et al. Mesenchymal stem cells improve healing of the cornea after alkali injury. Graefes Arch Clin Exp Ophthalmol 2015;253:1121-1135.

28. Holan V, Trosan P, Cejka C, et al. A Comparative Study of the Therapeutic Potential of Mesenchymal Stem Cells and Limbal Epithelial Stem Cells for Ocular Surface Reconstruction. Stem Cells Transl Med 2015;4:1052-1063.

29. Yao L, Li ZR, Su WR, et al. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. PLoS One 2012;7:e30842.

30. Ke Y, Wu Y, Cui X, et al. Polysaccharide hydrogel combined with mesenchymal stem cells promotes the healing of corneal alkali burn in rats. PLoS One 2015;10:e0119725.

31. Acar U, Pinarli FA, Acar DE, et al. Effect of allogeneic limbal mesenchymal stem cell therapy in corneal healing: role of administration route. Ophthalmic Res 2015;53:82-89.

32. Bu P, Vin AP, Sethupathi P, et al. Effects of activated omental cells on rat limbal corneal alkali injury. Exp Eye Res 2014;121:143-146.

33. Choi JA, Choi JS, Joo CK. Effects of amniotic membrane suspension in the rat alkali burn model. Mol Vis 2011;17:404-412.

34. Jiang A, Li C, Gao Y, et al. In vivo and in vitro inhibitory effect of amniotic extraction on neovascularization. Cornea 2006;25:S36-40.

35. Herretes S, Suwan-Apichon O, Pirouzmanesh A, et al. Use of topical human amniotic fluid in the treatment of acute ocular alkali injuries in mice. Am J Ophthalmol 2006;142:271-278.

36. Mills CD. M1 and M2 Macrophages: Oracles of Health and Disease. Crit Rev Immunol 2012;32:463-488.

37. Uchiyama M, Shimizu A, Masuda Y, et al. An ophthalmic solution of a peroxisome proliferator-activated receptor gamma agonist prevents corneal inflammation in a rat alkali burn model. Mol Vis 2013;19:2135-2150.

38. Han Y, Shao Y, Lin Z, et al. Netrin-1 simultaneously suppresses corneal inflammation and neovascularization. Invest Ophthalmol Vis Sci 2012;53:1285-1295.

39. Den S, Sotozono C, Kinoshita S, et al. Efficacy of early systemic betamethasone or cyclosporin A after corneal alkali injury via inflammatory cytokine reduction. Acta Ophthalmol Scand 2004;82:195-199.

40. Jiang TS, Cai L, Ji WY, et al. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. Mol Vis 2010;16:1304-1316.

41. Figueroa-Ortiz LC, Martin Rodriguez O, Garcia-Ben A, et al. Neovascular growth in an experimental alkali corneal burn model. Arch Soc Esp Oftalmol 2014;89:303-307.

42. Liu X, Lin Z, Zhou T, et al. Anti-angiogenic and anti-inflammatory effects of SERPINA3K on corneal injury. PLoS One 2011;6:e16712.

43. Hoffart L, Matonti F, Conrath J, et al. Inhibition of corneal neovascularization after alkali burn: comparison of different doses of bevacizumab in monotherapy or associated with dexamethasone. Clin Experiment Ophthalmol 2010;38:346-352.

44. Maurice DM. The structure and transparency of the cornea. J Physiol 1957;136:263-286.

45. Goldman JN, Benedek GB. The relationship between morphology and transparency in the nonswelling corneal stroma of the shark. Invest Ophthalmol 1967;6:574-600.

46. Saika S, Ooshima A, Shima K, et al. Collagen types in healing alkali-burned corneal stroma in rabbits. Jpn J Ophthalmol 1996;40:303-309.

Figure Legends

Fig. 1. Biomicroscopic and histopathological examination of corneal alkali injury. (A) Representative photographs demonstrate the 1N NaOH induced corneal injury progression. (B) Opacity score with the use of a clinical grading standard system on a scale from 0 to 4. The results are presented as the mean ± SEM, n=8~34. (C, D) Representative images of hematoxylin-eosin staining of injured cornea. 20 x magnification, scale bar 100 μm.

Figure 2. The infiltration of neutrophils and macrophages. Neutrophil infiltration was measured using myeloperoxidase concentration in the cornea extract (A) and real-time qRT-PCR assays for neutrophil elastase mRNA (B). CD68 (C) and Emr1 (D) expression was used as a proxy measure of macrophages. (E) CD163 expression represents M2 macrophages. Real-time qRT-PCR were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control. Neutrophil elastase staining of the (F) center (G) and periphery of the cornea. 20 x magnification, scale bar 100 μm. CD63 staining of the (H) center and (I) periphery of the cornea. 60 x magnification, scale bar 20 μm.

Figure 3. The expression of cytokines and chemokines in the cornea after alkali injury. Quantification of the mRNA expression levels of (**A**) interleukin (IL)-1β, (**B**) IL-1α, (**C**) IL-6, (D) IL-10, (E) tumor necrosis factor (TNF)-α, (F) C-X-C motif ligand (CXCL)1, (G) C-C motif ligand (CCL)2, (H) CCL3 mRNA. mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate

dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control.

Figure 4. (A) Quantification of neovascularization. Values are expressed in mean ± standard deviation, n=11. (B, C, D, E) The expression of neovascularization and lymphangiogenesis related genes. Quantification of the mRNA expression levels of (B) thrombomodulin (CD141; Thbd), (C) lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1), (D) vascular endothelial growth factor-A (VEGF) (E) VEGF receptor 1. mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control. CD141 staining of the (F) center and (G) periphery of the cornea. 60 x magnification, scale bar 20 μm. Arrow: CD141 positive blood vessel.

Figure 5. The expression of fibrosis related genes. Quantification of the mRNA expression levels of (A) transforming growth factor-β1 (TGF-β1) (B) α-smooth muscle actin (α-SMA), (C) Collagen type I (Col1a1), (D) Collagen type III (Col3a1) were measured with real-time qRT-PCR and were normalized to the level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control.

Figure 6. Histology of fibrosis related proteins. Collagen type I staining of the (A) center and (B) periphery of the cornea. The α-SMA staining of the (C) center and (D) periphery of the cornea. Collagen type III staining of the (E) center and (F) periphery of the cornea. 20 x magnification, scale bar 100 μm.

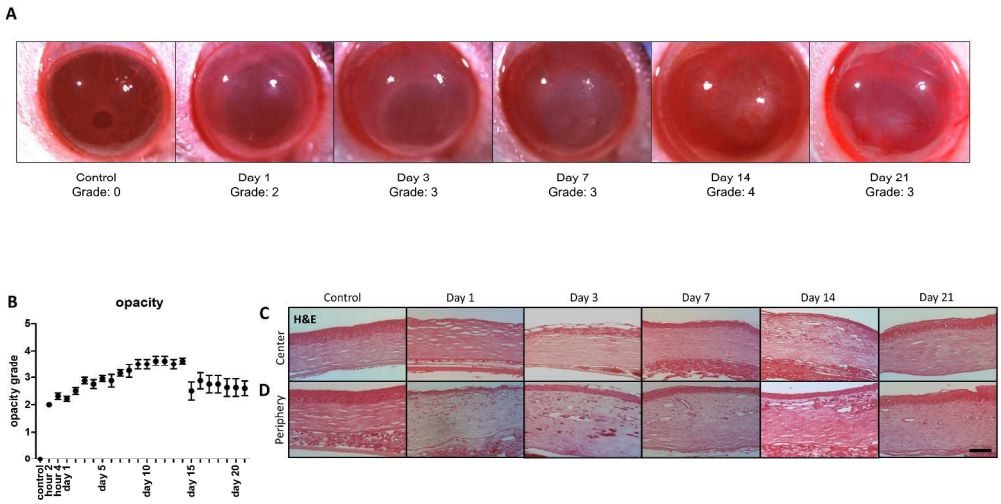


Figure 1

Fig. 1. Biomicroscopic and histopathological examination of corneal alkali injury. (A) Representative photographs demonstrate the 1N NaOH induced corneal injury progression. (B) Opacity score with the use of a clinical grading standard system on a scale from 0 to 4. The results are presented as the mean ± SEM, n=8~34. (C, D) Representative images of hematoxylin-eosin staining of injured cornea. 20 x magnification, scale bar 100 μm.

338x190mm (300 x 300 DPI)

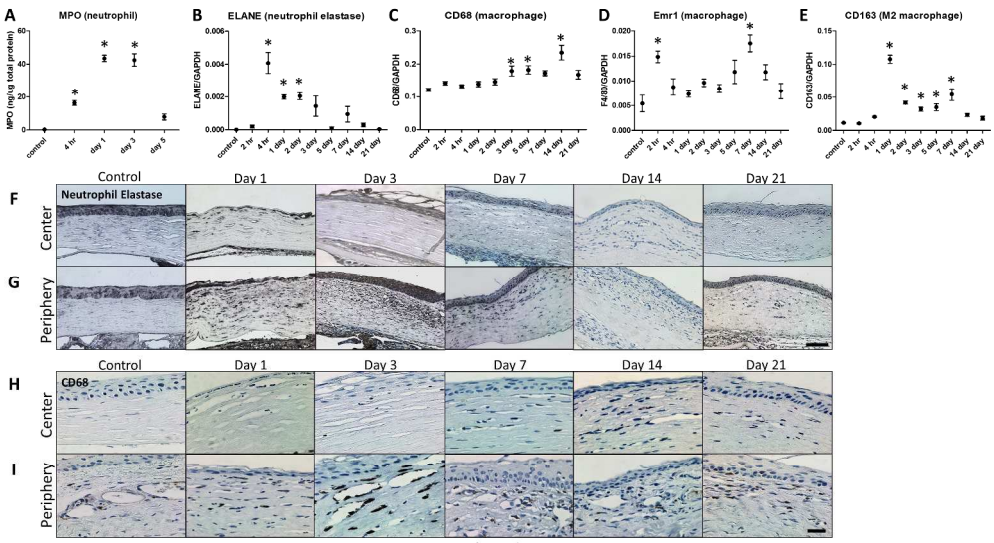


Figure 2

Figure 2. The infiltration of neutrophils and macrophages. Neutrophil infiltration was measured using myeloperoxidase concentration in the cornea extract (A) and real-time qRT-PCR assays for neutrophil elastase mRNA (B). CD68 (C) and Emr1 (D) expression was used as a proxy measure of macrophages. (E) CD163 expression represents M2 macrophages. Real-time qRT-PCR were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control. Neutrophil elastase staining of the (F) center (G) and periphery of the cornea. 20 x magnification, scale bar 100 µm. CD63 staining of the (H) center and (I) periphery of the cornea. 60 x magnification, scale bar 20 µm.

338x190mm (300 x 300 DPI)

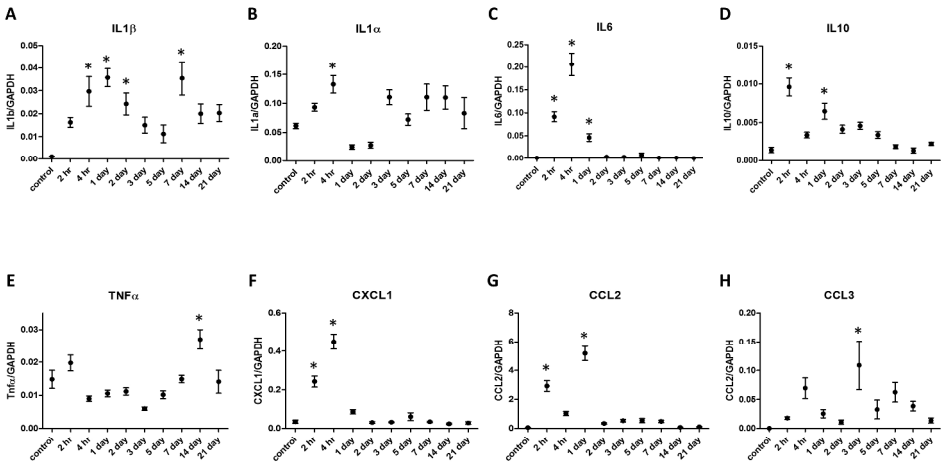


Figure 3

Figure 3. The expression of cytokines and chemokines in the cornea after alkali injury. Quantification of the mRNA expression levels of (A) interleukin (IL)-1β, (B) IL-1α, (C) IL-6, (D) IL-10, (E) tumor necrosis factor (TNF)-α, (F) C-X-C motif ligand (CXCL)1, (G) C-C motif ligand (CCL)2, (H) CCL3 mRNA. mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control.

338x190mm (300 x 300 DPI)

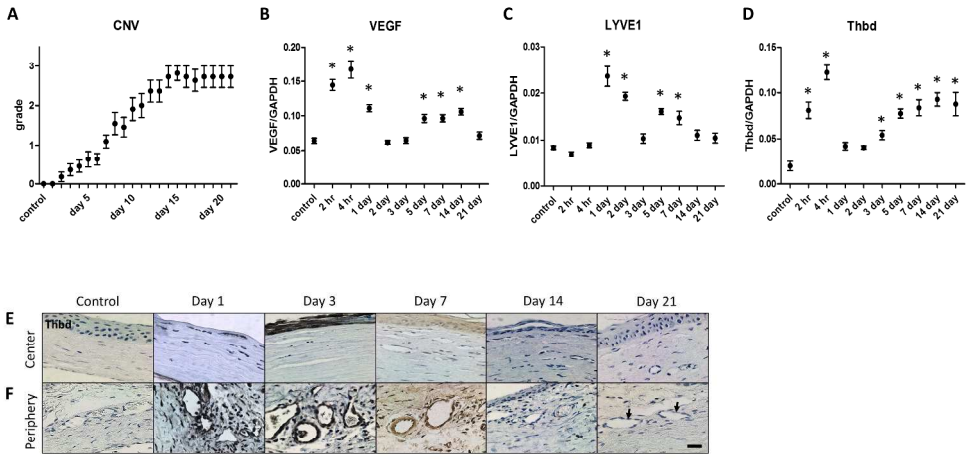


Figure 4

Figure 4. (A) Quantification of neovascularization. Values are expressed in mean ± standard deviation, n=11. (B, C, D, E) The expression of neovascularization and lymphangiogenesis related genes. Quantification of the mRNA expression levels of (B) thrombomodulin (CD141; Thbd), (C) lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1), (D) vascular endothelial growth factor-A (VEGF) (E) VEGF receptor 1. mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control. CD141 staining of the (F) center and (G) periphery of the cornea. 60 x magnification, scale bar 20 µm. Arrow: CD141 positive blood vessel.

338x190mm (300 x 300 DPI)

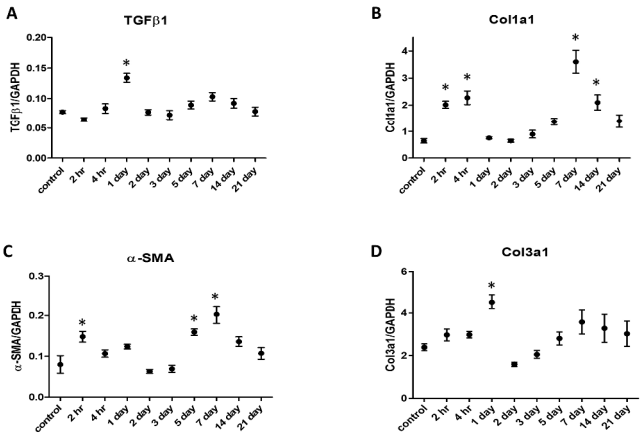


Figure 5

Figure 5. The expression of fibrosis related genes. Quantification of the mRNA expression levels of (A) transforming growth factor-β1 (TGF-β1) (B) α-smooth muscle actin (α-SMA), (C) Collagen type I (Col1a1), (D) Collagen type III (Col3a1) were measured with real-time qRT-PCR and were normalized to the level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SEM, n=8. *p<0.05 compared with the control.

338x190mm (300 x 300 DPI)

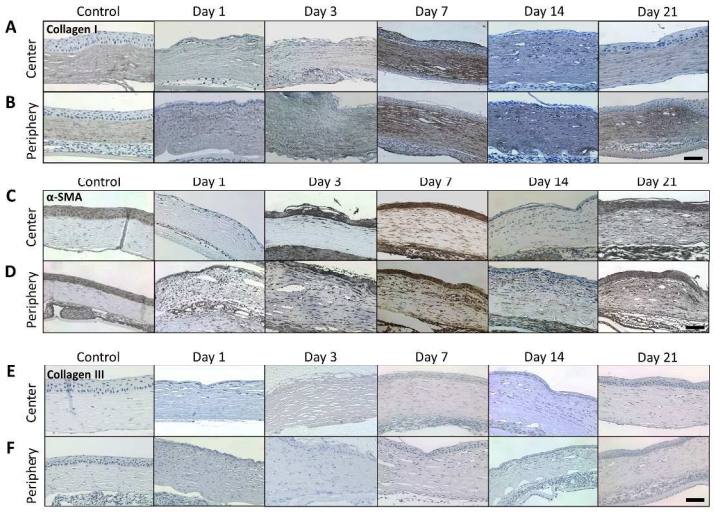


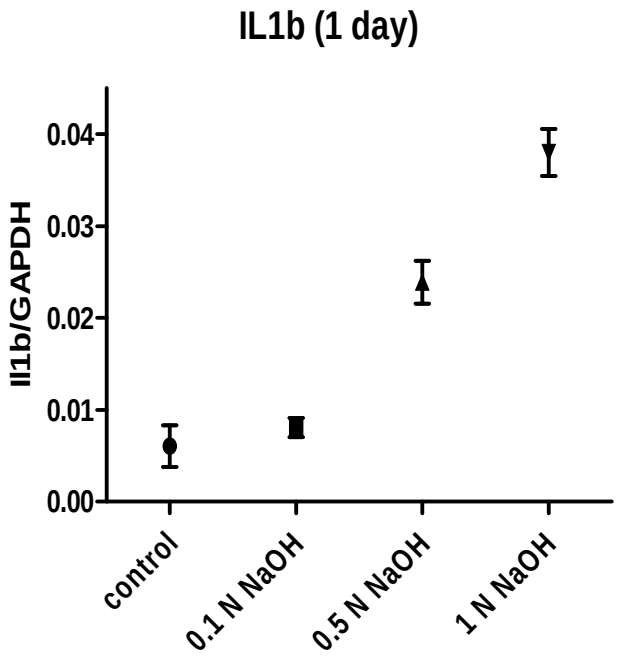
Figure 6

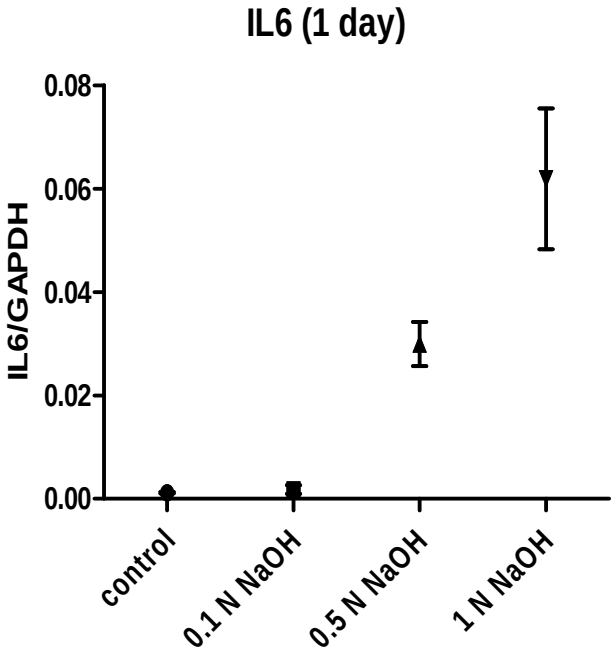
Figure 6. Histology of fibrosis related proteins. Collagen type I staining of the (A) center and (B) periphery of the cornea. The α-SMA staining of the (C) center and (D) periphery of the cornea. Collagen type III staining of the (E) center and (F) periphery of the cornea. 20 x magnification, scale bar 100 μm.

338x190mm (300 x 300 DPI)

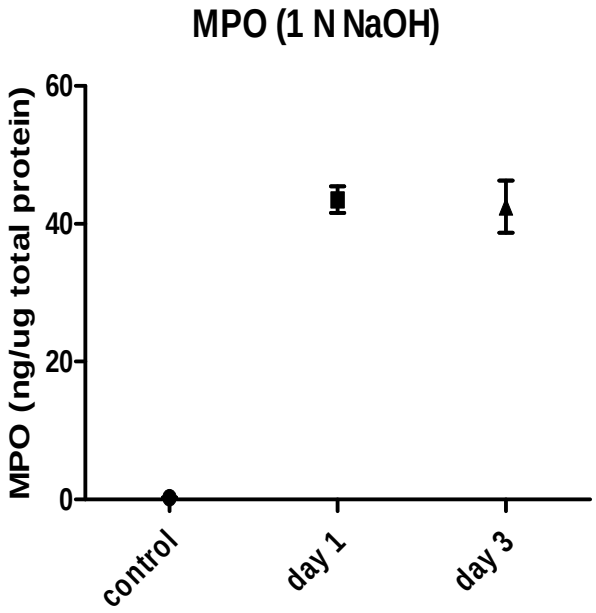
Appendix 3 Final Report

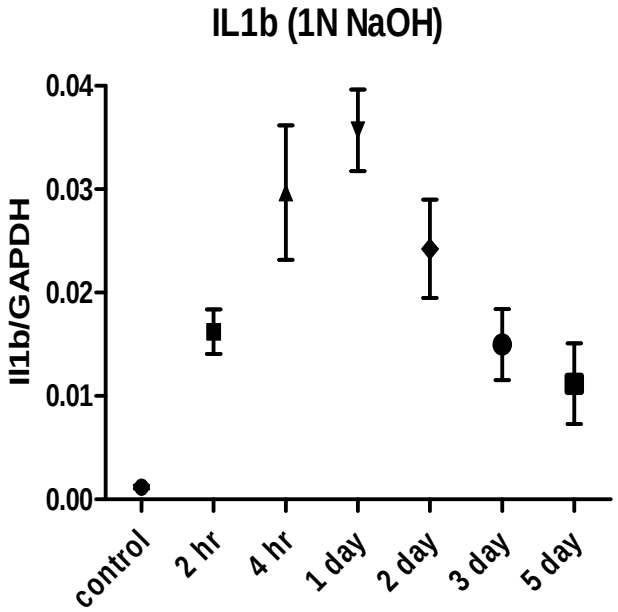
NaOH concentration dependent increase of cytokine expression

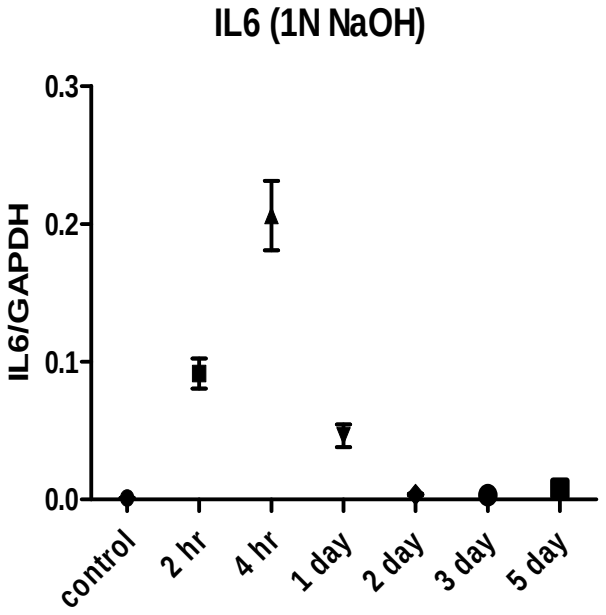




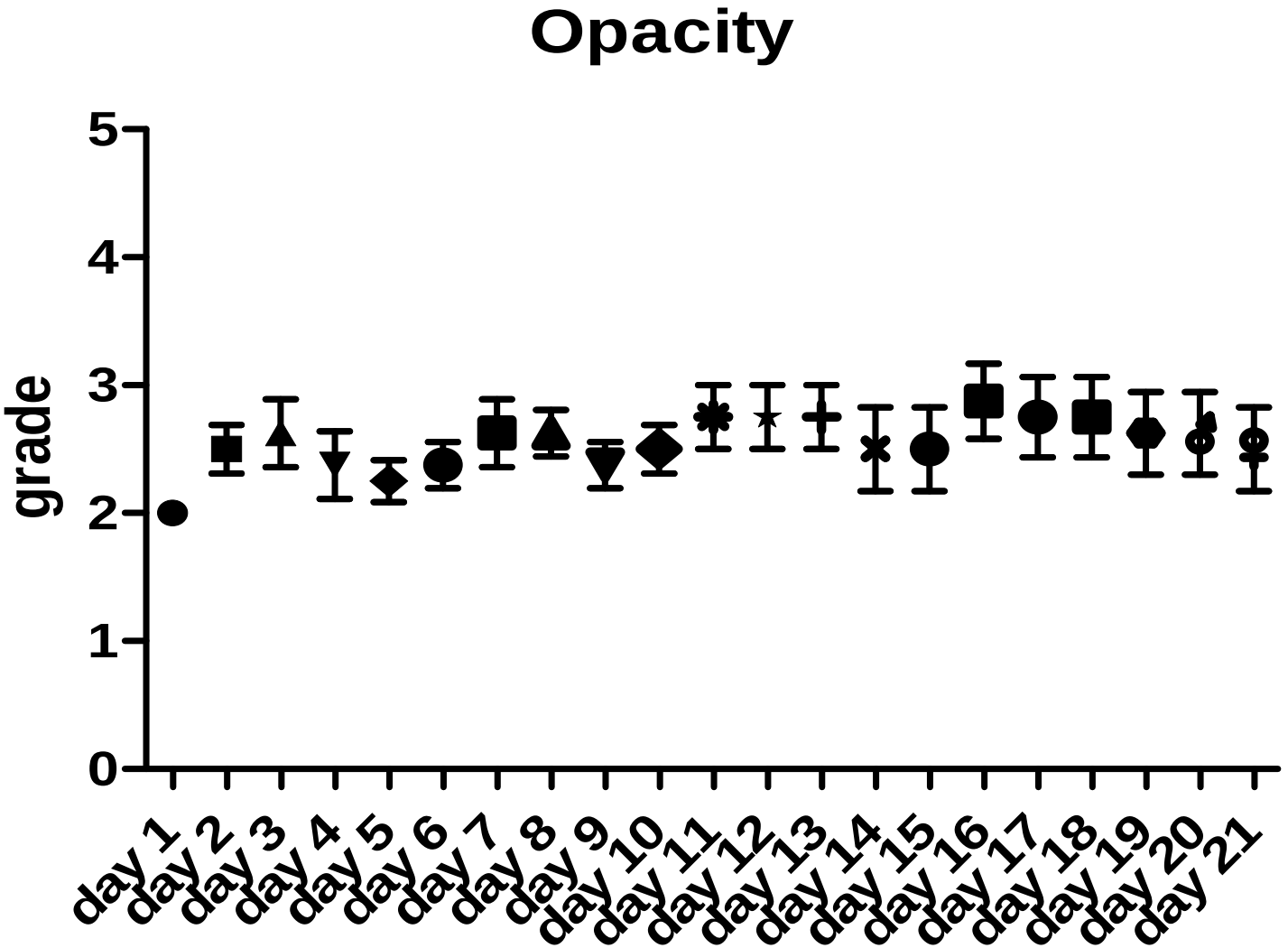
1 N NaOH time course



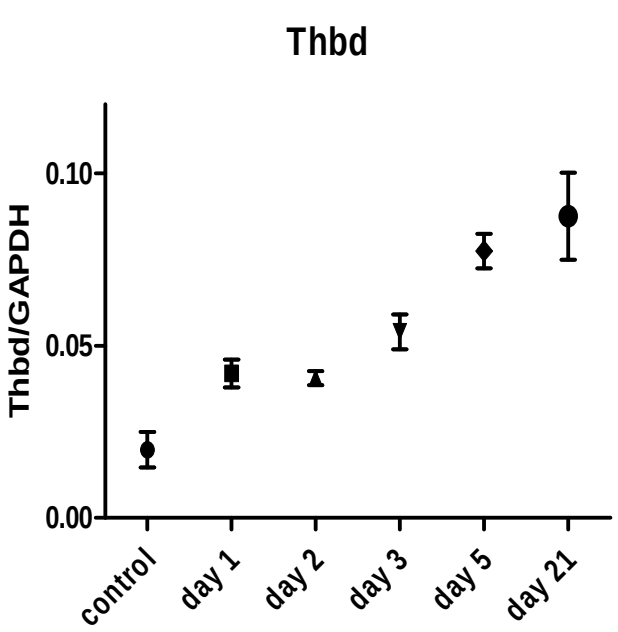




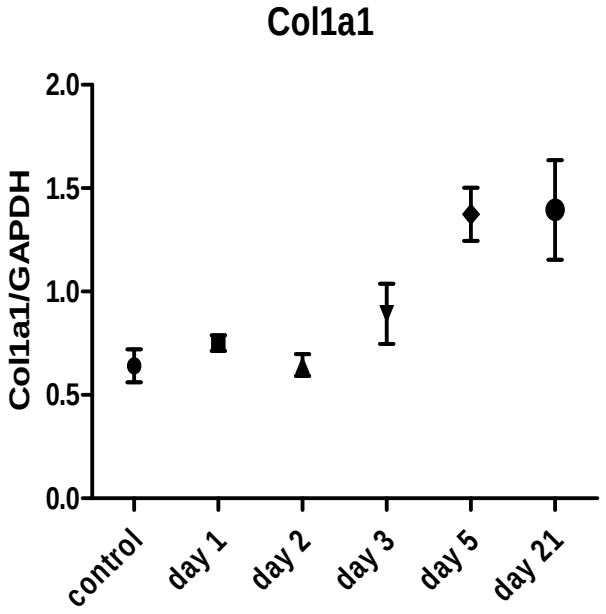
1 N NaOH time course



Neovascularization and fibrosis by 1 N NaOH injury



Thbd (thrombomodulin) alpha 1)



Col1a1 (collagen, type I,

Appendix 4 Final Report

Corneal Injury Model

AC Injection: TSG-6 / PBS

0.5 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

  6-5-15 through 6-12-15

- **Number of animals used:**

  8 rats with 0.5 N NaOH injury + TSG-6 AC injection (5 uL; 0.5 ug/uL)

  8 rats with 0.5 N NaOH injury + PBS AC injection (5 uL)

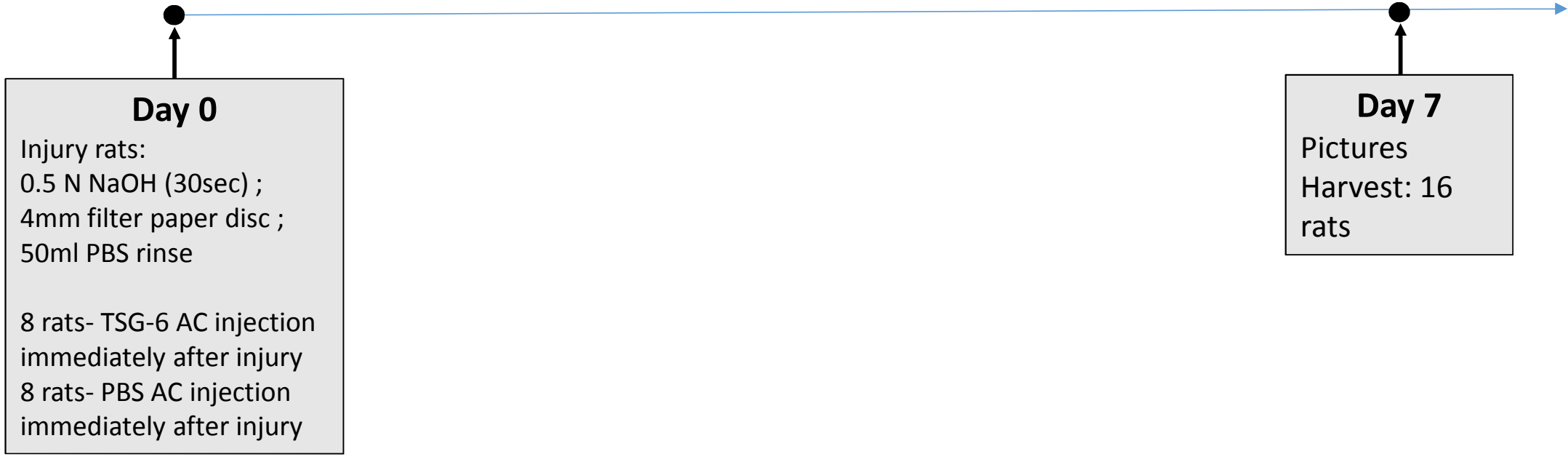
  = 16 Lewis rats total

- **Harvest day / number of animals:**

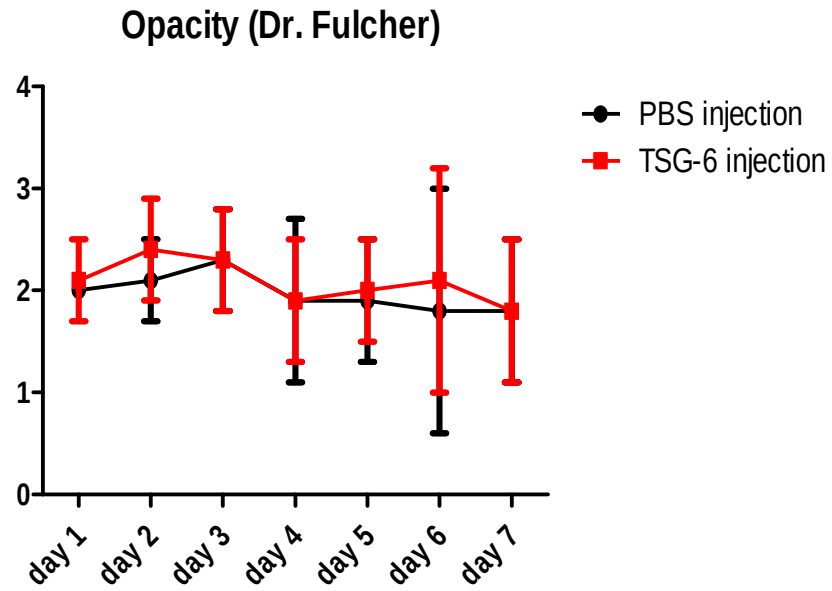
  Day 7 harvest / 16 rats

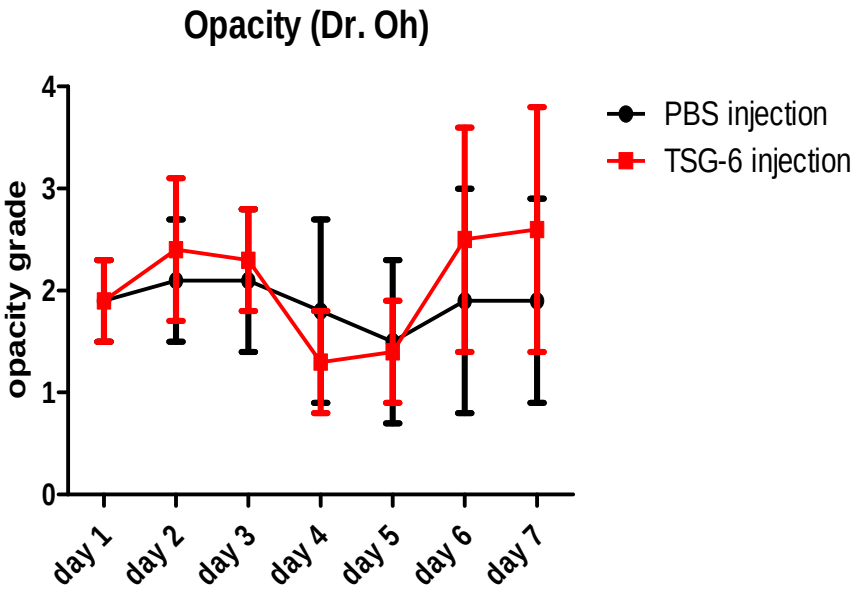
Alkali Injury of Rat Cornea

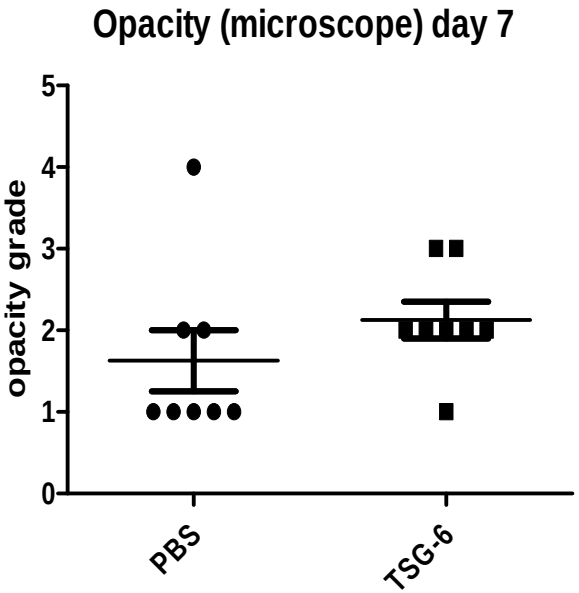
data reflects 6-5-15 injury date

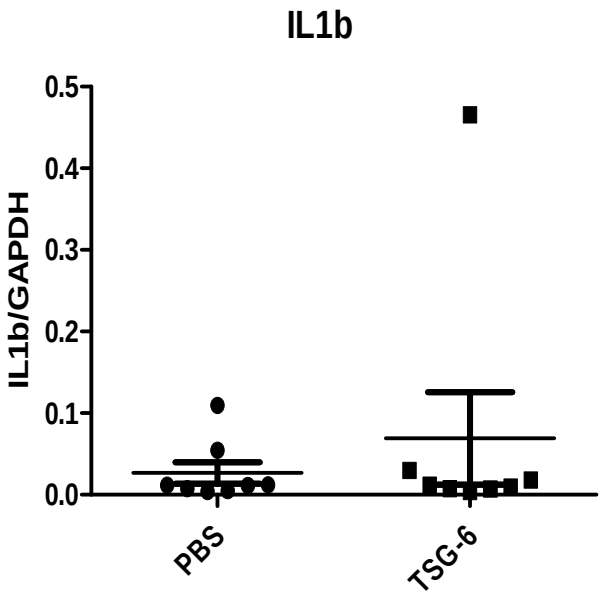


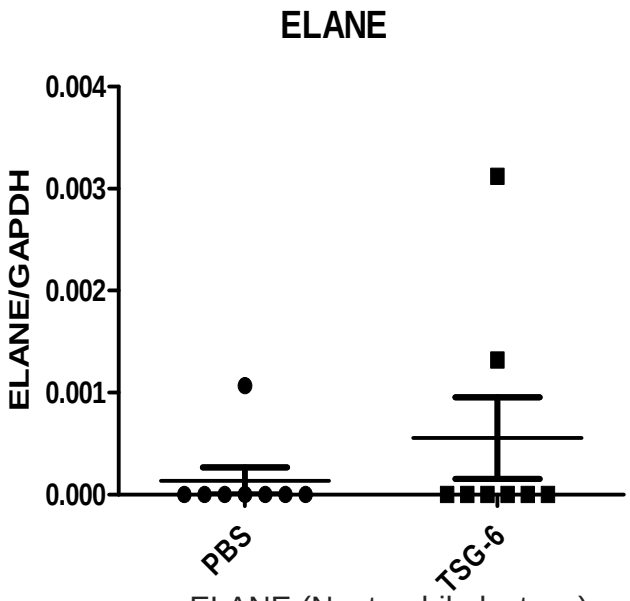
* Photographic data was recorded daily for each rat until time of harvest


Opacity (Dr. Fulcher)
PBS injection
TSG-6 injection
0
1
2
3
4
day 1
day 2
day 3
day 4
day 5
day 6
day 7

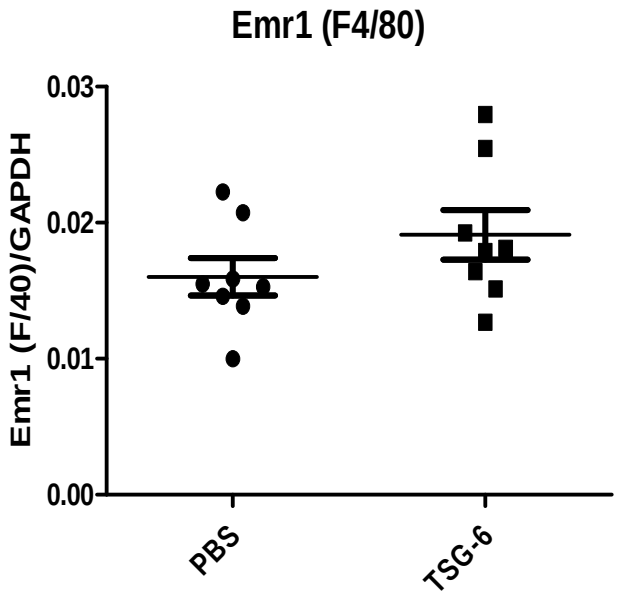

Opacity (Dr. Oh)
opacity grade
PBS injection
TSG-6 injection
0
1
2
3
4
day 1
day 2
day 3
day 4
day 5
day 6
day 7


Opacity (microscope) day 7
opacity grade
0
1
2
3
4
5
PBS
TSG-6

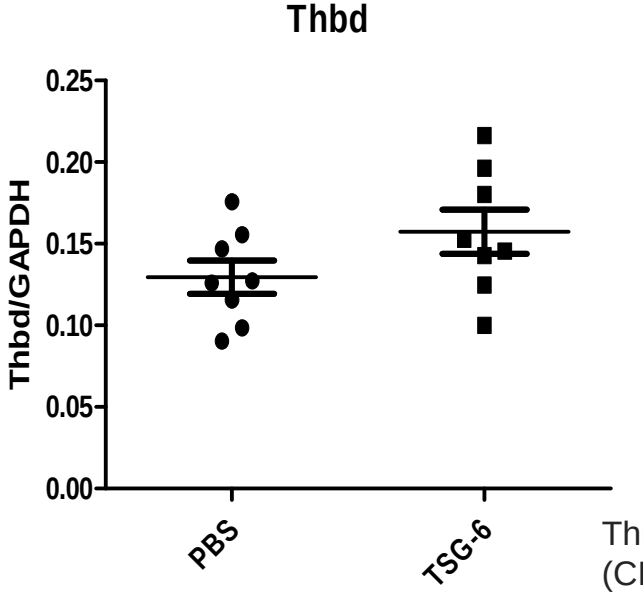




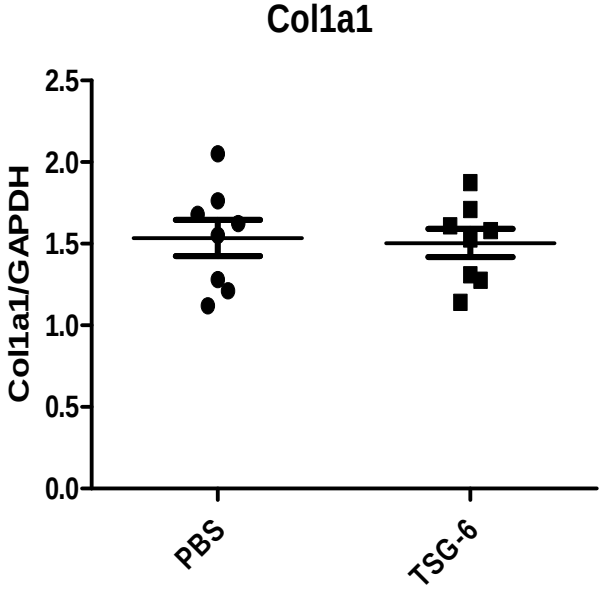
ELANE (Neutrophil elastase)



Emr1 (EGF-like module-containing mucin-like hormone receptor-like 1)



Thrombomodulin (CD141)



Corneal Injury Model

AC Injection: TSG-6 / PBS

1 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

  6-19-15 through 6-26-15

- **Number of animals used:**

  8 rats with 1 N NaOH injury + TSG-6 AC injection (5 uL; 0.5 ug/uL)

  7 rats with 1 N NaOH injury + PBS AC injection (5 uL)

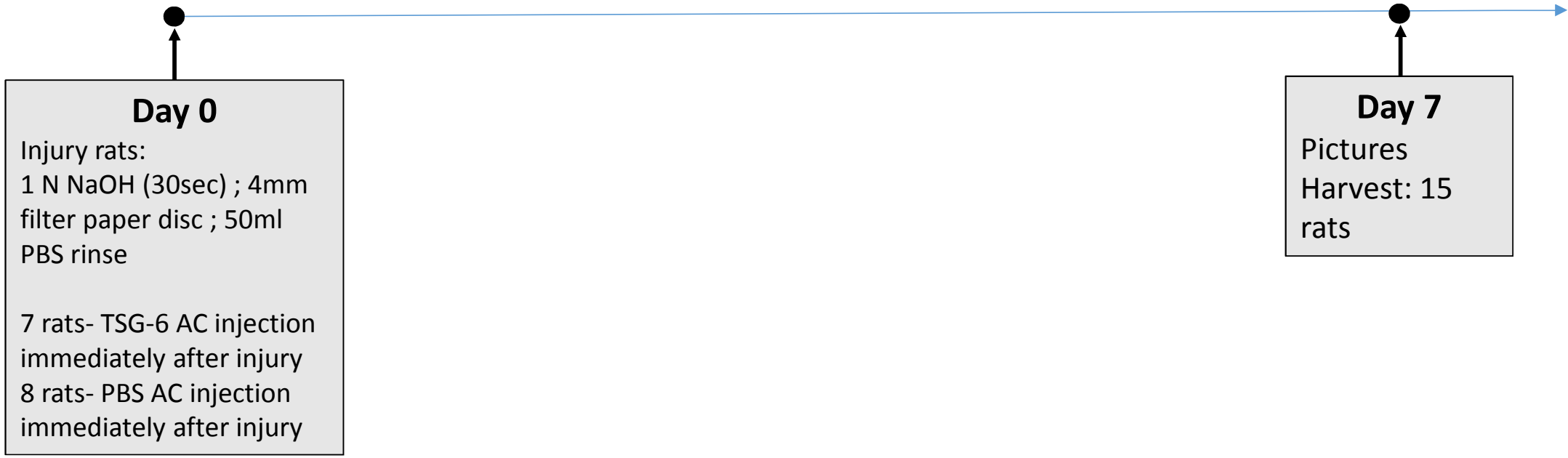
  = 15 Lewis rats total

- **Harvest day / number of animals:**

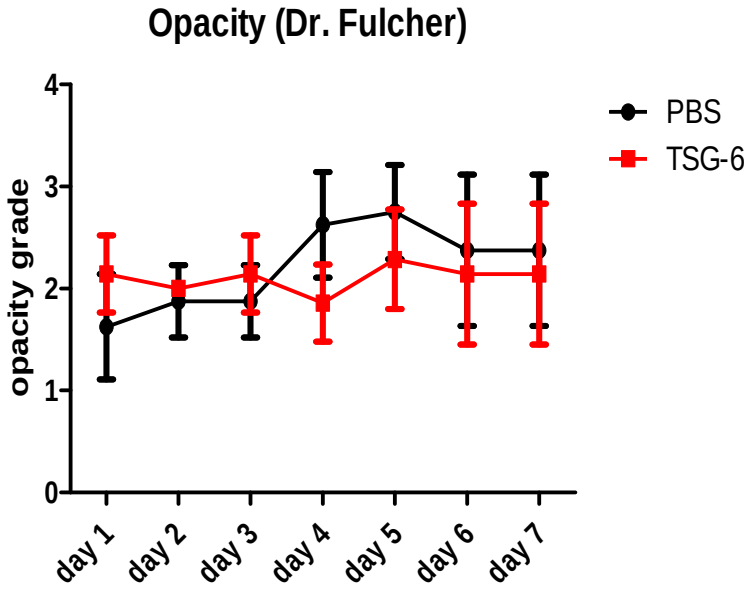
  Day 7 harvest / 15 rats

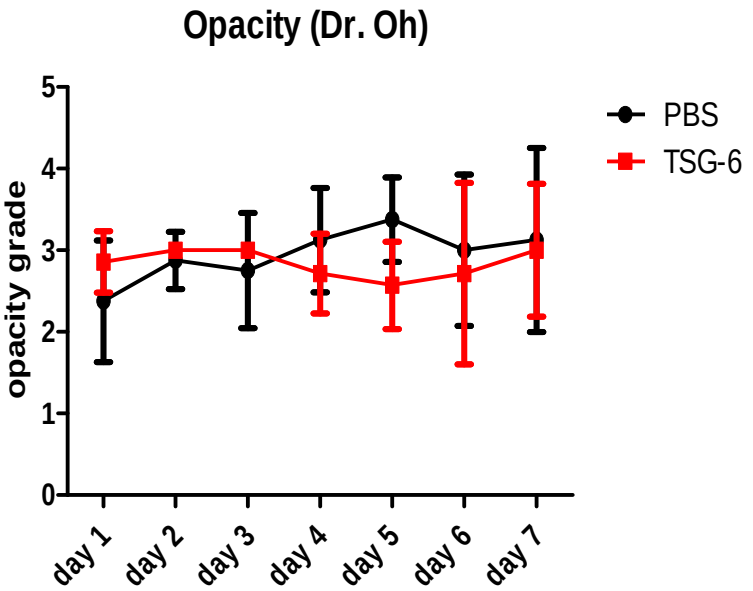
Alkali Injury of Rat Cornea

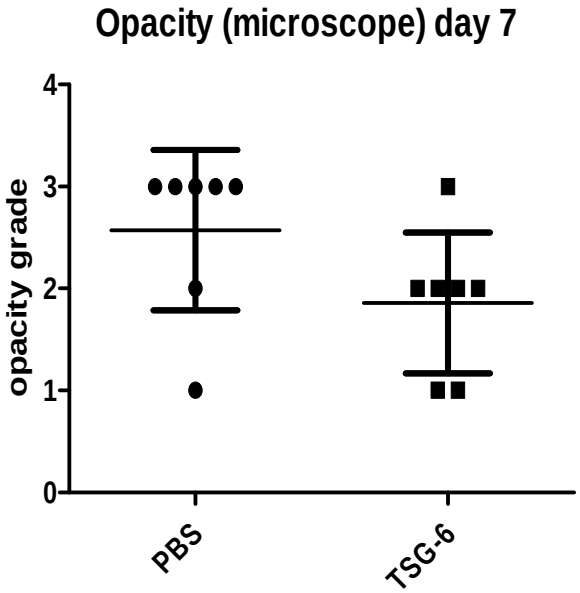
data reflects 6/19-26

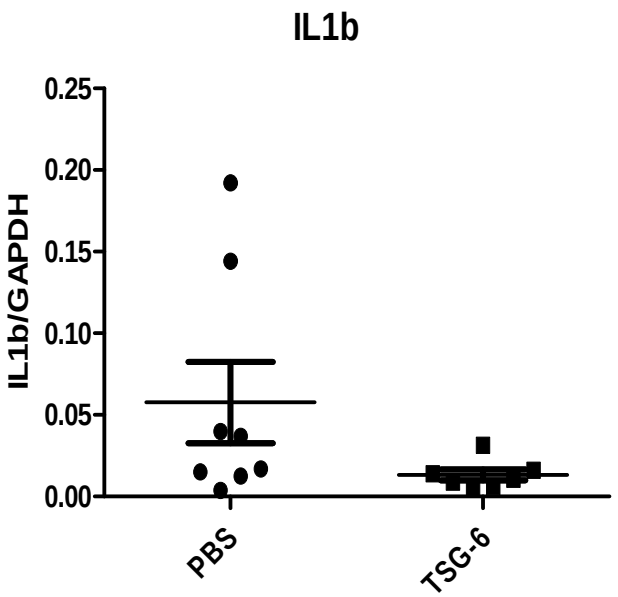


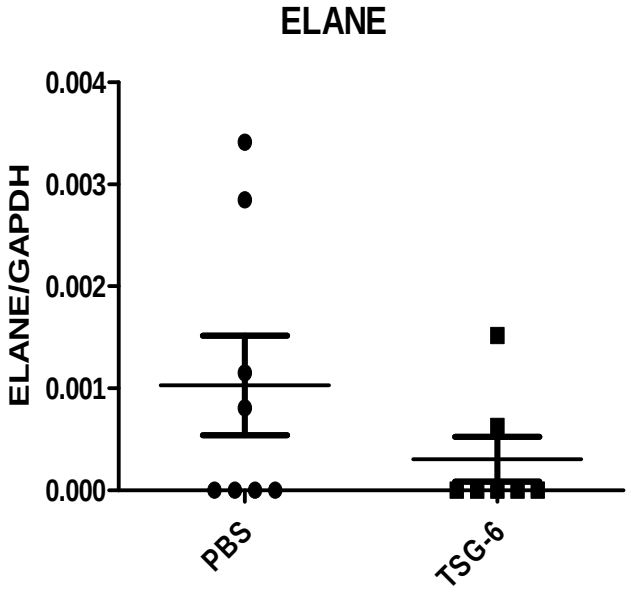
* Photographic data was recorded daily for each rat until time of harvest


Opacity (Dr. Fulcher)
opacity grade
0
1
2
3
4
day 1
day 2
day 3
day 4
day 5
day 6
day 7
PBS
TSG-6

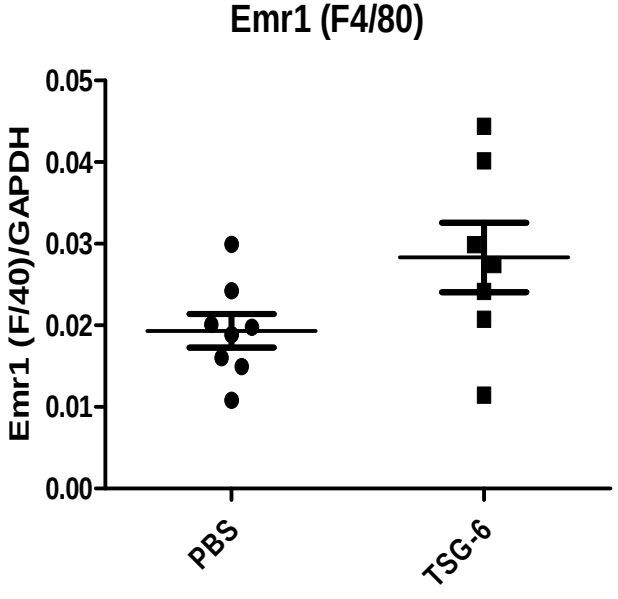

Opacity (Dr. Oh)
opacity grade
0
1
2
3
4
5
day 1
day 2
day 3
day 4
day 5
day 6
day 7
PBS
TSG-6


Opacity (microscope) day 7
opacity grade
0
1
2
3
4
PBS
TSG-6

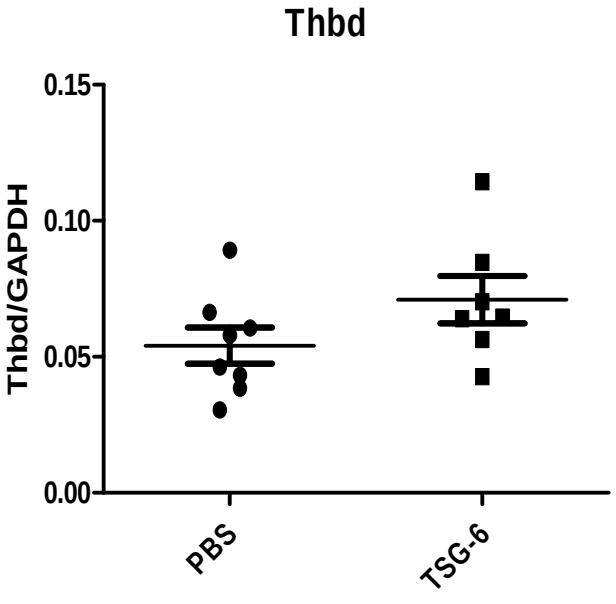




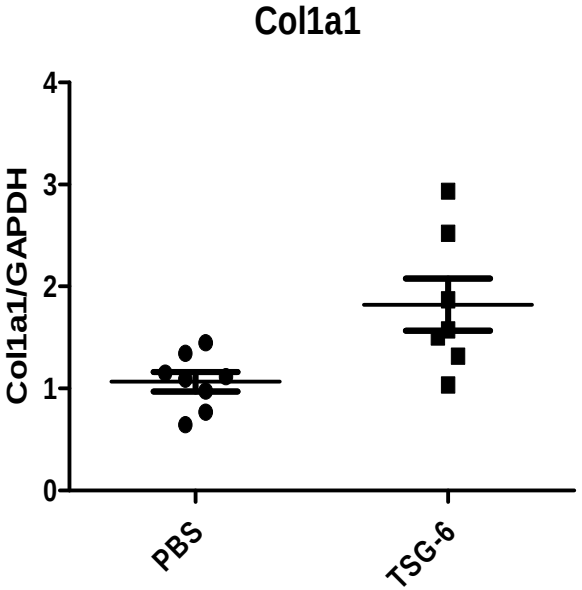
ELANE (Neutrophil elastase)



Emr1 (EGF-like module-containing mucin-like hormone receptor-like 1)



Thrombomodulin (CD141)



Corneal Injury Model

Topical: TSG-6 / PBS

1.0 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

  8-6-15 through 8-13-15

- **Method of Treatment:**

  Silicone ring (30 min) filled w/TSG-6 or PBS immediately following injury, then 5 µl drop three times per day (TID)

- **Number of animals used:**

  8 rats with 1.0 N NaOH injury + TSG-6 topical

  8 rats with 1.0 N NaOH injury + PBS topical

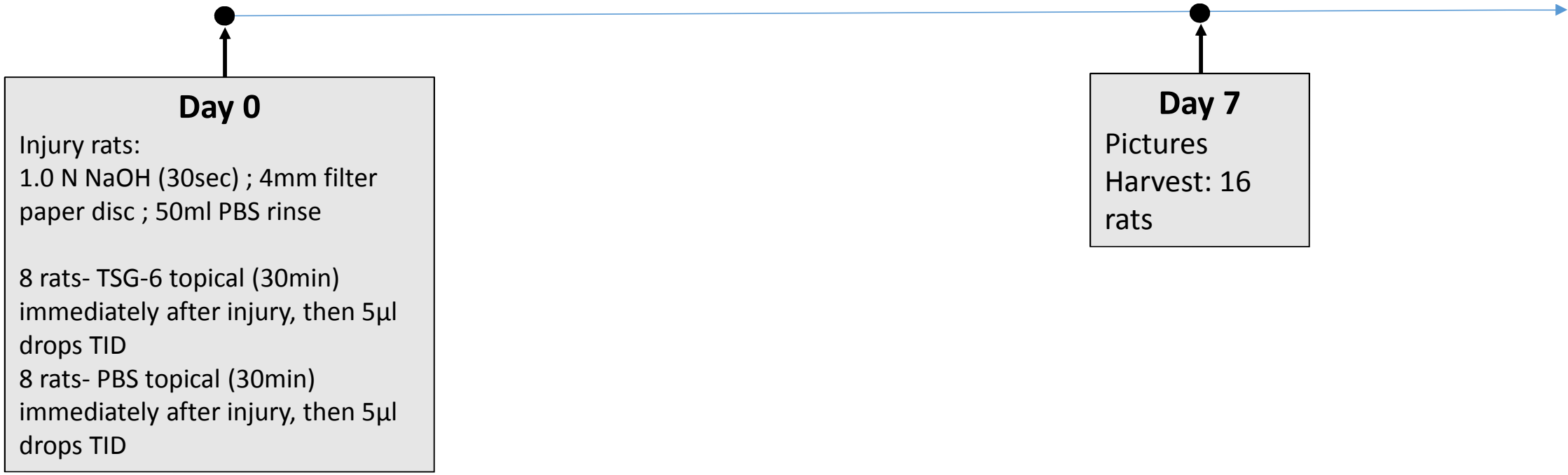
  = 16 Lewis rats total

- **Harvest day / number of animals:**

  Day 7 harvest / 16 rats

Alkali Injury of Rat Cornea

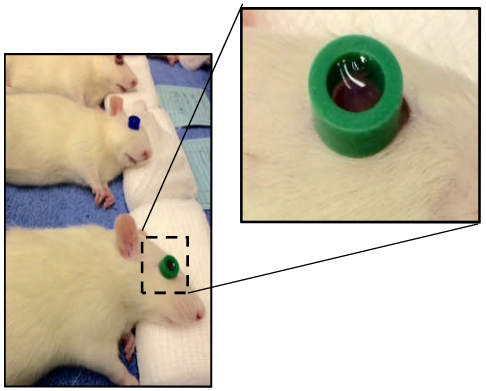
data reflects 8-6-15 injury date



* Photographic data was recorded daily for each rat until time of harvest

1.0 N NaOH Injury; 30 seconds

90µl PBS or TSG-6 topically; 30 minutes



Close eyelids using single suture; suture removal the following morning

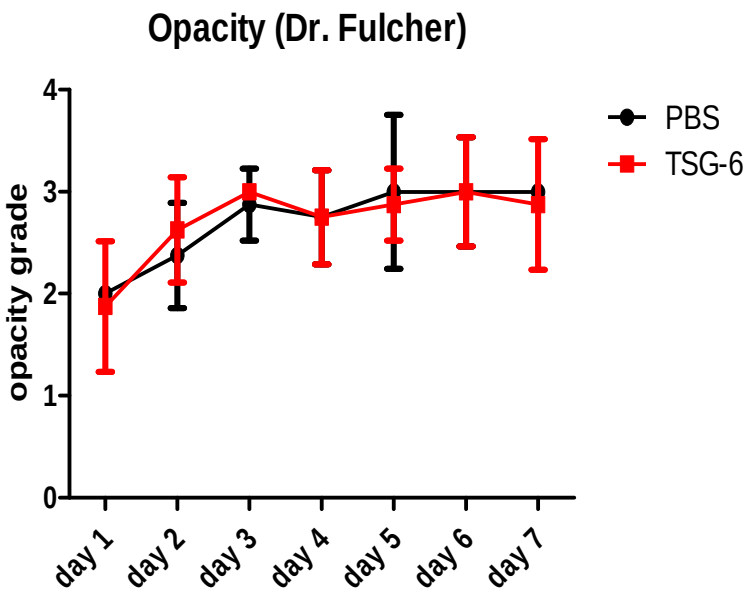
Daily:

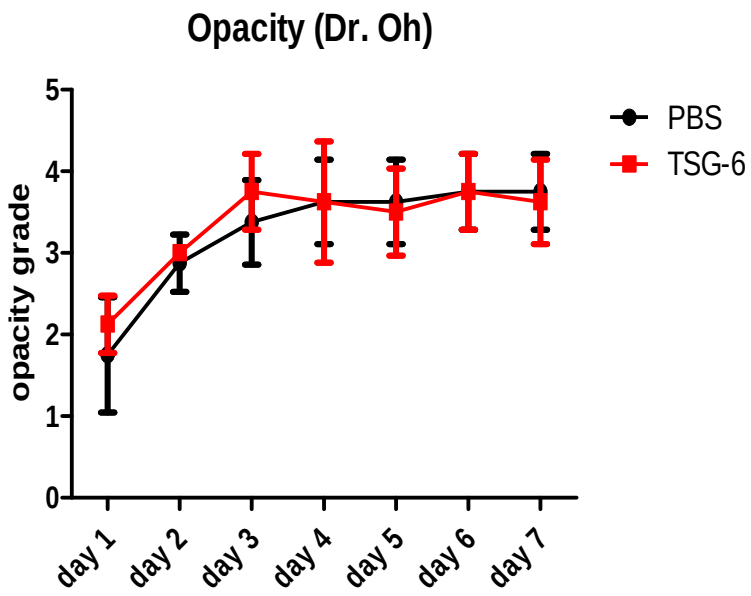
Morning: iso/oxy anesthesia for photographs
5µl of TSG-6 or PBS topically

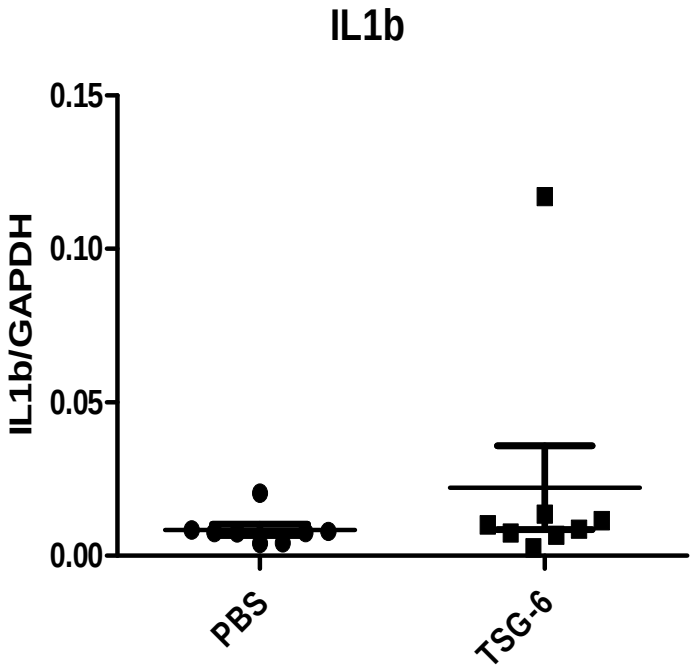
Afternoon: 5µl of TSG-6 or PBS topically

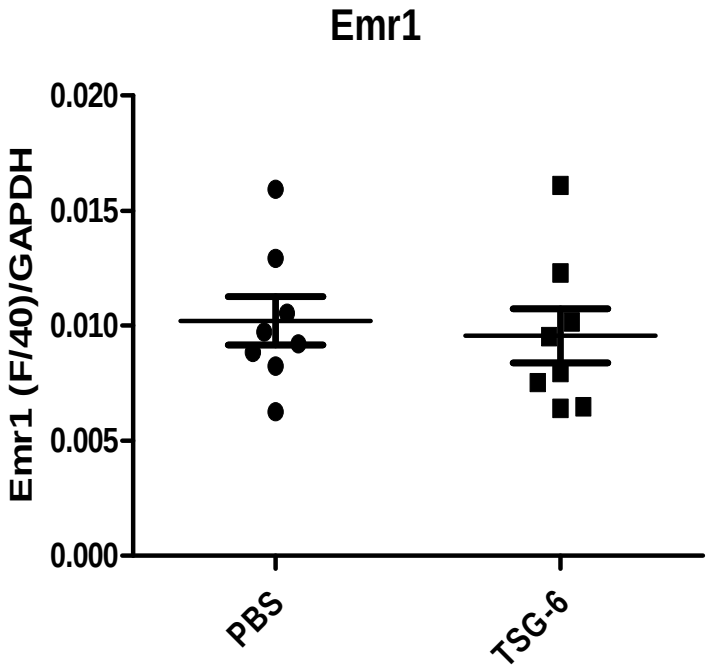
Evening: 5µl of TSG-6 or PBS topically

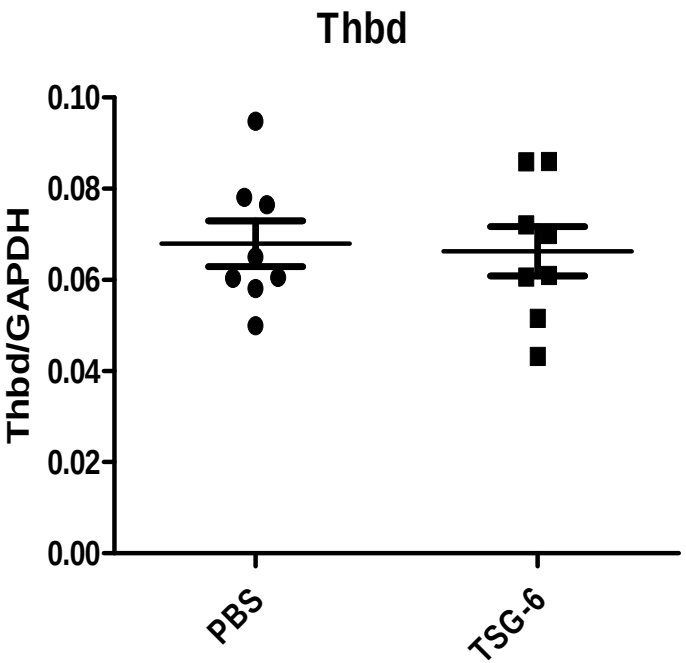
Day 7 harvest

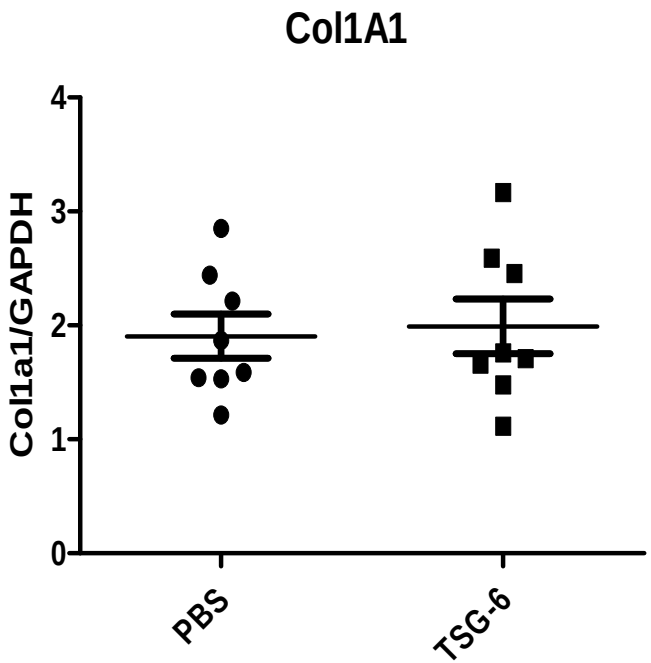

Opacity (Dr. Fulcher)
opacity grade
0
1
2
3
4
day 1
day 2
day 3
day 4
day 5
day 6
day 7
PBS
TSG-6


Opacity (Dr. Oh)
opacity grade
0
1
2
3
4
5
day 1
day 2
day 3
day 4
day 5
day 6
day 7
PBS
TSG-6


IL1b
IL1b/GAPDH
0.15
0.10
0.05
0.00
PBS
TSG-6


Emr1
Emr1 (F/40)/GAPDH
0.020
0.015
0.010
0.005
0.000
PBS
TSG-6


Thbd
Thbd/GAPDH
0.10
0.08
0.06
0.04
0.02
0.00
PBS
TSG-6


Col1A1
Col1a1/GAPDH
4
3
2
1
0
PBS
TSG-6

Corneal Injury Model

IV Injection: TSG-6 / PBS

1.0 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

  7-7-15 through 7-12-15

- **Number of animals used:**

  7 rats with 1.0 N NaOH injury + TSG-6 IV injection (200 ug/rat)

  7 rats with 1.0 N NaOH injury + PBS IV injection (264 uL)

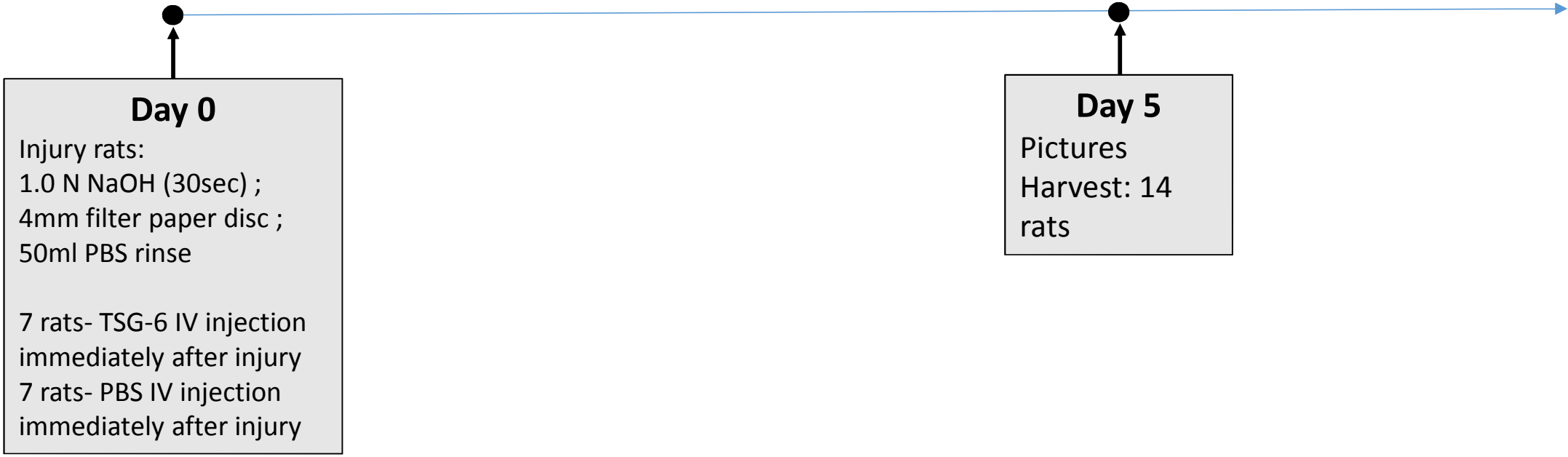
  = 14 Lewis rats total

- **Harvest day / number of animals:**

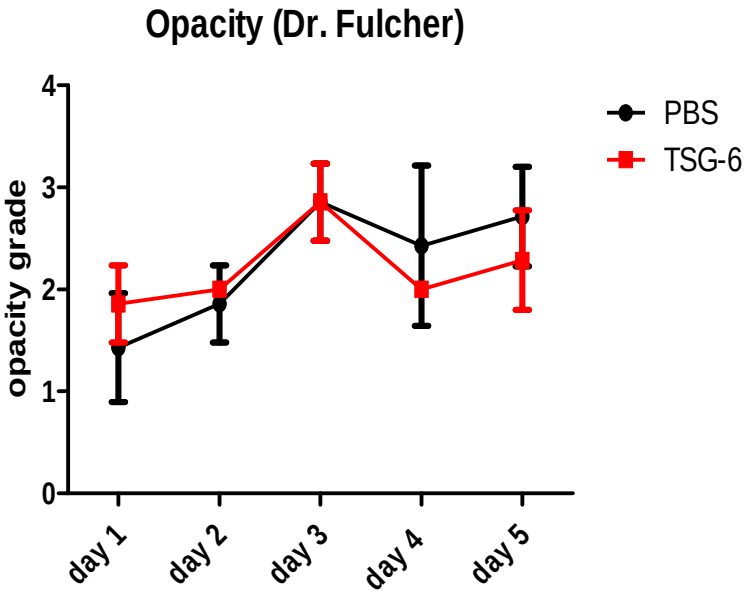
  Day 5 harvest / 14 rats

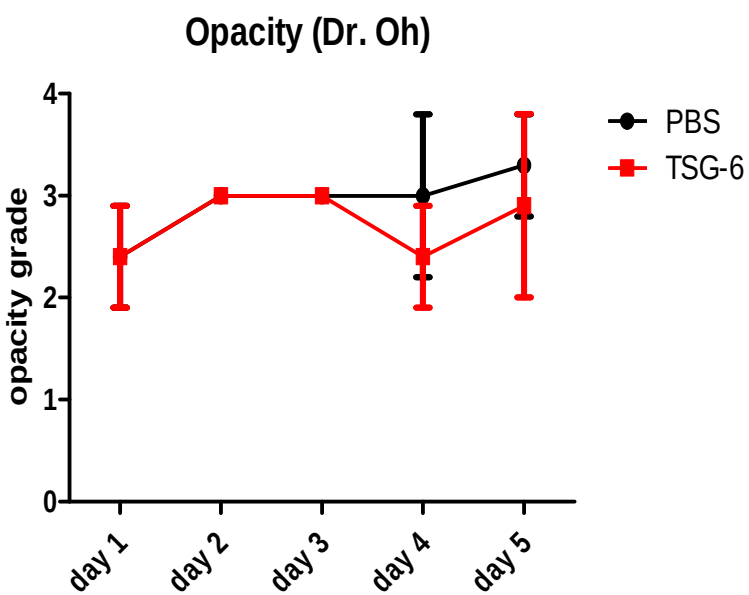
Alkali Injury of Rat Cornea

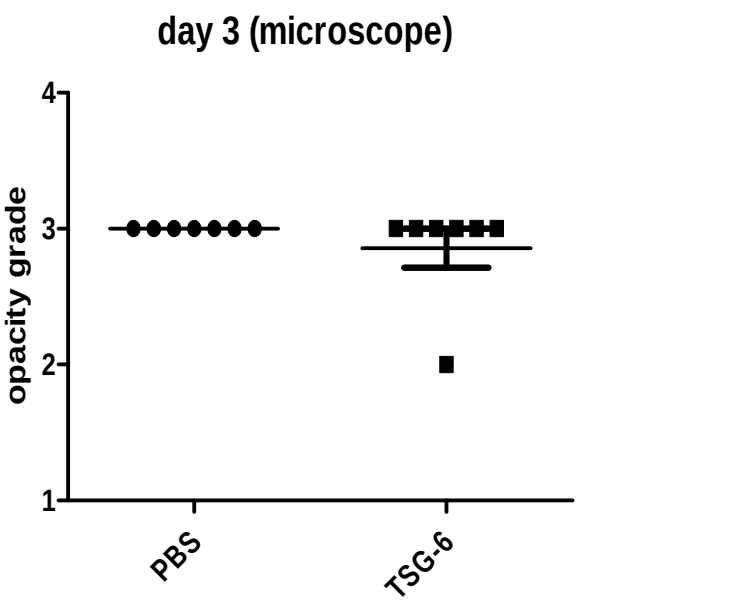
data reflects 7-7-15 injury date

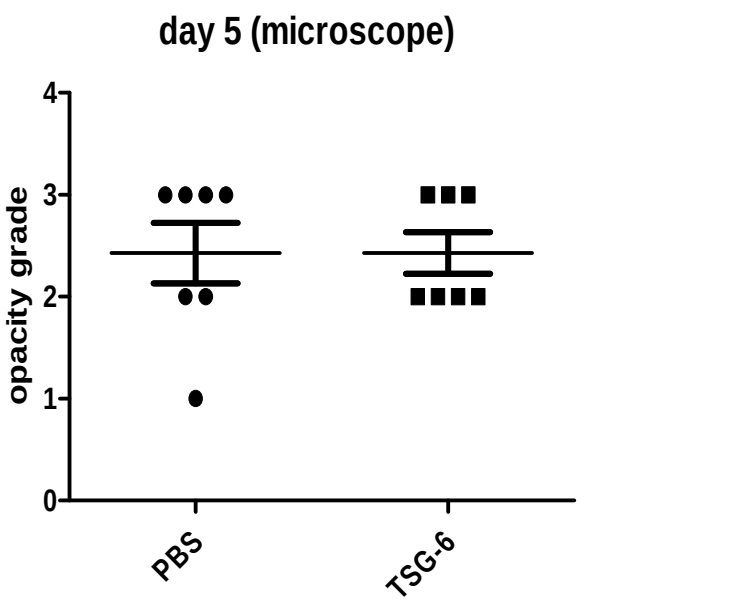


* Photographic data was recorded daily for each rat until time of harvest

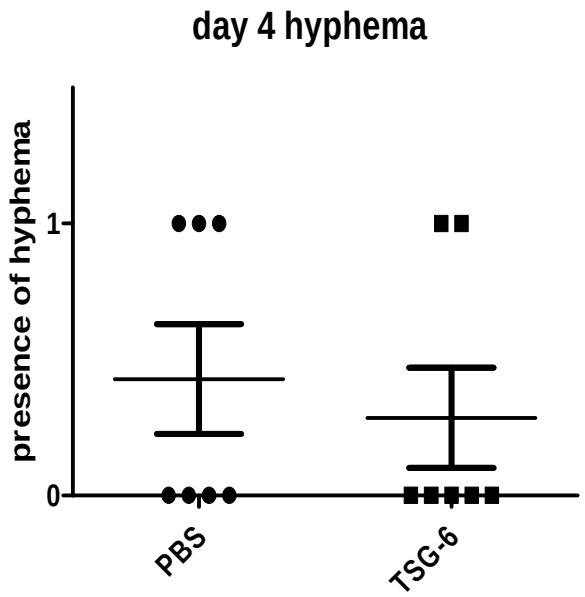

Opacity (Dr. Fulcher)
opacity grade
0
1
2
3
4
day 1
day 2
day 3
day 4
day 5
PBS
TSG-6

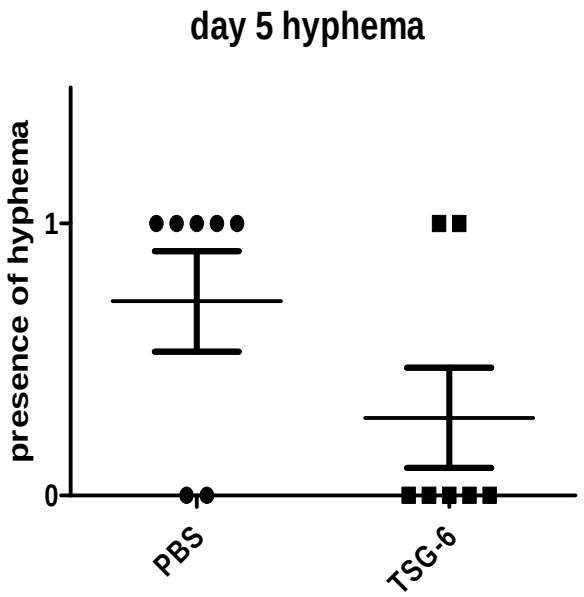

Opacity (Dr. Oh)
opacity grade
0
1
2
3
4
day 1
day 2
day 3
day 4
day 5
PBS
TSG-6


day 3 (microscope)
opacity grade
1
2
3
4
PBS
TSG-6

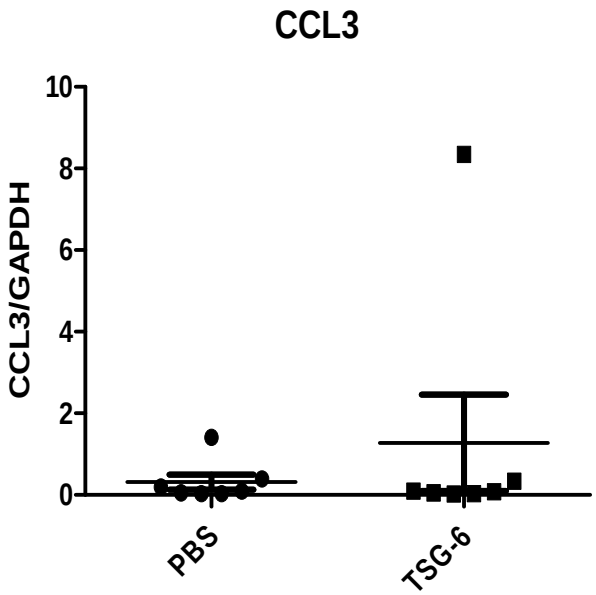

day 5 (microscope)
opacity grade
0
1
2
3
4
PBS
TSG-6

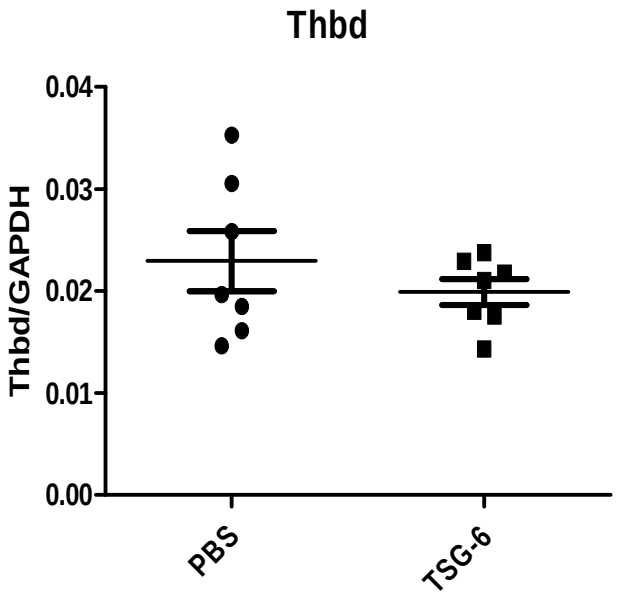
1N NaOH, IV, day 5





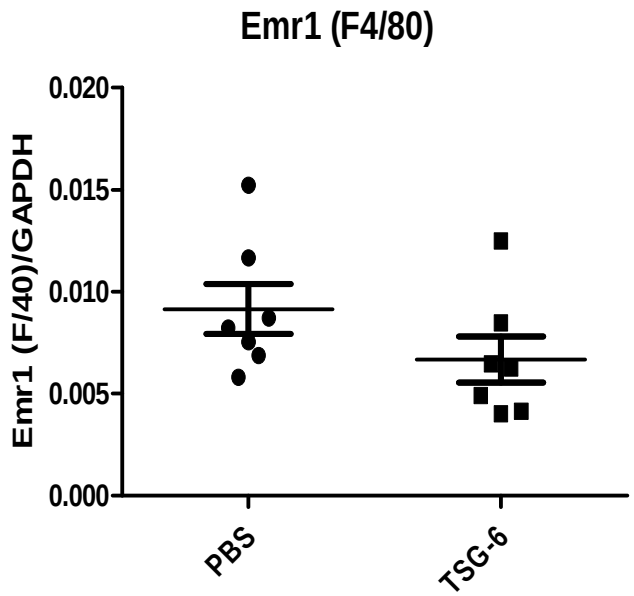
1N NaOH, IV, day 5

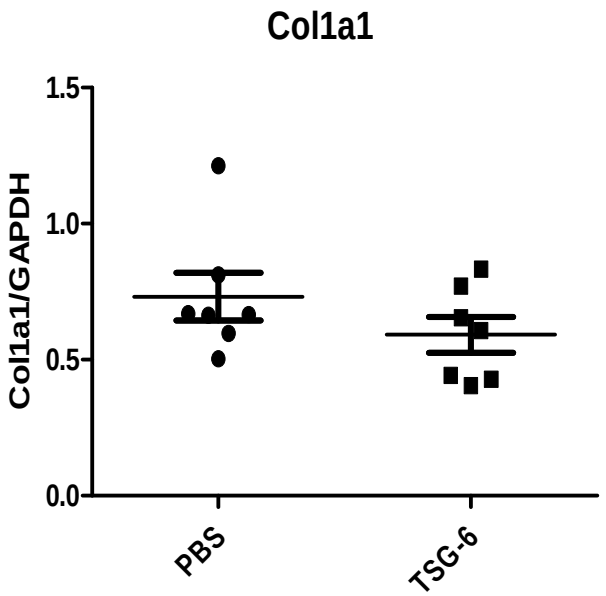




Thrombomodulin
(CD141)

1N NaOH, IV, day 5





Emr1 (EGF-like module-containing mucin-like hormone receptor-like 1)

Appendix 5

Presented at the American Society of Cataract and Refractive Surgery Annual Symposium, 5-9 May 2017, Los Angeles, CA

The Efficacy of TSG-6 for Acute Corneal Alkali Injury in a Rat Model

Samuel Fulcher MD[1]
Hosoon Choi PhD[1]
Casie Phillips[1]
Joo Youn Oh MD PhD[2]
Roxanne Reger MS[3]

[1]Central Texas Veterans Health Care System; [2]Seoul National University;
[3]Texas A&M Institute for Regenerative Medicine

Financial Disclosure

No authors have any financial interests to disclose

IACUC approved protocol

This work was funded by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0495, and supported in part with resources of the Central Texas Veterans Health Care System, Temple, Texas. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense, and do not necessarily reflect the positions or policies of the Veterans Health Administration. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office.

Chemical Injuries of the Cornea

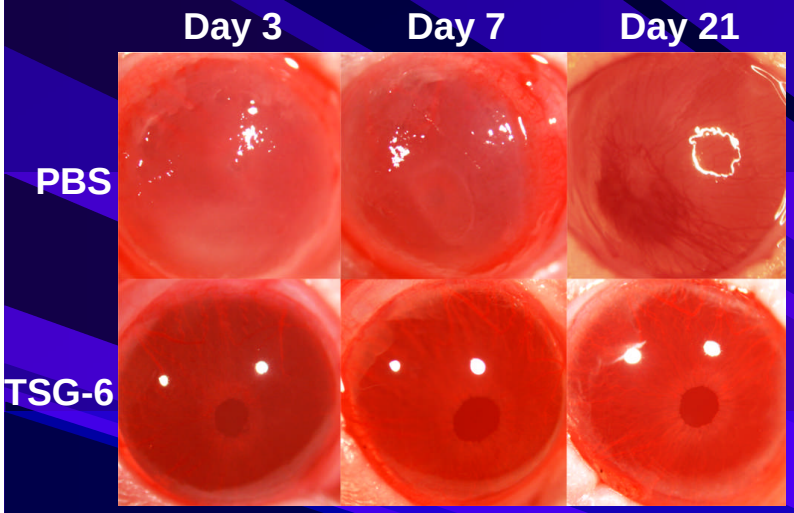
Tissue Damage:

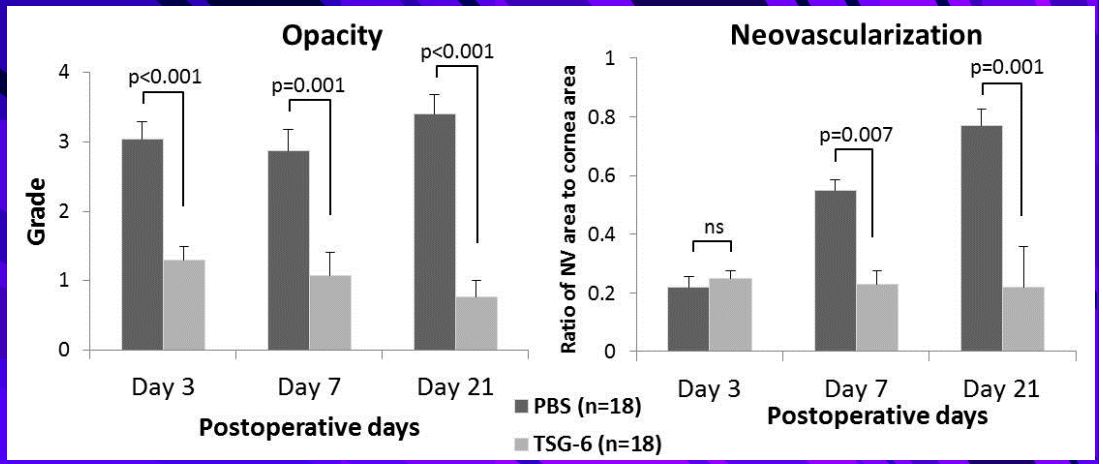
- … Direct chemical injury
- … Compounded by inflammation
- … Steroids and NSAIDS may amplify

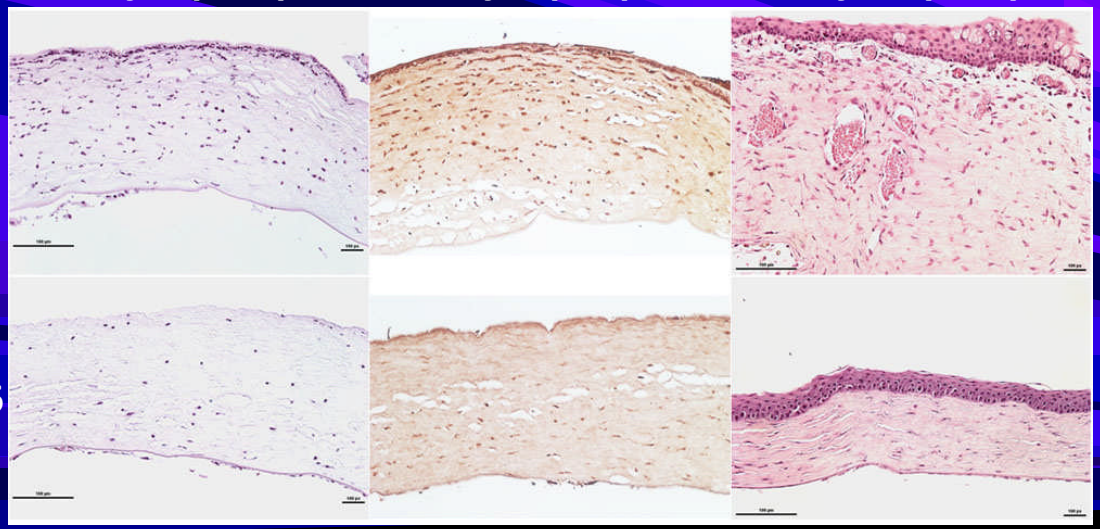
A Potential Solution:

- … The protein **"TSG-6"**
  - … TNF-stimulated gene 6 protein
  - … modulator of inflammation
  - … novel mode of action

MOUSE ETHANOL/SCRAPING MODEL







Anterior chamber (AC) TSG-6

Reduced opacity
Reduced neovascularization
Decreased invasion of inflammatory cells

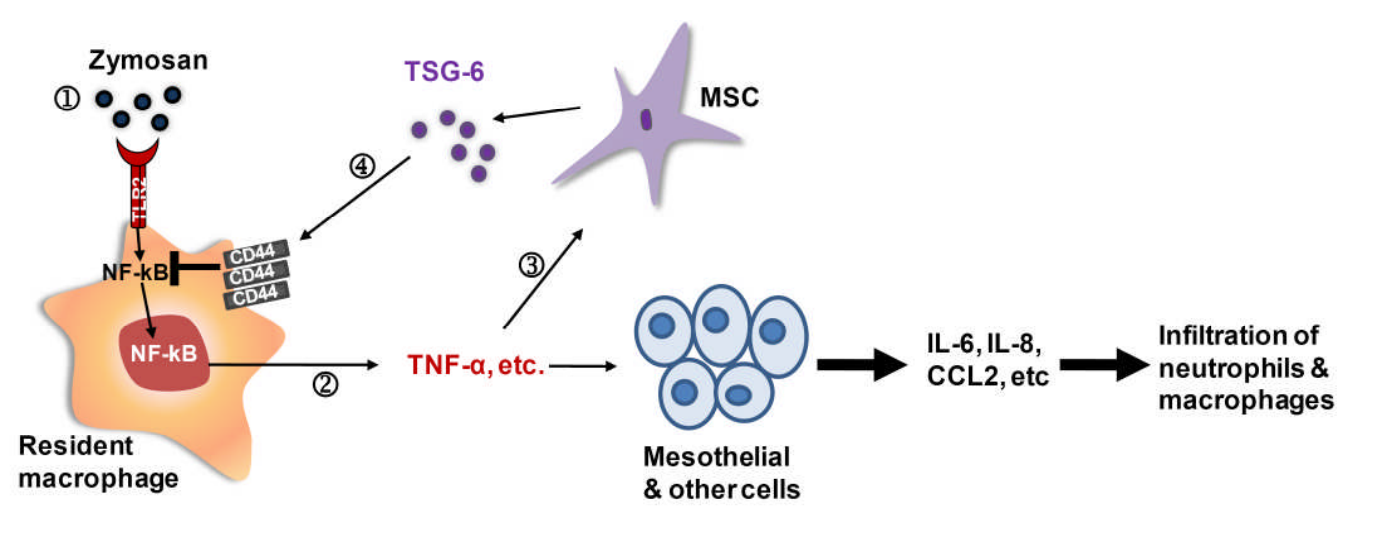
Oh et al., PNAS 2010

Unique Mechanism of TSG-6

… A natural negative feedback loop for inflammation

…Released by Mesenchymal Stem Cells (MSCs)
blocks macrophage NF-κB pathway via CD44 receptor
"first responders" to tissue injury

…no known toxic effects in eye or other tissues

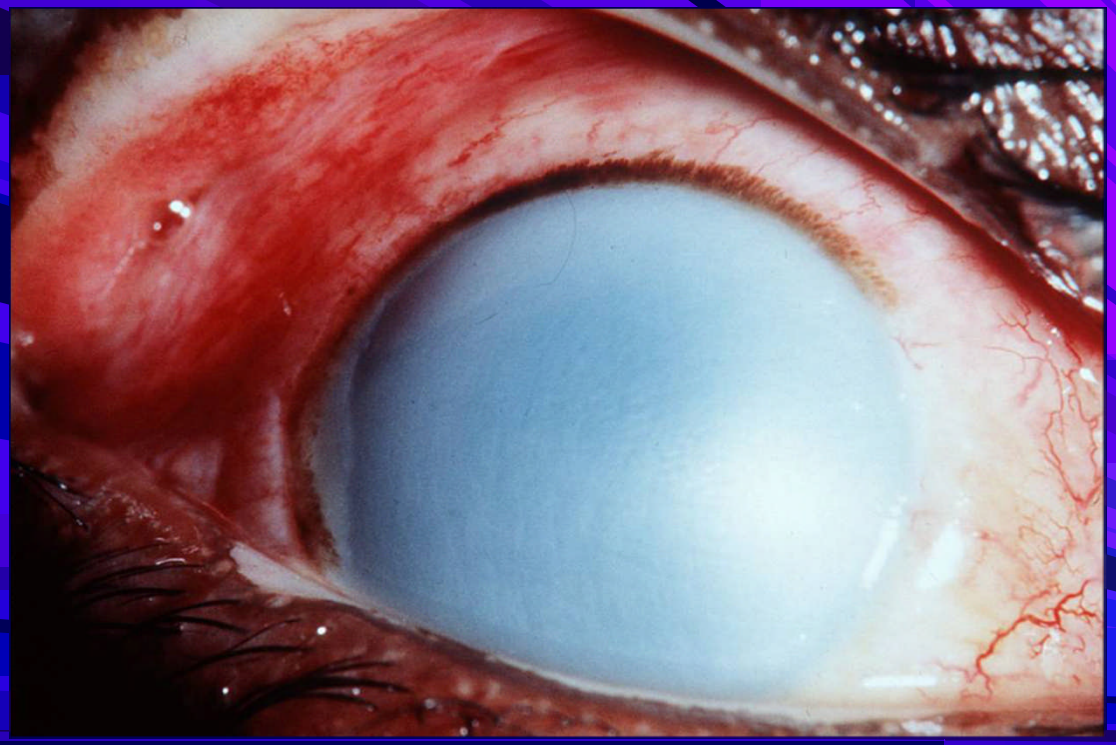


Choi et al., Blood 2011

A More Difficult Injury: Corneal Alkali Burn

…alkali penetrates rapidly and deeply

…requires prolonged irrigation to reduce pH



Experimental Design

Lewis Rats (8 per group)

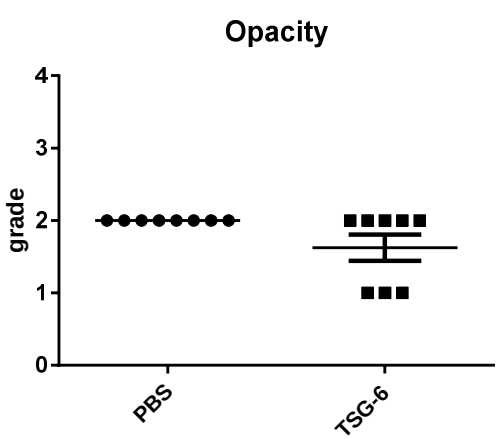
0.5N NaOH, 4 mm paper, 30 sec exposure, extensive rinse

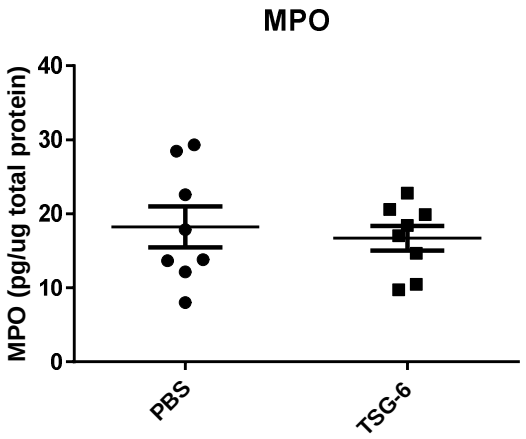
TSG-6: Topical qtts tid 0.75 ug/ml
AC 4 ug
IV 200 ug
PBS controls

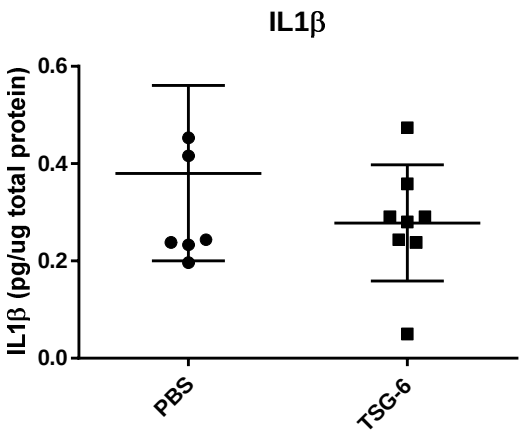
Inflammatory marker assays

Opacity Grading

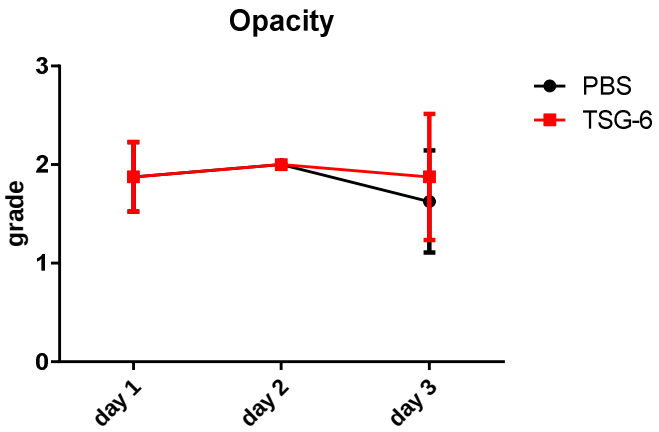
TOPICAL TSG-6
Day 1

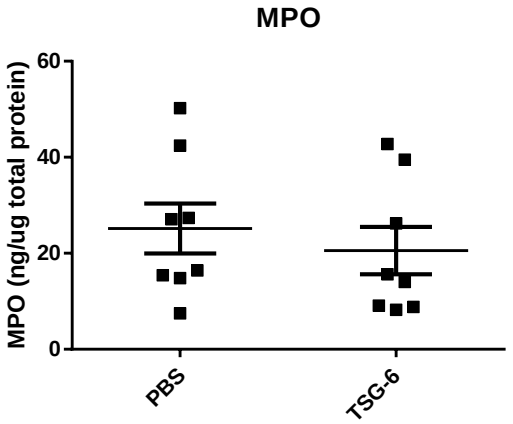




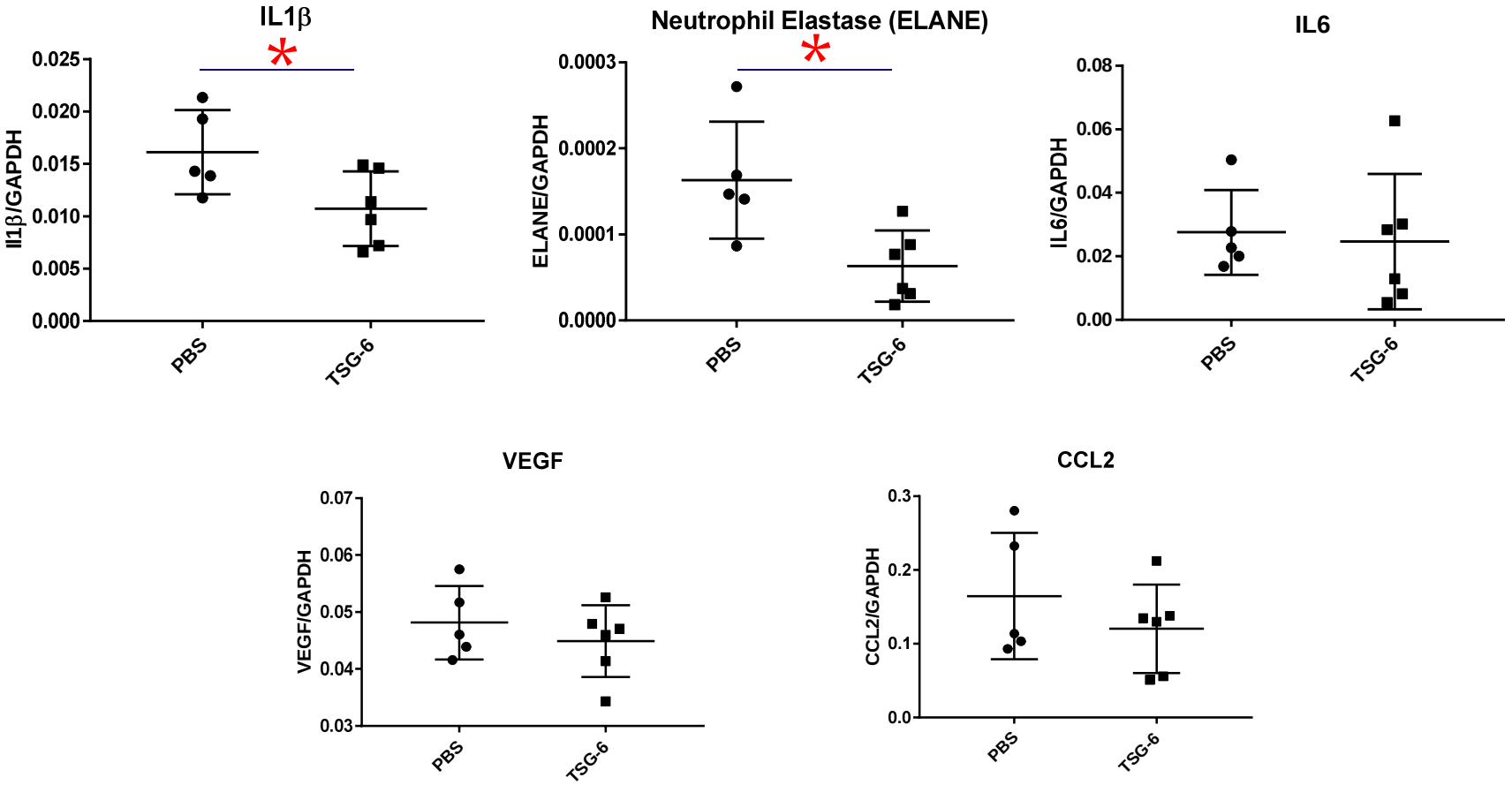


TOPICAL TSG-6
Day 3

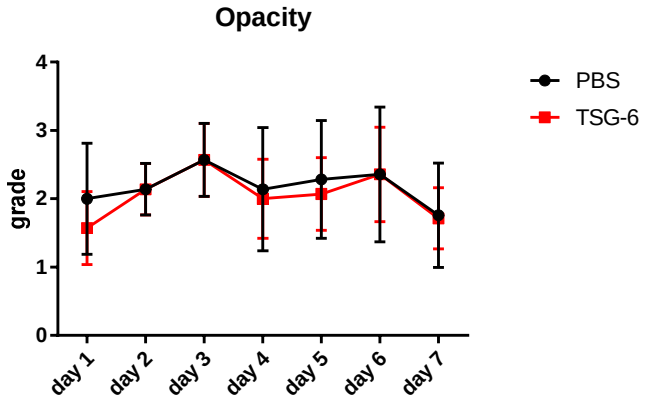


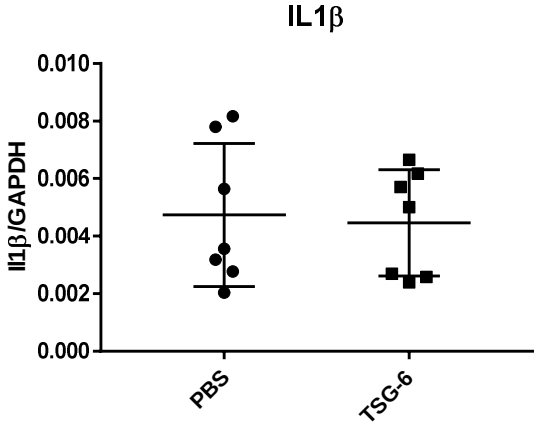


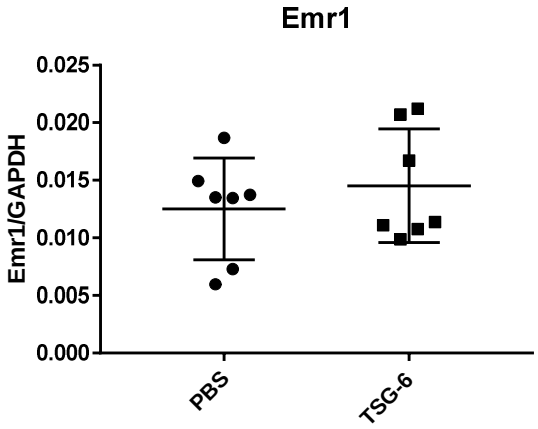
IV TSG-6
Day 1

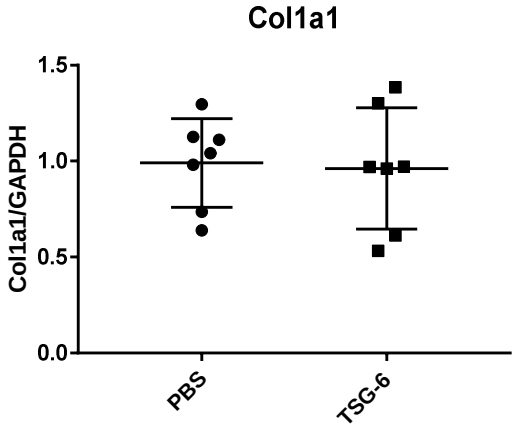

IL1β
*
Il1β/GAPDH
0.025
0.020
0.015
0.010
0.005
0.000
PBS
TSG-6
Neutrophil Elastase (ELANE)
*
ELANE/GAPDH
0.0003
0.0002
0.0001
0.0000
PBS
TSG-6
IL6
IL6/GAPDH
0.08
0.06
0.04
0.02
0.00
PBS
TSG-6
VEGF
VEGF/GAPDH
0.07
0.06
0.05
0.04
0.03
PBS
TSG-6
CCL2
CCL2/GAPDH
0.3
0.2
0.1
0.0
PBS
TSG-6

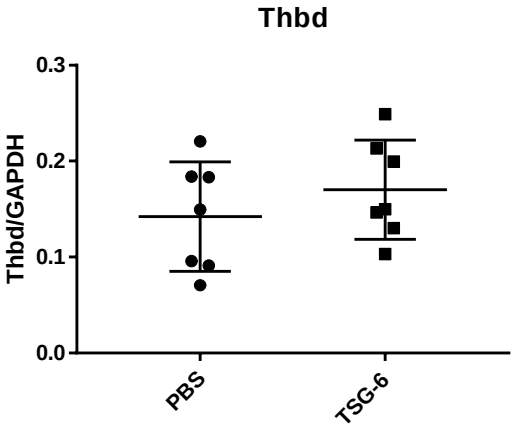
IV TSG-6
Day 7


Opacity
grade
4
3
2
1
0
day 1
day 2
day 3
day 4
day 5
day 6
day 7
PBS
TSG-6

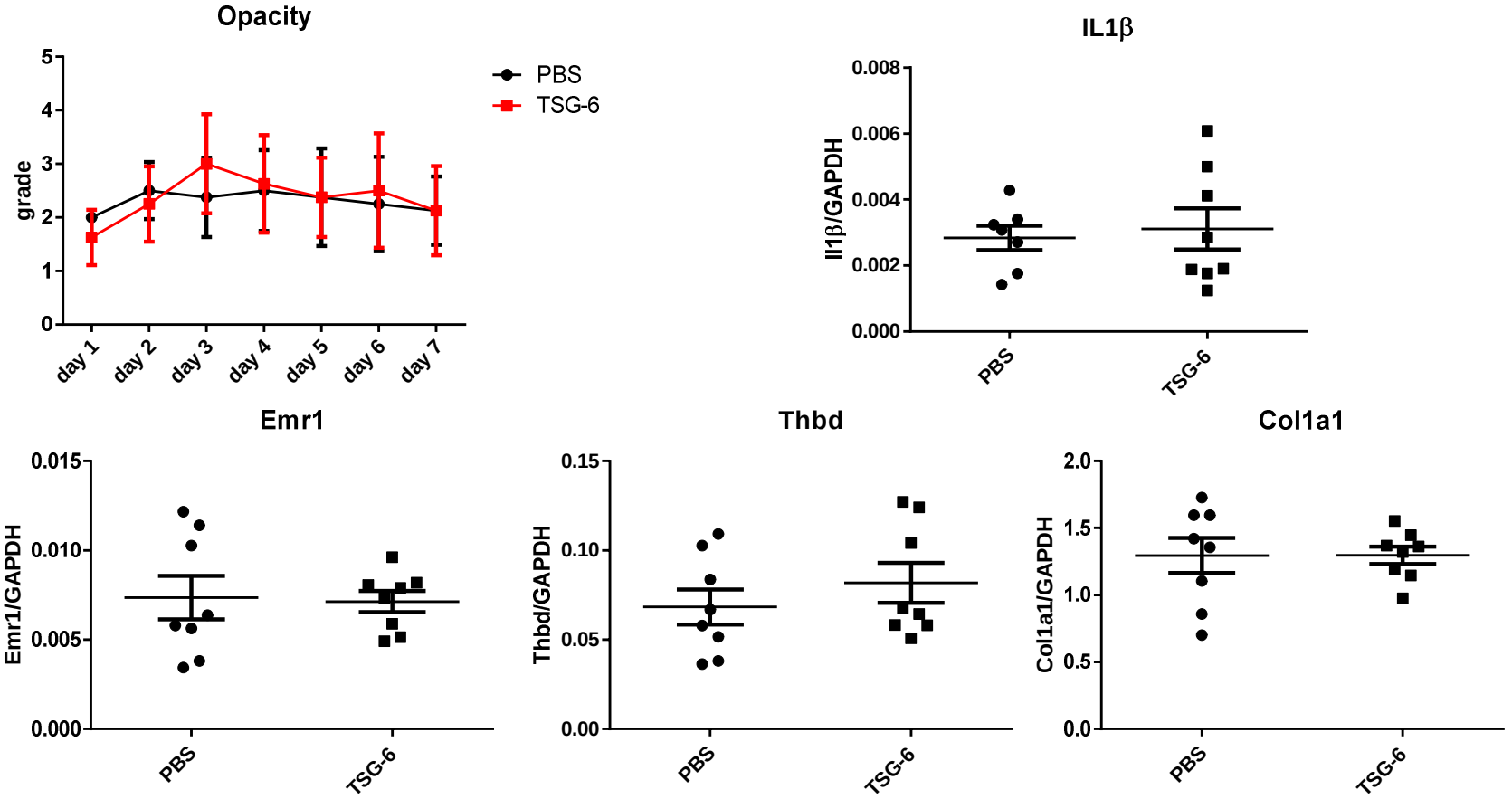

IL1β
Il1β/GAPDH
0.010
0.008
0.006
0.004
0.002
0.000
PBS
TSG-6


Emr1
Emr1/GAPDH
0.025
0.020
0.015
0.010
0.005
0.000
PBS
TSG-6

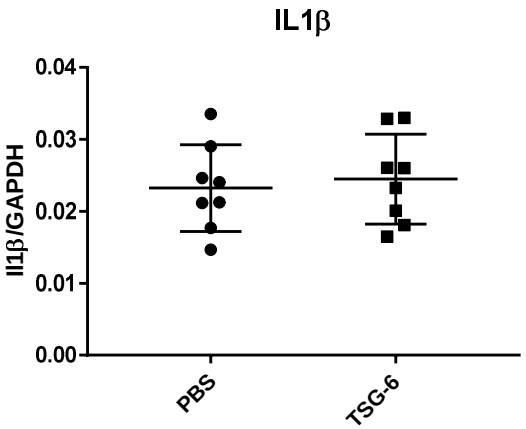

Col1a1
Col1a1/GAPDH
1.5
1.0
0.5
0.0
PBS
TSG-6

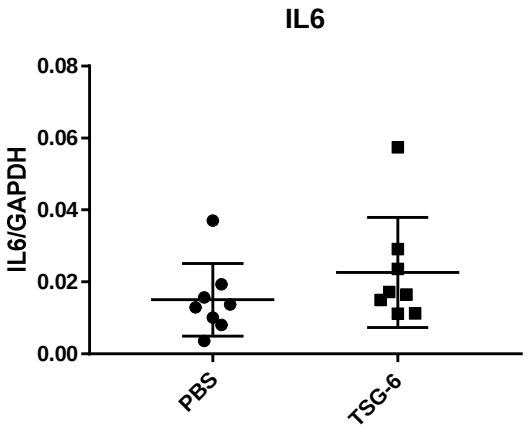

Thbd
Thbd/GAPDH
0.3
0.2
0.1
0.0
PBS
TSG-6

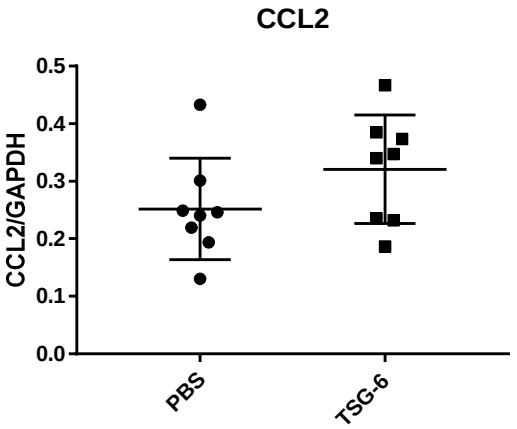
MULTI DOSE IV TSG-6
0, 6, 24 HOURS
DAY 7

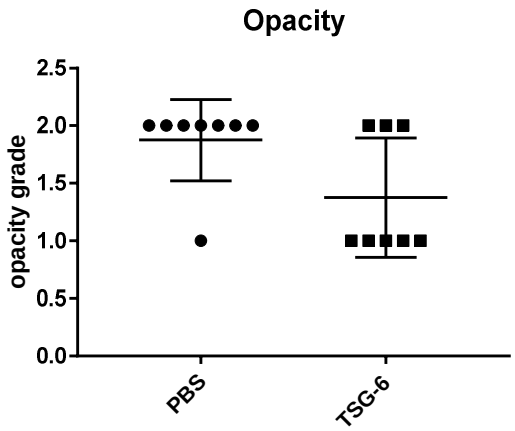


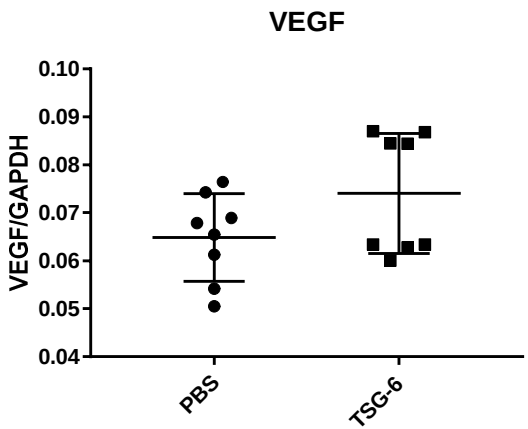
HIGH DOSE IV TSG-6 400 ug
DAY 1

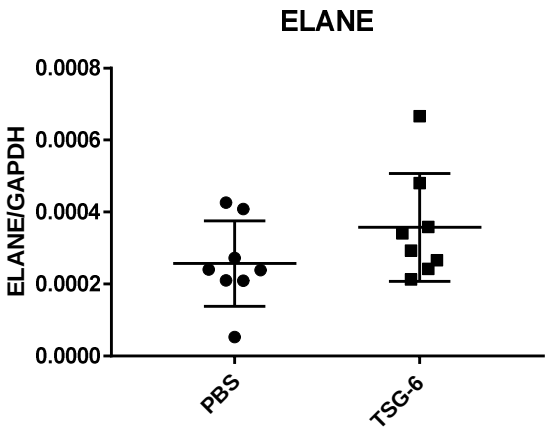




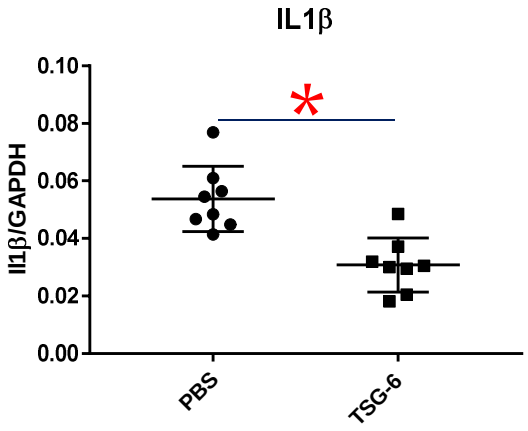


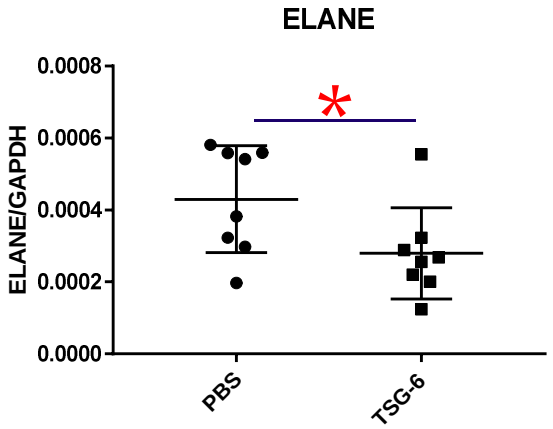


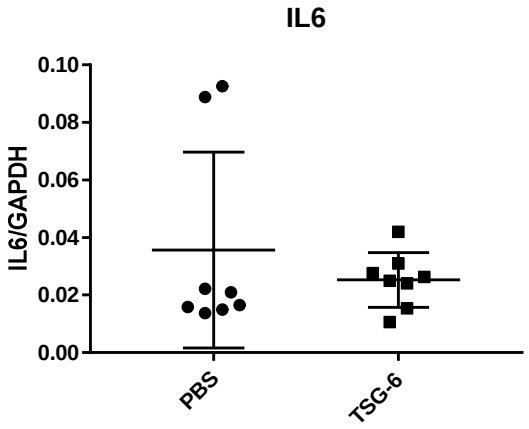


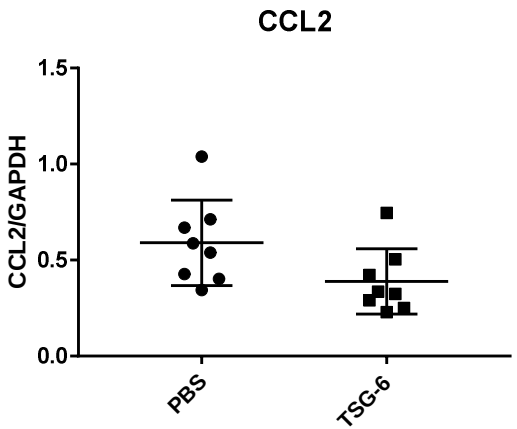


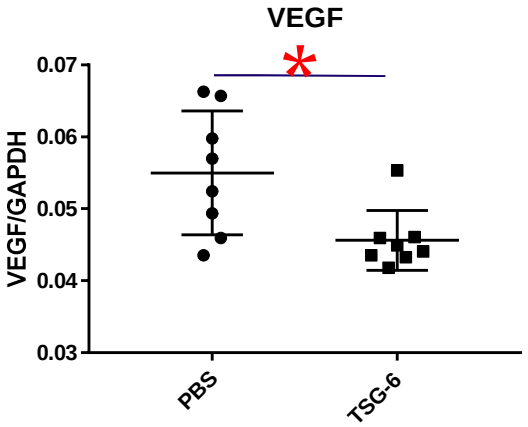
AC TSG-6
Day 1


IL1β
Il1β/GAPDH
0.10
0.08
0.06
0.04
0.02
0.00
*
PBS
TSG-6

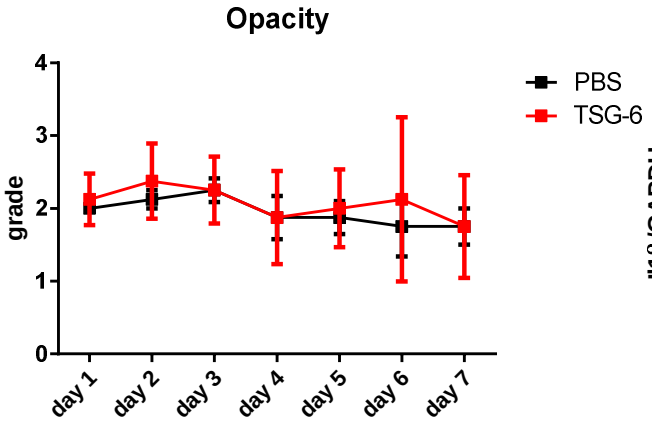

ELANE
ELANE/GAPDH
0.0008
0.0006
0.0004
0.0002
0.0000
*
PBS
TSG-6

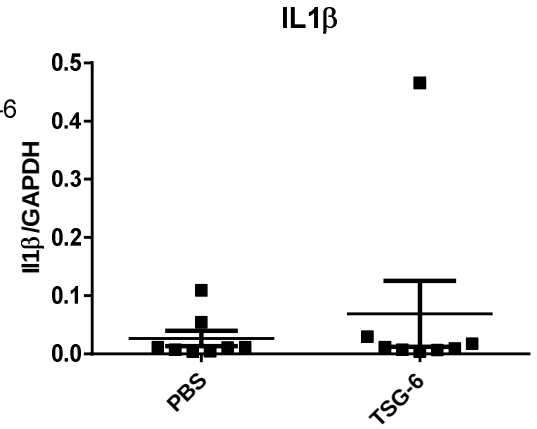

IL6
IL6/GAPDH
0.10
0.08
0.06
0.04
0.02
0.00
PBS
TSG-6

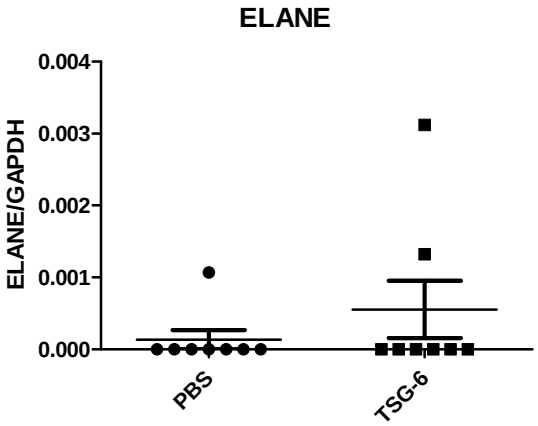

CCL2
CCL2/GAPDH
1.5
1.0
0.5
0.0
PBS
TSG-6

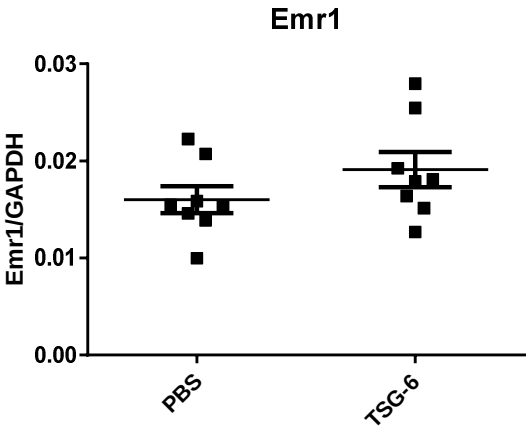

VEGF
VEGF/GAPDH
0.07
0.06
0.05
0.04
0.03
*
PBS
TSG-6

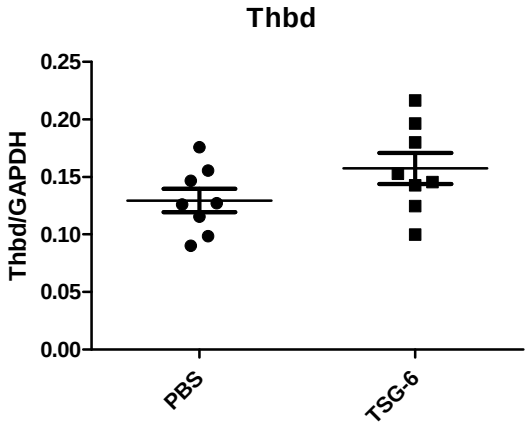
AC TSG-6
Day 7

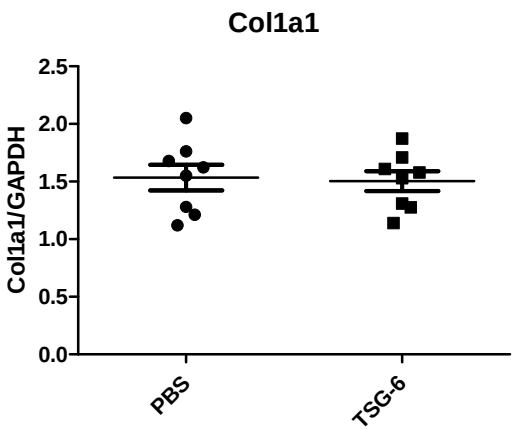

Opacity
grade
4
3
2
1
0
day 1
day 2
day 3
day 4
day 5
day 6
day 7
PBS
TSG-6


IL1β
Il1β/GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
PBS
TSG-6


ELANE
ELANE/GAPDH
0.004
0.003
0.002
0.001
0.000
PBS
TSG-6


Emr1
Emr1/GAPDH
0.03
0.02
0.01
0.00
PBS
TSG-6


Thbd
Thbd/GAPDH
0.25
0.20
0.15
0.10
0.05
0.00
PBS
TSG-6


Col1a1
Col1a1/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
PBS
TSG-6

RESULTS

AC & IV TSG-6 reduce inflammatory markers at Day 1

BUT (a) Topical TSG-6 demonstrated no effect

(b) Corneal opacity was not reduced

Conclusions and Future Studies

Rat model for corneal alkali injury

- …AC/IV TSG-6 reduced inflammation Day 1
- …Topical TSG-6 ineffective
- …No benefit yet on corneal opacity

TSG-6 holds promise in chemical corneal injury

- …A natural protein
- …Unique mechanism of action
- …High dose, multiple dose
- …Possible synergies with new anti-apoptotic peptides

Appendix 6

Time course window of IV TSG-6

IV Injection Delayed Treatment Day 1

NaOH 0.5 N (30 sec); 50 mL saline flush + 50 mL saline rinse (15 minutes)

- Intravenous injection (4 hour after injury; delayed treatment):

  200 µg / rat

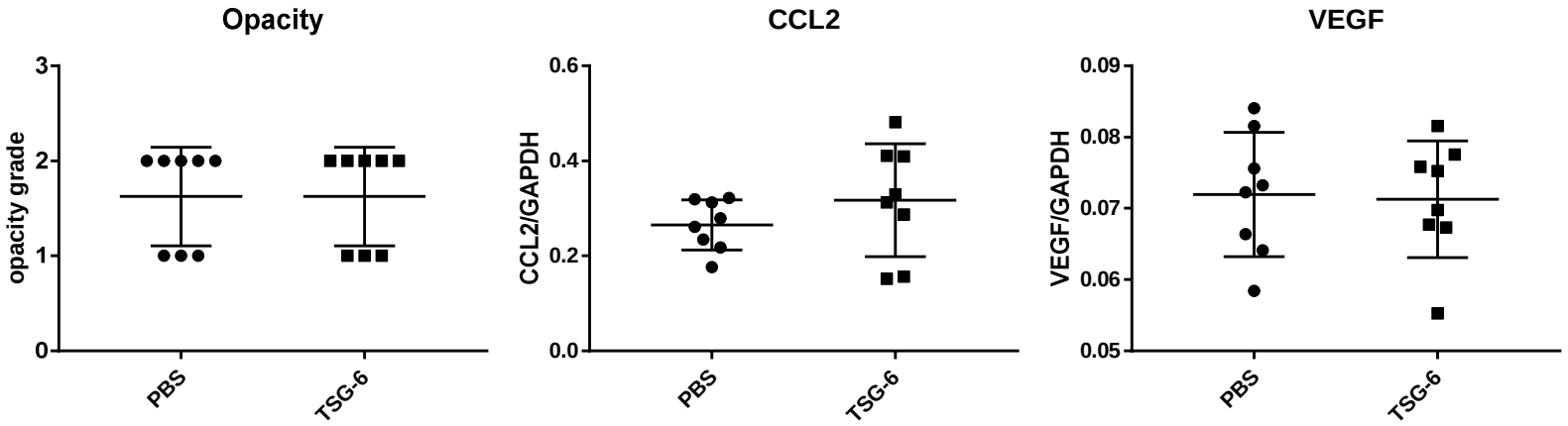
- Rats:

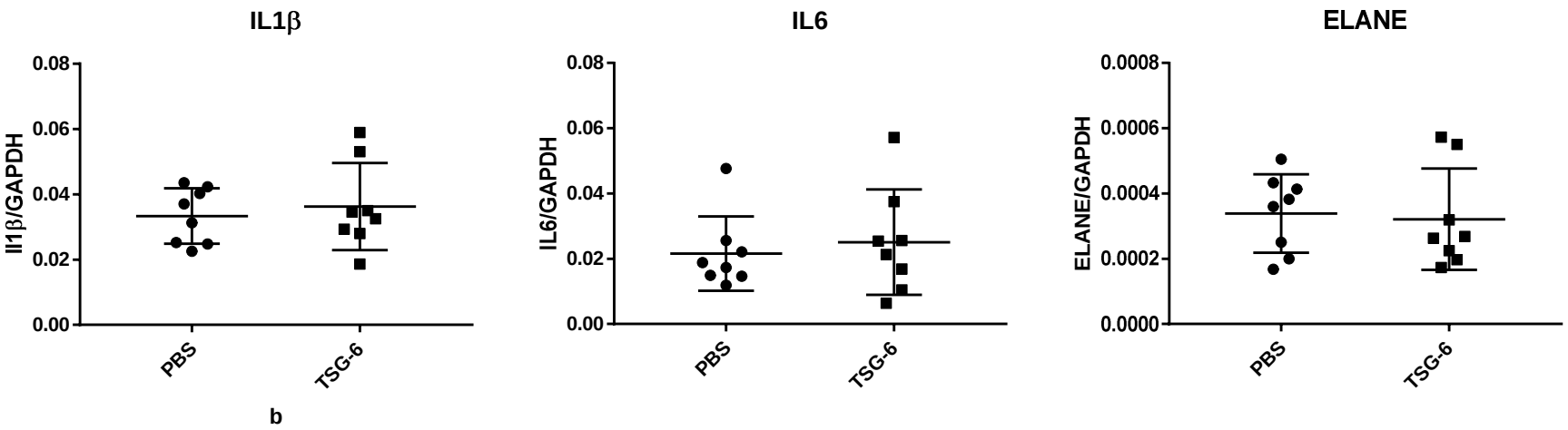
  8 rats / PBS

  8 rats / TSG-6 = 16 Lewis rats total

- Harvest day / number of animals:

  Day 1 harvest / 16 rats


Opacity
opacity grade
3
2
1
0
PBS
TSG-6
CCL2
CCL2/GAPDH
0.6
0.4
0.2
0.0
PBS
TSG-6
VEGF
VEGF/GAPDH
0.09
0.08
0.07
0.06
0.05
PBS
TSG-6


IL1β
Il1β/GAPDH
0.08
0.06
0.04
0.02
0.00
PBS
TSG-6
IL6
IL6/GAPDH
0.08
0.06
0.04
0.02
0.00
PBS
TSG-6
ELANE
ELANE/GAPDH
0.0008
0.0006
0.0004
0.0002
0.0000
PBS
TSG-6

b

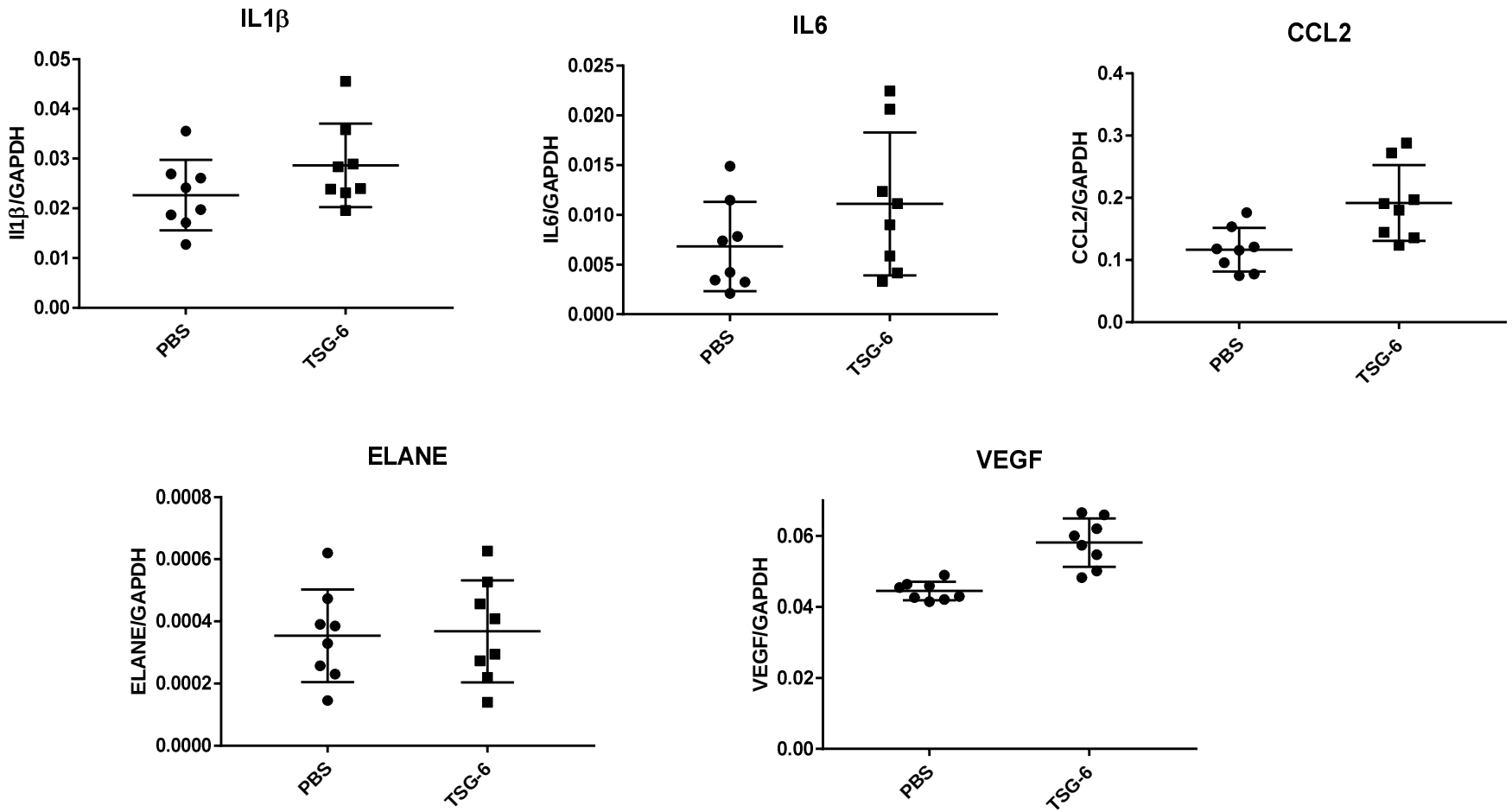
Appendix 7

Investigation of Synergistic Effect of Topical and IV TSG-6

IV Injection + topical

NaOH 0.5 N (30 sec); 50 mL saline flush + 50 mL saline rinse (15 minutes)

- Intravenous injection :
  200 µg / rat
- Topical treatment:
  5 µl / drop
  1 drop, 3x/day
- Rats:
  8 rats / PBS
  8 rats / TSG-6 = 16 Lewis rats total
- Harvest day / number of animals:
  Day 1 harvest / 16 rats


IL1β
Il1β/GAPDH
0.05
0.04
0.03
0.02
0.01
0.00
PBS
TSG-6
IL6
IL6/GAPDH
0.025
0.020
0.015
0.010
0.005
0.000
PBS
TSG-6
CCL2
CCL2/GAPDH
0.4
0.3
0.2
0.1
0.0
PBS
TSG-6
ELANE
ELANE/GAPDH
0.0008
0.0006
0.0004
0.0002
0.0000
PBS
TSG-6
VEGF
VEGF/GAPDH
0.06
0.04
0.02
0.00
PBS
TSG-6

Appendix 8 Final Report

Presented at the Annual Symposium of the American Cataract and Refractive Surgery 13-17 April 2018, Washington DC

Efficacy of the Anti-Inflammatory Protein TSG-6 for Acute Corneal Alkali Injury in a Rat Model

Samuel Fulcher MD[1]
Casie Phillips[1]
Roxanne Reger MS[2]
Joo Youn Oh MD PhD[3]
Hosoon Choi PhD[1]

The authors have no financial interests to disclose

[1]Central Texas Veterans Health Care System; [2]Texas A&M Institute for Regenerative Medicine; [3]Seoul National University

Chemical Injuries of the Cornea

Tissue Damage:

- … Direct chemical injury
- … Compounded by inflammation
- … Steroids and NSAIDS may amplify

A Potential Solution:

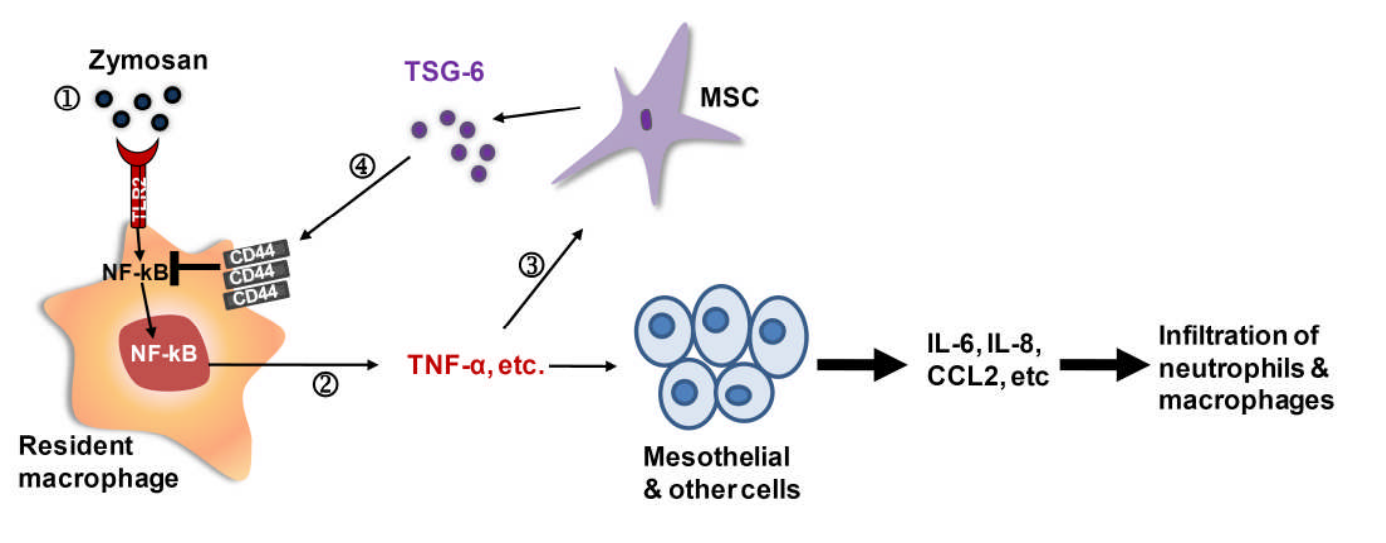
- … The protein "TSG-6"
  - … TNF-stimulated gene 6 protein
  - … modulator of inflammation
  - … novel mode of action

Unique Mechanism of TSG-6

… A natural negative feedback loop for inflammation

…Released by Mesenchymal Stem Cells (MSCs)
blocks macrophage NF-κB pathway via CD44 receptor
"first responders" to tissue injury

…no known toxic effects in eye or other tissues



Choi et al., Blood 2011

Experimental Design

Lewis Rats (8 per group)

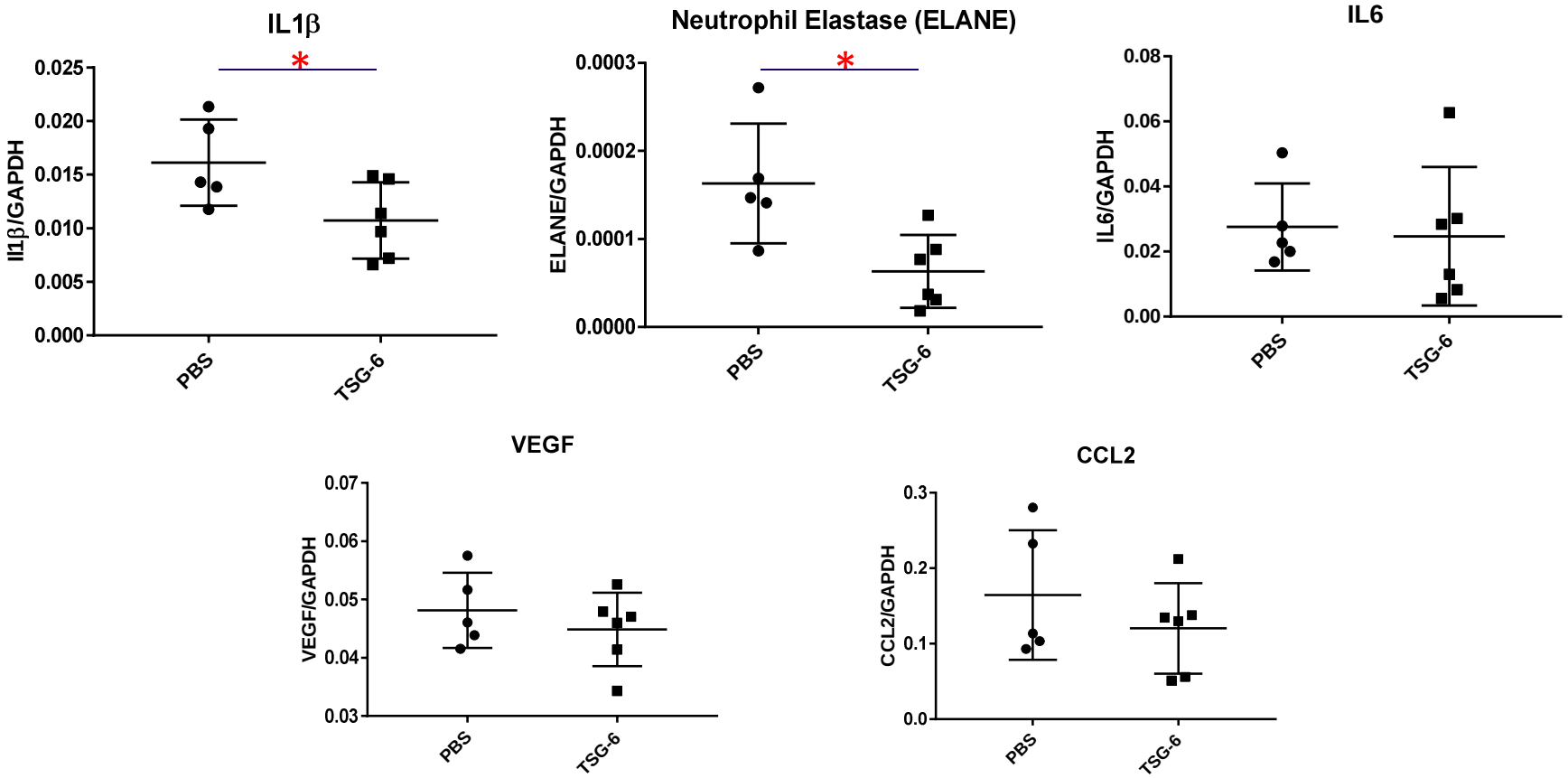
0.5N NaOH, 4 mm paper, 30 sec exposure, extensive rinse

TSG-6: Topical qtts tid 0.75 ug/ml
AC 5.5 ug
IV 200 ug, immediate and delayed 4 hours
PBS controls

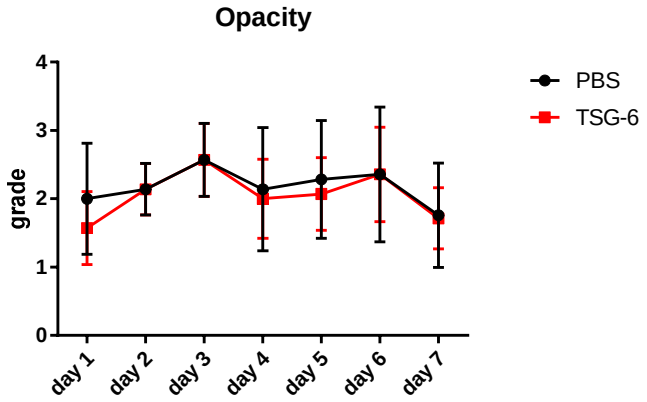
Inflammatory marker assays days 1,7

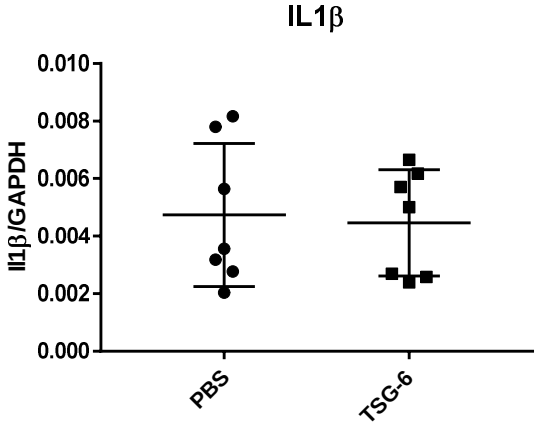
Opacity Grading days 1-7

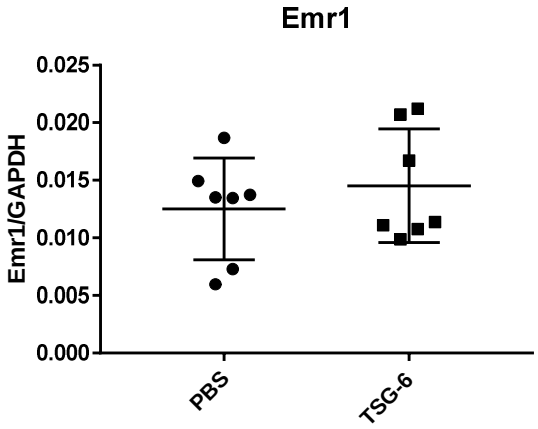
DAY 1 IV TSG-6
IL1B, ELANE
REDUCED

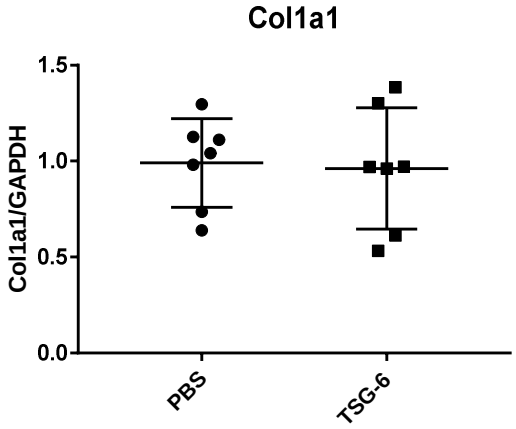


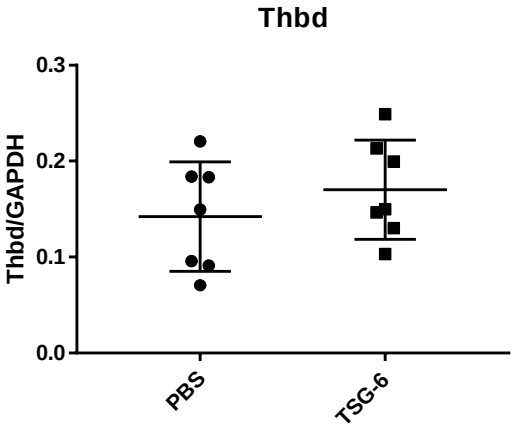
Day 7 IV TSG-6
NO DIFFERENCES



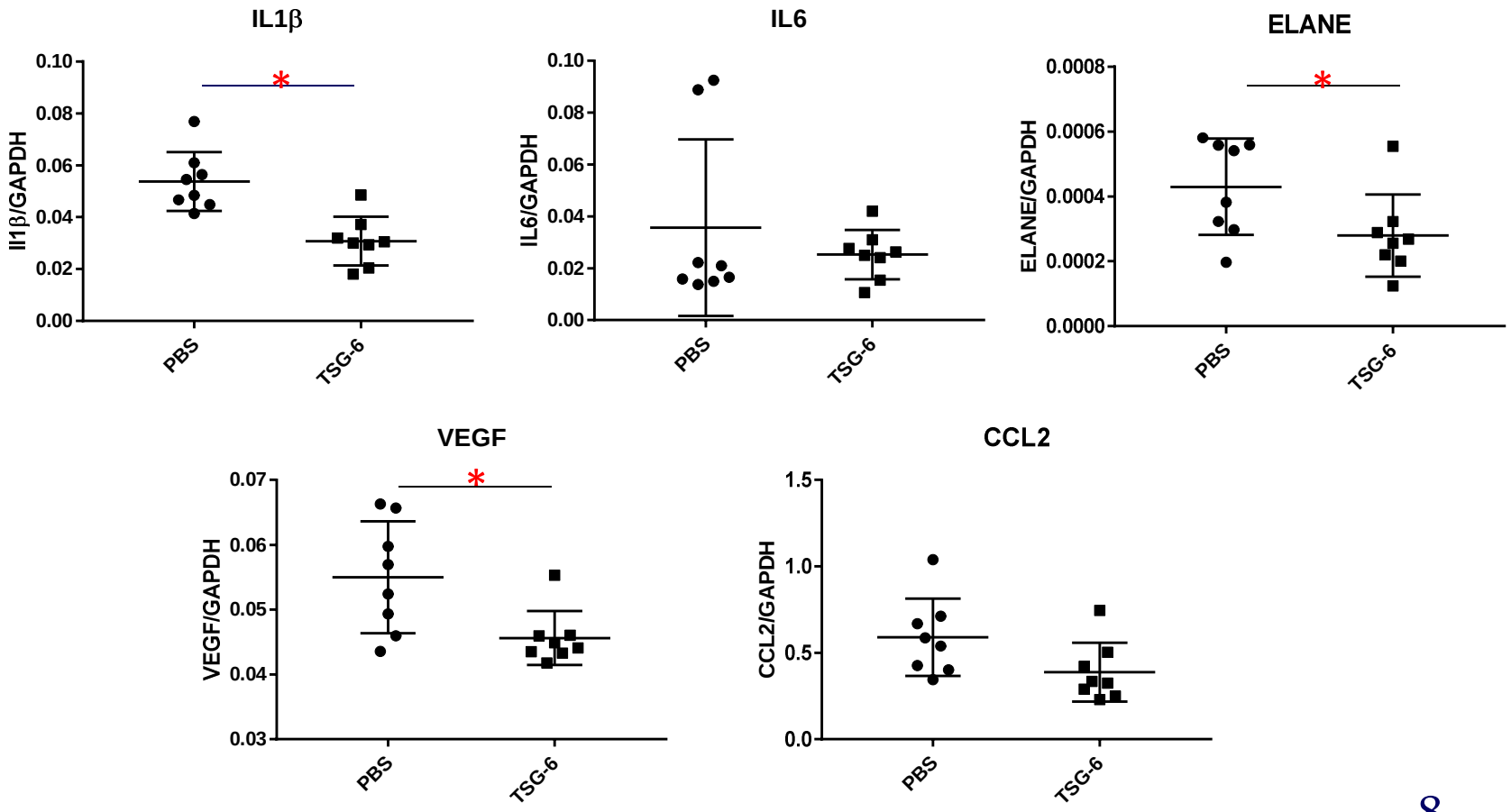




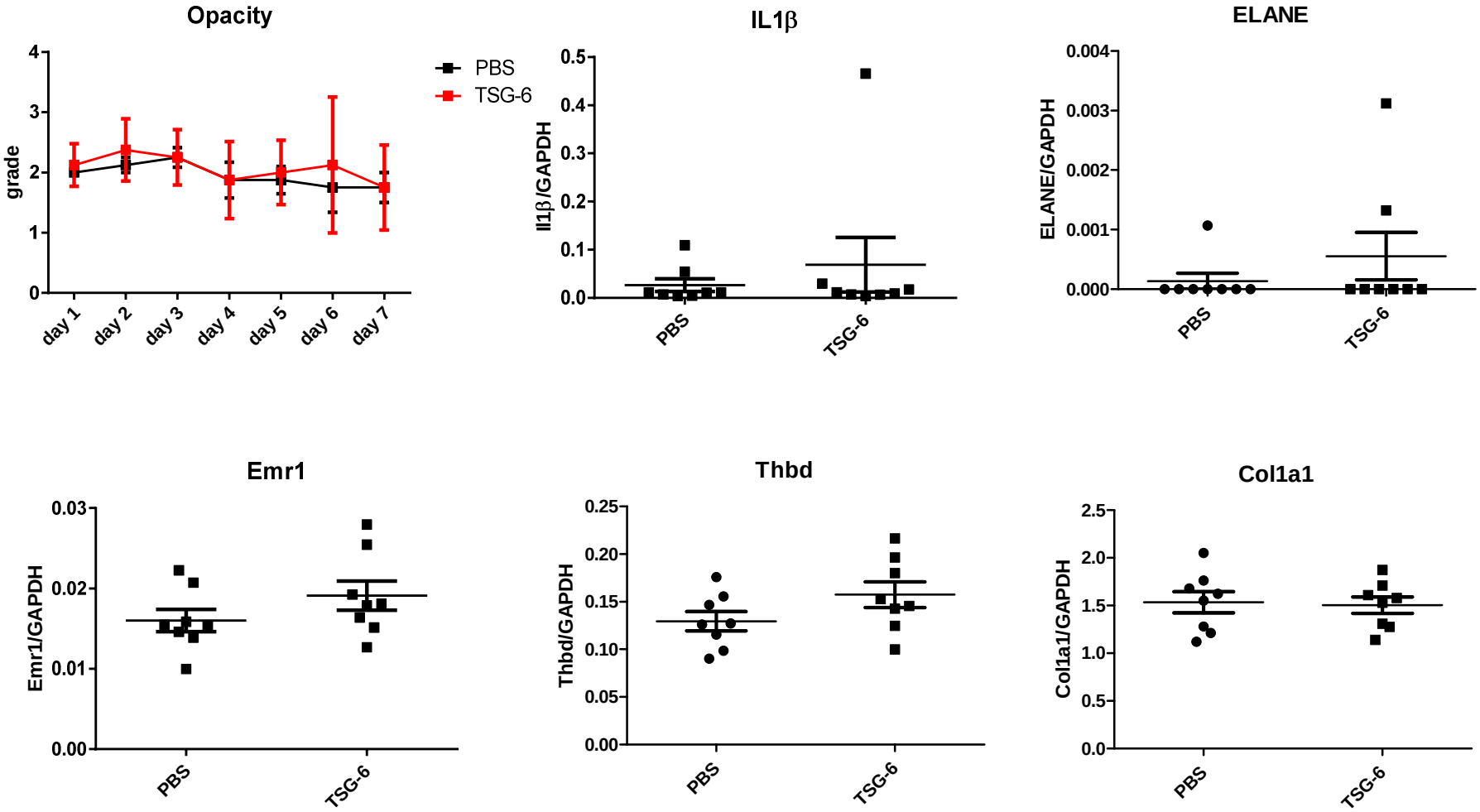




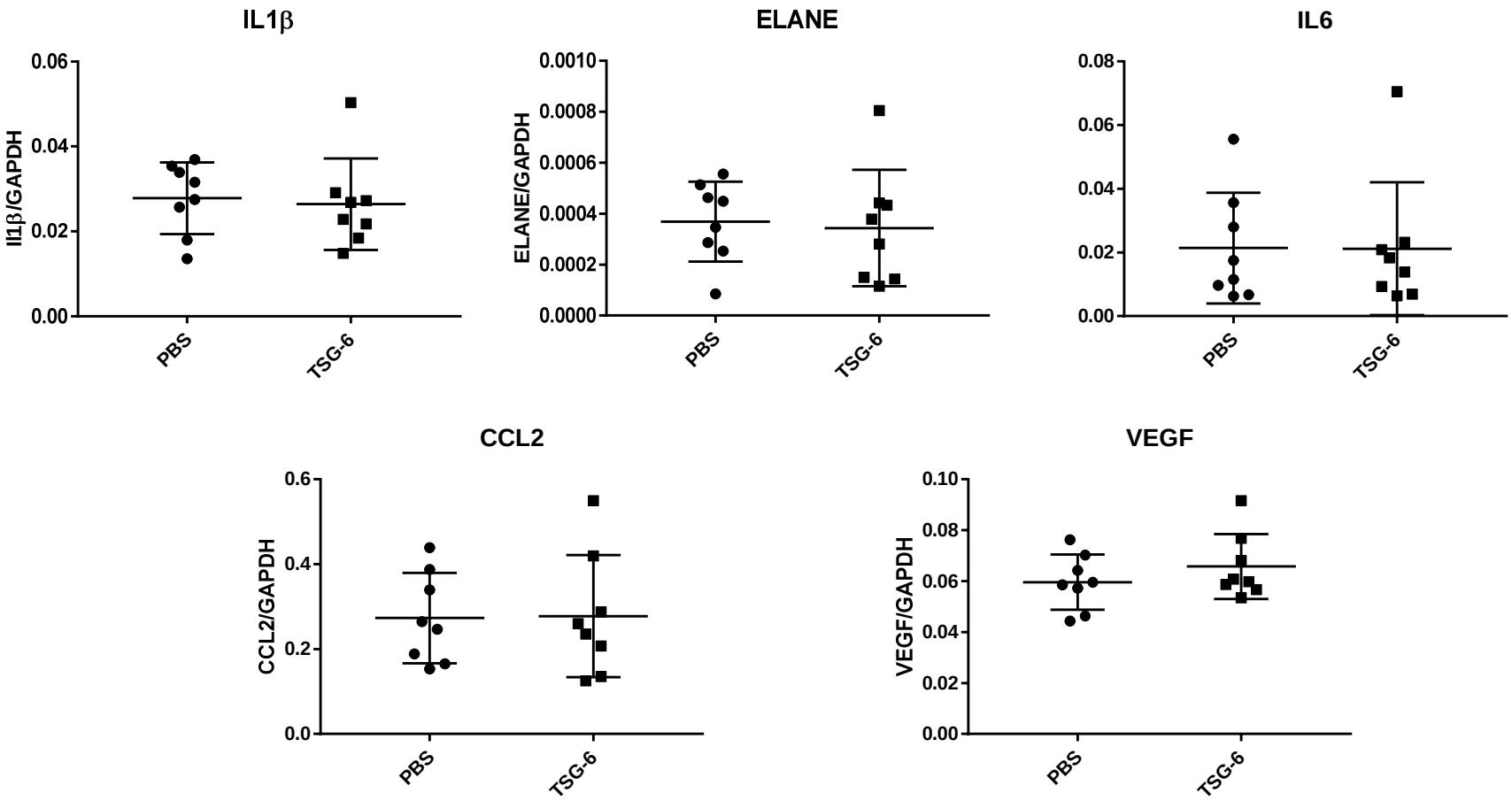
DAY 1 AC TSG-6
IL1B, ELANE,VEGF
REDUCED



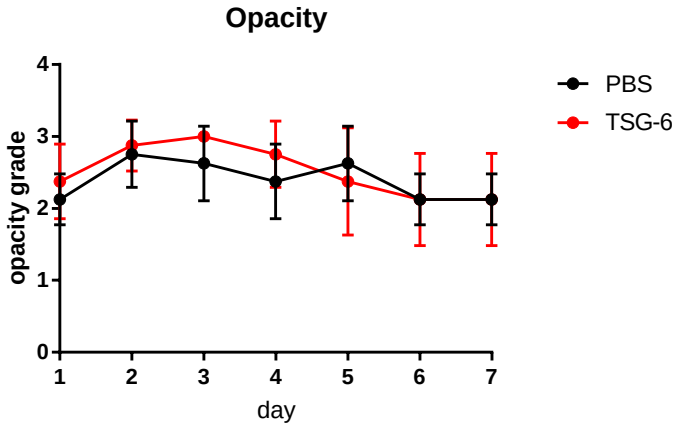
DAY 7 AC TSG-6
NO DIFFERENCES

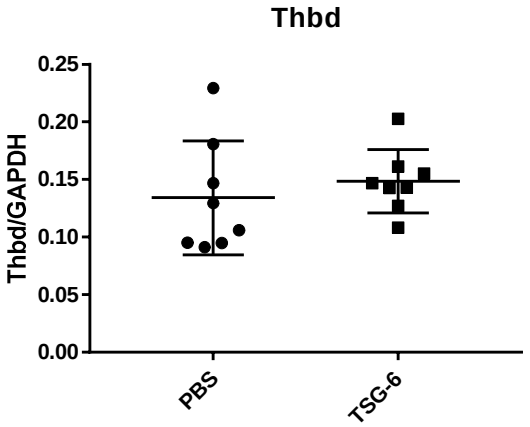


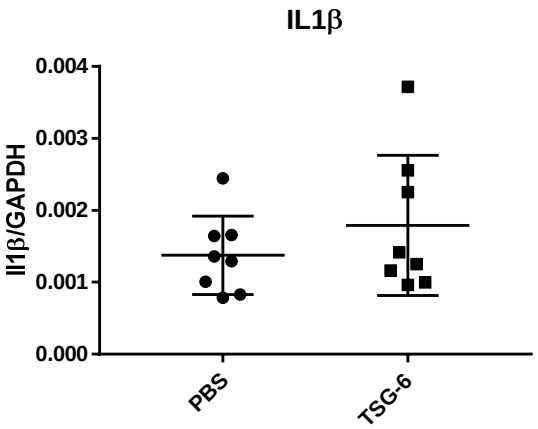
DAY 1 TOPICAL TSG-6
NO DIFFERENCES

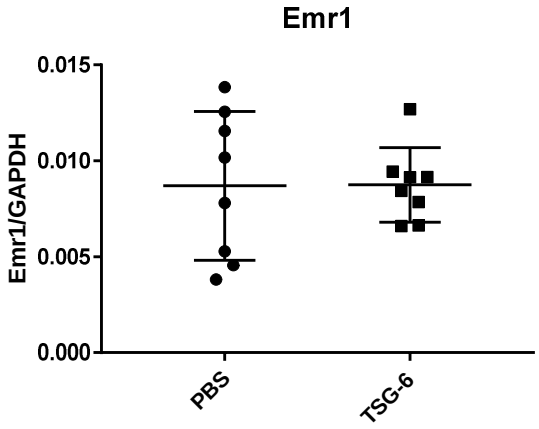


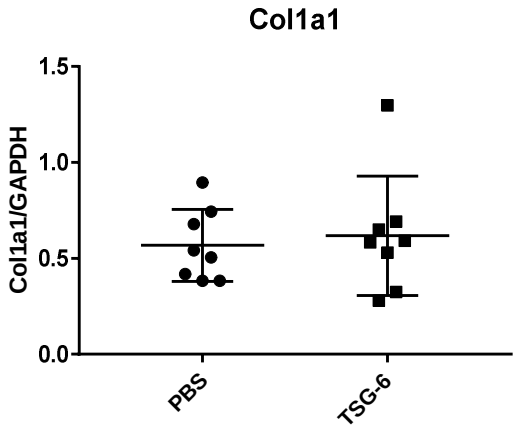
DAY 7 TOPICAL TSG-6
NO DIFFERENCES











Conclusions and Future Studies

Rat model for corneal alkali injury

…AC/IV TSG-6 reduces inflammatory signal at Day 1

…Topical TSG-6 ineffective at all time points

…No benefit yet on corneal opacity

Exosomal delivery of TSG-6 may improve results

…Enhanced bioavailability

…Unique mechanism of action

…Possible synergies with new anti-apoptotic peptides

This IACUC approved protocol was funded by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0495, and supported in part with resources of the Central Texas Veterans Health Care System, Temple, Texas. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense, and do not necessarily reflect the positions or policies of the Veterans Health Administration. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office.

Efficacy of the Anti-inflammatory Protein TSG-6 for Corneal Alkali Injury in a Rat Model

Hosoon Choi, PhD [1,*], Casie Phillips, BS [1], Joo Youn Oh, MD, PhD [2], Luke Potts, MD, PhD [3], Roxanne L. Reger, MS [4], Darwin J. Prockop, MD, PhD [4], and Samuel Fulcher, MD [5]

[1] Central Texas Veterans Research Foundation, Temple, Texas, United States of America

[2] Department of Ophthalmology, Seoul National University Hospital, Daehak-ro, Jongno-gu, Seoul, Republic of Korea

[3] Department of Ophthalmology and Surgery, Scott and White Eye Institute, Temple, Texas, United States of America

[4] Institute for Regenerative Medicine, College of Medicine, Texas A&M University, College Station, Texas, United States of America

[5] Department of Surgery, Central Texas Veterans Health Care System, Temple, Texas, United States of America

*to whom correspondence may be addressed.

1901 S. 1st St. (Room 3R25)
Temple, TX 76504-7451
Phone: 254-743-2469

E-mail: Hosoon.Choi@va.gov

Keywords: corneal alkali injury, TSG-6, inflammation, corneal neovascularization, fibrosis

Running title: TSG-6 for Corneal Alkali Injury

Abstract

Purpose. To investigate the therapeutic efficacy of tumor necrosis factor (TNF)-α stimulated gene/protein 6 (TSG-6) in a rat model of corneal alkali injury. *Methods.* Corneal alkali injury was produced by placing a NaOH soaked filter paper disk on the central cornea of the right eye of an anesthetized male Lewis (LEW/Crl) rat. Recombinant human TSG-6, or an equal volume of phosphate-buffered saline (PBS), was administered intravenously (IV), by anterior chamber (AC) injection, or as a topical drop. The affected eyes were photographed daily using a dissecting microscope and documented for clinical time course analysis of corneal opacification. Corneal tissue was excised at pre-determined therapeutic endpoints, with subsequent qRT-PCR or histological analyses. *Results.* The continuous monitoring of corneal alkali injury progression revealed insufficient *in vivo* effectiveness for TSG-6 as IV injection, AC injection, or topical application. Corneal opacification and neovascularization were not diminished and gene expression was not impacted by these treatments. However, both IV and AC administration of TSG-6 significantly suppressed pro-inflammatory cytokines compared to PBS-treated eyes. *Conclusion.* We conclude that the therapeutic potential of TSG-6 is insufficient in a rat corneal alkali injury model. Based on its ability to suppress pro-inflammatory cytokines, we suggest that further exploration of IV or AC administration of TSG-6 may prove beneficial in future therapeutic studies of corneal alkali injury.

1. Introduction

Ocular chemical burns account for approximately 7-18% of all ocular traumas, and may cause irreversible vision loss depending on the severity of injury.(Singh et al. 2013) Injuries which involve strongly basic (alkaline) or acidic solutions are among the most severe,(Peate 2007) and require immediate medical attention and suitable treatment to prevent the short- and long-term debilitating sequelae.(Singh et al. 2013) Alkaline agents tend to be more severe due to their lipophilic and hydrophilic properties which allow these solutions to penetrate cell membranes and enter the anterior chamber more rapidly than acids.(Poon et al. 2015) The infiltration of alkaline chemicals generates a cascade of effects including saponification of cells, tissue damage, and proteolytic enzyme secretion, which consequently perpetuates a destructive inflammatory cycle, often resulting in permanent scarification, opacification, and visual deficiencies.(Singh et al. 2013) Commonly encountered alkalis include: ammonia (found in fertilizers and cleaning agents), sodium hydroxide (lye; found in drain cleaners), and calcium hydroxide (lime; in cement, plaster, and mortar).(Liao and Rossignol 2000, Kuckelkorn et al. 2002) This type of exposure can also originate from the chemical components necessary for vehicle airbag inflation(Barnes, Wong, and Affeldt 2012) and from toxic agents in potential chemical warfare tactics in global combat or local acts of terrorism.(Rall and Pechura 1993, Hurst 2008) Alkali injuries are four times as prevalent as those from acidic substances,(Willmann and Melanson 2018, Singh et al. 2013, Blais 1996) yet an effective medical countermeasure against the ocular effects of

alkali exposure does not exist. Therefore, a more effective therapeutic approach is needed to mitigate the adverse health effects of ocular exposure to alkaline agents.

Medical management for alkali-induced ocular injuries includes immediate irrigation of the ocular surface to remove the chemical agent and prevent any further damage to the cornea.(Singh et al. 2013) Clinical evaluation of corneal opacity and limbal ischemia, as described in the Roper-Hall classification system,(Roper-Hall 1965) can be helpful in guiding clinical decision making for the appropriate therapeutic approach and determining a suitable prognosis.

Numerous medical therapeutic modalities are presently available for treating corneal alkali burns and include the use of non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, tear substitutes, corticosteroids, anti-glaucoma medications, and citrate drops.(Tuft and Shortt 2009) Examples of surgical procedures for vision rehabilitation include conjunctival autografting, limbal epithelial transplants, and corneal transplantation.(Eslani et al. 2014, Fish and Davidson 2010) However the extensive effort to reestablish the ocular surface often requires multiple procedures over several years, and is often complicated by chronic ocular surface disease, graft failure, or infection.(Kilic Muftuoglu, Aydin Akova, and Cetinkaya 2015, Miri, Al-Deiri, and Dua 2010)

Severe sequelae of chemical injuries (i.e. corneal melt, graft failure, limbal stem cell deficiency, glaucoma) often present later in the course of treatment, and acute therapy plays an important role in the mitigation of these outcomes.(Wagoner 1997, Brodovsky et al. 2000) In the acute phase of injury, tissue damage induces an upsurge of inflammation, which can lead to corneal ulceration, perforation, and

neovascularization with resultant permanent visual impairment or blindness.(Brodovsky et al. 2000, Wagoner 1997, Kuo 2004) The limited success of the currently accessible therapeutics is due, in part, to their inability to effectively reduce acute inflammation to prevent the adverse sequelae of corneal alkali injuries.

At present, there are many treatment modalities that target acute inflammation, yet they are unreliable, insufficient, and/or exhibit adverse side effects.(Brodovsky et al. 2000, Kuo 2004) Corticosteroids are widely used to suppress the inflammation in the acute phase of corneal alkali burn; however these drugs may delay corneal wound healing,(Sugar and Chandler 1974, Aquavella, Gasset, and Dohlman 1964) increase potential for infection,(Mitsui and Hanabusa 1955, Thygeson, Hogan, and Kimura 1953) and can aid in the formation of cataracts and glaucoma.(Awan 1975, Feroze and Khazaeni 2018)

In this study, we investigated the therapeutic effect of tumor necrosis factor (TNF)-α stimulated gene/protein-6 (TSG-6) in a rat model of corneal alkali injury. TSG-6 is a 30 kD glycoprotein expressed by a variety of cells in response to stimulation by pro-inflammatory cytokines.(Milner and Day 2003) This multifunctional protein was shown to prevent inflammation and tissue damage in rodent models of arthritis,(Mindrescu et al. 2002) myocardial infarction,(Lee et al. 2009) peritonitis,(Choi et al. 2011) traumatic brain injury,(Watanabe et al. 2013) and corneal ethanol injury.(Oh et al. 2012, Roddy et al. 2011, Oh et al. 2010) Based on the success of TSG-6 in previous inflammatory models, and the well documented inflammatory response after corneal alkali burn, this study was designed to explore the therapeutic potential of TSG-6 on rat corneas injured by sodium hydroxide.

2. Materials and Methods

*2.1 Animal Model of Corneal Alkali Injury***.** All experiments adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Our animal use protocol was approved and monitored by the Institutional Animal Care and Use Committee (IACUC) of Central Texas Veterans Health Care System (CTVHCS) and Baylor Scott & White Healthcare. All animal-related research was conducted in accordance with the regulations of the Department of Agriculture and the laws of the United States. Experiments were performed on eight-week-old, male Lewis rats (LEW/Crl) with an average body weight of 250g (Charles River Laboratories; Wilmington, MA). Rats were housed as pairs in an environmentally controlled room with a 12/12 (light/dark) cycle. Food and water supply were provided *ad libitum*.

2.2. Standard Corneal Alkali Burn Injury. After a minimum five-day acclimation period, a corneal alkali burn was generated. Rats were anesthetized by intraperitoneal (IP) injection of an anesthetic cocktail containing 80mg/kg of ketamine and 6 mg/kg of xylazine. Depth of anesthesia was determined by pedal withdrawal reflex and blink response. Ophthalmic ointment was applied to the unaffected (left) eye to prevent desiccation and a subcutaneous (SQ) injection of buprenorphine (0.03 mg/kg) was administered for preemptive analgesia. Once the proper surgical plane was reached, each rat was placed under a surgical dissecting microscope and a single drop of topical

analgesic (0.5% proparacaine hydrochloride ophthalmic solution; Sandoz Inc. Princeton, NJ) was applied to the right eye. The corneal alkali burn injury was produced with a 4-mm diameter circular filter paper disk soaked in a solution of 0.5 N NaOH, and applied to the central cornea of the right eye for 30 seconds, as previously described.(Choi et al. 2017) Immediately after filter paper removal from the cornea, the eye was continually flushed with sterile saline for 2 minutes (50 mL). A steady drip of sterile saline was then applied for an additional 15 minutes (50 mL) of irrigation. Treatment was then administered under the surgical dissecting microscope and tarsorrhaphy performed, if applicable. Buprenorphine was administered SQ 4-8 hours after injury and twice daily for two days to follow.

2.3. Treatment of Corneal Alkali Burn with TSG-6. For each experiment, sixteen rats were evenly distributed into two groups based on weight: TSG-6 (n=8) and PBS (control; n=8). A single drop of topical antibiotic (Vigamox; 0.5% moxifloxacin hydrochloride ophthalmic solution; Alcon Laboratory, Inc. Fort Worth, TX) was applied to prevent microbial contamination. Immediately after injury, treatment were administered via one of the following routes: 1) Intravenous (IV) injection- 250 µL of either rhTSG-6 (recombinant human TSG-6; 200 µg; R & D Systems, Minneapolis, MN) or PBS into the lateral tail vein with an insulin syringe and 26 G x ½" needle; 2) Anterior chamber (AC) injection- 7 µL of either rhTSG-6 (5 µg) or PBS with an insulin syringe and 31 G x 5/16" needle; or 3) Topical- 5 µL of either rhTSG-6 (4 µg) or PBS applied by pipet drop three times per day. The treatment protocols are described in Figure 1. Briefly, IV and AC treatments were one-time injections after initial injury and flush, whereas topical

treatment was applied three times daily until the pre-determined harvest date. A tarsorrhaphy was not performed on animals receiving topical treatments due to the frequency of treatment administration. There were no adverse effects (i.e. corneal abrasions, etc.) noted from absence of the suture. All rats were euthanized either 1 day or 7 days after initial injury by a ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail IP adjunct thoracotomy. Corneal tissue of the affected eye was excised for immunohistochemical analysis or RNA isolation.

2.4. Biomicroscopic Evaluation and Photographs. All injured eyes were microscopically examined at 24-hour intervals and photographs were taken using a digital camera attached to a dissecting microscope. Before each biomicroscopic examination, rats were mildly sedated with inhalant anesthesia (isoflurane). Corneal clarity was graded independently by three experienced ophthalmologists, who were blinded to the treatment conditions. Corneas were evaluated based on the opacification scoring system (scale of 0-4) previously described by Gupta et. al,(Gupta, Kalaivani, and Tandon 2011) where 0= no opacity; 1= slight superficial opacity, iris details visible; 2= moderate stromal opacity, pupillary margin visible, iris details not visible; 3= significant stromal opacity, pupillary margin not visible; and 4= completely opaque, pupil and anterior chamber not visible.

2.5. Histopathology and Immunohistochemistry. Excised corneas for histological and immunohistochemical processing were fixed in 10% buffered formalin and embedded into paraffin blocks. Fixed samples were sectioned at 4 μm and subjected to either

hematoxylin and eosin (H&E) or immunohistochemical staining. Antigens for immunohistochemical analyses were retrieved with a steamer in epitope retrieval solution (10 mM sodium citrate pH 6). Rabbit polyclonal anti-neutrophil elastase antibody (1:150) (ab21595, Abcam, Cambridge, MA) was used for neutrophil infiltration detection. Anti-rabbit IgG (1:5000; Abcam) was used as the secondary antibody. DAB staining was performed using a DAB Peroxidase Substrate kit (Vector Laboratories, Burlingame, CA) and the slides were counterstained with Hematoxylin QS (Vector Laboratories).

2.6. Real-time qRT-PCR. The isolation of total RNA from the cornea specimens was performed using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) per the manufacturer's protocol. The RNA concentration and purity was measured spectrophotometrically, based on the absorbance at 260 and 280 nm, using NanoDrop ND-1000 V 3.2.1 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Real-time amplification was performed with QuantiTect Probe RT-PCR Kit (Qiagen) and analyzed on 7900HT Fast Real-Time PCR system (Applied Biosystems. Foster City, CA) using TaqMan gene expression assays for interleukin-1β (IL-1β; Rn00580432_m1), IL-6 (Rn01410330_m1), monocyte chemoattractant protein-1 (MCP-1/CCL2; Rn00580555_m1), vascular endothelial growth factor A (VEGF-A; Rn01511601_m1), neutrophil elastase (ELANE; Rn01535456_g1), F4/80 (Emr1; Rn01527631_m1), thrombomodulin (Thbd, CD141; Rn00582226_s1), and type I collagens (Col1a1; Rn01463848_m1). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe (Rn01775763_g1) were used as internal controls.

2.7. Statistical Analysis. Data are expressed as mean ± SD. All analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Real-time qRT-PCR data were evaluated with unpaired 2-tailed Student *t* test. The Kruskal-Wallis one-way ANOVA with Dunn's post-hoc tests were used for comparative analysis of opacification scores. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

All eyes subjected to alkali burn showed corneal opacification and increased measures of inflammation and corneal neovascularization, as previously described for this moderate-grade alkali burn rat model.(Choi et al. 2017) In this study, we investigated the therapeutic effects of intravenous (IV), anterior chamber (AC), and topical administration of TSG-6 against a unilateral corneal alkali burn injury.

3.1. Effects of IV-administered TSG-6 in a Corneal Alkali Burn Model. To investigate the therapeutic effects of IV-administered rhTSG-6 in a corneal alkali burn model, a one-time injection of rhTSG-6 (200 μg rhTSG-6 in 250 μL PBS) was administered immediately following the initial alkali burn and saline rinse. Corneal opacity was evaluated daily and a moderate-grade (the mean score of 2) injury was observed for both PBS- and TSG-6-treated groups. There was no remarkable difference in corneal clarity between the two groups over the 7-day acute injury phase (Figure 2 A, 3 A, 3 B). Corneal tissue was extracted 1 day or 7 days after injury to examine the effects of TSG-

6 on acute inflammation, fibrosis, neovascularization, and macrophage and/or neutrophil infiltration. One day after alkali burn, IV administration of rhTSG-6 significantly reduced expression of the pro-inflammatory cytokine, IL-1β (Figure 2 D), and decreased neutrophil infiltration (ELANE) when compared to PBS-treated controls (Figure 2 B, G, H, I, J). Other markers of inflammation (IL-6, CCL2) however, did not demonstrate statistically significant differences between groups on day 1 (Figure 2 E, F). Corneal neovascularization progressively increased post-injury, (Choi et al. 2017) yet there was no significant difference in VEGF expression between treatment and control groups (Figure 2 C).

The limited success of IV-administered rhTSG-6 in reducing acute inflammation at day 1 suggests that this treatment approach may prove advantageous in mitigating the injurious sequelae throughout the 7-day acute injury phase. Quantification of macrophage infiltration (Emr1) and neovascularization (Thbd) revealed a slight increase for TSG-6-treated rats compared to PBS controls on day 7, however these differences were not statistically significant (Figure 3 D, E). Since corneal alkali injury induces stromal fibrosis which may lead to scar formation,(Eslani et al. 2014) Col1a1 was used as a marker of fibrosis at day 7, with no therapeutic benefit observed for TSG-6 compared to PBS controls (Figure 3 F). Although the pro-inflammatory cytokine IL-1β is known to increase throughout the 7-day acute injury phase(Choi et al. 2017) TSG-6 did not show any benefit when compared to PBS in reducing this expression level on day 7 (Figure 3 C). In both the treatment and control groups, corneal epithelial denudation was observed at the center and periphery of the cornea on day 1. Corneal re-

epithelialization was completed by day 7 in both PBS-treated and TSG-6–treated corneas (Figure 3 A).

3.2. Effects of AC-administered TSG-6 in a Corneal Alkali Burn Model. Based on the success of AC-administered TSG-6 in an ethanol-induced corneal injury model,(Oh et al. 2010) we tested the effects of a one-time AC injection of rhTSG-6 (5 μg of rhTSG-6 in 7 μL of PBS) in our corneal alkali burn model. Although TSG-6 markedly reduced inflammation following ethanol exposure to the cornea,(Oh et al. 2010, Oh et al. 2012) there was limited benefit in this alkali injury model. The corneal clarity on day 1 was slightly worse in TSG-6-treated corneas than PBS-treated cornea, however the data were not statistically significant (Figure 4 A). One day after initial alkali burn, qRT-PCR revealed a significant reduction of VEGF and IL-1β expression levels following TSG-6 injection (Figure 4 C, D). TSG-6 was also able to reduce measures of ELANE, IL-6, and CCL2 at day 1, though these data are not statistically significant (Figure 4 B, E, F). Corneal clarity throughout the 7-day acute phase progression was consistent for both PBS- and TSG-6-treated corneas, exhibiting a moderate-grade level of injury (Figure 5 A). There was no apparent difference between treatment and control groups for Col1a1, IL-1β, Emr1, and Thbd on day 7 (Figure 5 B, C, D, E).

3.3. Effects of Topically-administered TSG-6 in a Corneal Alkali Burn Model. Topical application of TSG-6 is important to investigate because of the applicability in a clinical setting. A single drop (4μg of rhTSG-6 in 5 μL of PBS) of rhTSG-6 was applied to the ocular surface 3 times per day until tissue harvest at day 1 or 7. Topically-administered

rhTSG-6 showed statistically significant improvements in corneal wound healing in an ethanol-induced injury,(Roddy et al. 2011) however topical administration of rhTSG-6 in our alkali burn model showed no benefit at day 1 or 7. Corneal opacity was scored as a moderate-grade injury for both groups throughout the 7-day injury progression (Figure 6 A, 7 A). Expression levels for ELANE, VEGF, IL-1β, IL-6, and CCL2 were all similar for TSG-6 and PBS controls on day 1 (Figure 6 B, C, D, E, F). On day 7 after alkali burn, the mRNA levels of Col1a1, Emr1, and Thbd for TSG-6-treated corneas were equivalent to PBS controls, and IL-1β showed a slight increase for TSG-6-treated corneas (Figure 7 B, C, D, E). The minimal increase in inflammation was not statistically significant.

4. Discussion

An ocular injury generated by chemical exposure can lead to permanent vision loss and chronic debility.(Wagoner 1997, Miri, Al-Deiri, and Dua 2010) The abundance of alkali solutions in household and industrial products accounts for the more frequent occurrence of alkali injury compared to acid injury. Alkaline agents have sufficient lipophilicity to rapidly penetrate the ocular surface and enter the anterior chamber within seconds of initial exposure.(Eslani et al. 2014) This saponification process induces a severe inflammatory cascade, which can result in acute, long-term, and/or recurring changes in corneal optical properties that impair vision and negatively impact quality of life.(Le et al. 2011) The sequelae of an alkali-burned cornea can be severe and particularly challenging to manage due to the extensive destruction of tissue, the loss of the corneal microenvironment, and the prolonged complex and difficult healing process,

and currently insufficient treatment options.(Singh et al. 2013, Tuft and Shortt 2009, Xiang et al. 2005) The mitigation of the post-injury inflammatory response in the acute recovery phase (days 0-7) can be critical in restoring corneal clarity and a normal ocular surface, and minimizing adverse sequelae.(Singh et al. 2013) Although there are several therapeutic approaches that target early inflammation, they are relatively inefficient and unreliable at effectively treating alkali-burned corneas. Thus, it is of utmost importance to investigate novel anti-inflammatory treatment options, such as TSG-6.

TSG-6 is a multifunctional protein with a unique mechanism of action that works by inhibiting inflammation at the earliest stage (Choi et al. 2011, Oh et al. 2012). Systemic or local administration of recombinant human TSG-6 protein demonstrates anti-inflammatory activity in various disease models.(Oh et al. 2010, Choi et al. 2011, Lee et al. 2009, Kota et al. 2013, Wisniewski and Vilcek 2004, Milner, Higman, and Day 2006, Bardos et al. 2001) TSG-6 suppresses neutrophil migration into the site of inflammation.(Milner, Higman, and Day 2006, Milner and Day 2003, Getting et al. 2002) The mechanism by which TSG-6 reduces neutrophil infiltration and mitigates inflammatory responses is not fully understood. Several reports have suggested TSG-6 acts on resident macrophages via modulating TLR2/NF-kB signaling to suppress chemokines/ cytokines produced by injured tissue.(Choi et al. 2011, Oh et al. 2012)

This study was designed to investigate the therapeutic effectiveness of TSG-6 in a rat model of corneal alkali injury based on the success of TSG-6 in ophthalmic models of mild chemical injury caused by alcohol and mechanical scraping. Since currently available anti-inflammatory approaches are unable to effectively reduce acute

inflammation without any adverse side effects,(Dinarello 2010) the development of an innovative, safe approach for effectively treating corneal alkali burns is both desirable and necessary for improving patient care, cost, and clinical outcome. This project is the first to investigate the treatment of corneal alkali injury with the anti-inflammatory protein TSG-6 and these data will contribute knowledge to help fill a long-standing scientific gap for improving corneal wound healing following alkali exposure of the cornea.

Despite its prior therapeutic efficacy in other models of inflammation and injury, the administration of TSG-6 via IV or AC injection revealed limited therapeutic benefit 1 day after alkali-induced corneal injury and was ineffective at day 7. A single IV- or AC-administered dose of rhTSG-6 exhibited a modest, but statistically significant reduction in inflammatory signaling for IL-1β and neutrophil elastase at day 1. Uncontrolled neutrophil infiltration can lead to stromal degradation and ulceration and subsequent permanent vision-impairing corneal opacification, fibrosis and neovascularization,(Wagoner 1997, Brown, Weller, and Akiya 1970) therefore the ability of IV- and AC-administered TSG-6 to reduce neutrophil infiltration at day 1 may suggest that it could minimize the damaging sequelae at the end of the acute injury phase on day 7. In testing this hypothesis, we were unable to detect any significant differences between TSG-6-treated corneas and PBS controls when various mRNA markers of corneal damage progression on day 7 post injury.

Furthermore, topical application of TSG-6 showed no improvement in corneal optical properties when compared to PBS-treated controls at days 1 and 7 post-injury. These data suggest that local and systemic administration of TSG-6 is insufficient to effectively mitigate the ocular effects of corneal alkali exposure.

One possible explanation for the observed therapeutic limitation in the present study is that the activity of TSG-6 may be reduced in an alkaline environment. Since TSG-6 has a very narrow range of pH for hyaluronic acid and aggrecan binding and shows a dramatic loss of function with increasing pH without gross structural change,(Heng et al. 2008, Parkar et al. 1998) the functional element of TSG-6 may be pH-sensitive. Due to the lack of full understanding of the mechanism of TSG-6 anti-inflammatory activity, it is unclear what effect pH has on TSG-6 activity in this context. However, it is possible that the extreme pH in the cornea after alkali injury may hinder the activity of TSG-6. Despite the extensive rinsing regimen applied in this study, the pH of the cornea and other anterior segment structures of the eye remained elevated due to the rapid tissue penetration of lipophilic alkali with resultant delay in the recovery of physiological pH. To avoid the alkaline damage of TSG-6, we tested delayed or multiple dose TSG-6 treatment strategies to minimize the potential adverse effect of alkaline pH on TSG-6, but these had no significant effect (data not shown). This observation may be explained by the fact that the therapeutic window for treatment of corneal inflammation with TSG-6 is very narrow (less than 2 hours).(Oh et al. 2010) In addition, the reduction of acute inflammation may be insufficient to prevent the extensive tissue destruction observed in this model of alkali injury. Similarly, steroid treatment shows very limited effects on the prognosis of alkali injury as contrasted to other forms of sterile corneal ulceration.(Donshik et al. 1978, Dan et al. 2008, Cole et al. 2007)

The results of this work add to the body of data which have previously demonstrated the beneficial anti-inflammatory effects of TSG-6 in other models of tissue

injury or human disease. It is apparent from our data that the severity of tissue damage which occurs in alkali ocular injury may limit outcomes in models of TSG-6 treatment.

5. Conclusion

In conclusion, our data demonstrate that TSG-6 has limited effect on corneal inflammation induced by alkali injury and limited therapeutic benefit to prevent injury progression.

Conflicts of Interest Statement

HC, CP, JYO, LP, RLR, and SF have no financial disclosures that would be potential conflicts of interest with this manuscript. DJP is an advisor and has a minor equity position in a biotech company (Temple Therapeutic LLC) with an interest in TSG-6.

Funding Support

This material is the result of work supported with resources and the use of facilities at the Central Texas Veterans Health Care System, with funding by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-14-1-0495. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

References

Aquavella, J. V., A. R. Gasset, and C. H. Dohlman. 1964. "Corticosteroids in Corneal Wound Healing." *Am J Ophthalmol* 58:621-6.

Awan, K. J. 1975. "Delayed cataract formation after alkali burn." *Can J Ophthalmol* 10 (3):423-6.

Bardos, T., R. V. Kamath, K. Mikecz, and T. T. Glant. 2001. "Anti-inflammatory and chondroprotective effect of TSG-6 (tumor necrosis factor-alpha-stimulated gene-6) in murine models of experimental arthritis." *Am J Pathol* 159 (5):1711-21.

Barnes, S. S., W. Wong, Jr., and J. C. Affeldt. 2012. "A case of severe airbag related ocular alkali injury." *Hawaii J Med Public Health* 71 (8):229-31.

Blais, B. R. 1996. "Treating chemical eye injuries." *Occup Health Saf* 65 (9):23-6.

Brodovsky, S. C., C. A. McCarty, G. Snibson, M. Loughnan, L. Sullivan, M. Daniell, and H. R. Taylor. 2000. "Management of alkali burns : an 11-year retrospective review." *Ophthalmology* 107 (10):1829-35.

Brown, S. I., C. A. Weller, and S. Akiya. 1970. "Pathogenesis of ulcers of the alkali-burned cornea." *Arch Ophthalmol* 83 (2):205-8.

Choi, H., R. H. Lee, N. Bazhanov, J. Y. Oh, and D. J. Prockop. 2011. "Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-kappaB signaling in resident macrophages." *Blood* 118 (2):330-8. doi: 10.1182/blood-2010-12-327353.

Choi, H., C. Phillips, J. Y. Oh, E. M. Stock, D. K. Kim, J. K. Won, and S. Fulcher. 2017. "Comprehensive Modeling of Corneal Alkali Injury in the Rat Eye." *Curr Eye Res* 42 (10):1348-1357. doi: 10.1080/02713683.2017.1317817.

412 Cole, N., E. B. Hume, I. Jalbert, A. K. Vijay, R. Krishnan, and M. D. Willcox. 2007.
413 "Effects of topical administration of 12-methyl tetradecanoic acid (12-MTA) on the
414 development of corneal angiogenesis." *Angiogenesis* 10 (1):47-54. doi:
415 10.1007/s10456-007-9063-3.
416 Dan, L., Y. Shi-long, L. Miao-li, L. Yong-ping, M. Hong-jie, Z. Ying, and W. Xiang-gui.
417 2008. "Inhibitory effect of oral doxycycline on neovascularization in a rat corneal
418 alkali burn model of angiogenesis." *Curr Eye Res* 33 (8):653-60. doi:
419 10.1080/02713680802245772.
420 Dinarello, C. A. 2010. "Anti-inflammatory Agents: Present and Future." *Cell* 140
421 (6):935-50. doi: 10.1016/j.cell.2010.02.043.
422 Donshik, P. C., M. B. Berman, C. H. Dohlman, J. Gage, and J. Rose. 1978. "Effect of
423 topical corticosteroids on ulceration in alkali-burned corneas." *Arch Ophthalmol*
424 96 (11):2117-20.
425 Eslani, M., A. Baradaran-Rafii, A. Movahedan, and A. R. Djalilian. 2014. "The ocular
426 surface chemical burns." *J Ophthalmol* 2014:196827. doi: 10.1155/2014/196827.
427 Feroze, K. B., and L. Khazaeni. 2018. "Glaucoma, Secondary Glaucoma, Steroid." In
428 *StatPearls*. Treasure Island (FL).
429 Fish, R., and R. S. Davidson. 2010. "Management of ocular thermal and chemical
430 injuries, including amniotic membrane therapy." *Curr Opin Ophthalmol* 21
431 (4):317-21. doi: 10.1097/ICU.0b013e32833a8da2.
432 Getting, S. J., D. J. Mahoney, T. Cao, M. S. Rugg, E. Fries, C. M. Milner, M. Perretti,
433 and A. J. Day. 2002. "The link module from human TSG-6 inhibits neutrophil

migration in a hyaluronan- and inter-alpha -inhibitor-independent manner." *J Biol Chem* 277 (52):51068-76. doi: 10.1074/jbc.M205121200.

Gupta, N., M. Kalaivani, and R. Tandon. 2011. "Comparison of prognostic value of Roper Hall and Dua classification systems in acute ocular burns." *Br J Ophthalmol* 95 (2):194-8. doi: 10.1136/bjo.2009.173724.

Heng, B. C., P. M. Gribbon, A. J. Day, and T. E. Hardingham. 2008. "Hyaluronan binding to link module of TSG-6 and to G1 domain of aggrecan is differently regulated by pH." *J Biol Chem* 283 (47):32294-301. doi: 10.1074/jbc.M804155200.

Hurst, C.G., Petrali, J.P., Barillo, D.J., Graham, J.S., Smith, W.J., Urbanetti, J.S., and Sidell, F.R. . 2008. "Vesicants." In *Medical Aspects of Chemical Warfare*, edited by Shirley D. ed Tuorinsky, 259-311. The office of the Surgeon General at TMM Publications).

Kilic Muftuoglu, I., Y. Aydin Akova, and A. Cetinkaya. 2015. "Clinical Spectrum and Treatment Approaches in Corneal Burns." *Turk J Ophthalmol* 45 (5):182-187. doi: 10.4274/tjo.99267.

Kota, D. J., L. L. Wiggins, N. Yoon, and R. H. Lee. 2013. "TSG-6 produced by hMSCs delays the onset of autoimmune diabetes by suppressing Th1 development and enhancing tolerogenicity." *Diabetes* 62 (6):2048-58. doi: 10.2337/db12-0931.

Kuckelkorn, R., N. Schrage, G. Keller, and C. Redbrake. 2002. "Emergency treatment of chemical and thermal eye burns." *Acta Ophthalmol Scand* 80 (1):4-10.

Kuo, I. C. 2004. "Corneal wound healing." *Curr Opin Ophthalmol* 15 (4):311-5.

Le, Q., Y. Chen, X. Wang, Y. Li, J. Hong, and J. Xu. 2011. "Vision-related quality of life in patients with ocular chemical burns." *Invest Ophthalmol Vis Sci* 52 (12):8951-6. doi: 10.1167/iovs.11-8355.

Lee, R. H., A. A. Pulin, M. J. Seo, D. J. Kota, J. Ylostalo, B. L. Larson, L. Semprun-Prieto, P. Delafontaine, and D. J. Prockop. 2009. "Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6." *Cell Stem Cell* 5 (1):54-63. doi: 10.1016/j.stem.2009.05.003.

Liao, C. C., and A. M. Rossignol. 2000. "Landmarks in burn prevention." *Burns* 26 (5):422-34.

Milner, C. M., and A. J. Day. 2003. "TSG-6: a multifunctional protein associated with inflammation." *J Cell Sci* 116 (Pt 10):1863-73. doi: 10.1242/jcs.00407.

Milner, C. M., V. A. Higman, and A. J. Day. 2006. "TSG-6: a pluripotent inflammatory mediator?" *Biochem Soc Trans* 34 (Pt 3):446-50. doi: 10.1042/BST0340446.

Mindrescu, C., A. A. Dias, R. J. Olszewski, M. J. Klein, L. F. Reis, and H. G. Wisniewski. 2002. "Reduced susceptibility to collagen-induced arthritis in DBA/1J mice expressing the TSG-6 transgene." *Arthritis Rheum* 46 (9):2453-64. doi: 10.1002/art.10503.

Miri, A., B. Al-Deiri, and H. S. Dua. 2010. "Long-term outcomes of autolimbal and allolimbal transplants." *Ophthalmology* 117 (6):1207-13. doi: 10.1016/j.ophtha.2009.10.028.

Mitsui, Y., and J. Hanabusa. 1955. "Corneal infections after cortisone therapy." *Br J Ophthalmol* 39 (4):244-50.

Oh, J. Y., H. Choi, R. H. Lee, G. W. Roddy, J. H. Ylostalo, E. Wawrousek, and D. J. Prockop. 2012. "Identification of the HSPB4/TLR2/NF-kappaB axis in macrophage as a therapeutic target for sterile inflammation of the cornea." *EMBO Mol Med* 4 (5):435-48. doi: 10.1002/emmm.201200221.

Oh, J. Y., G. W. Roddy, H. Choi, R. H. Lee, J. H. Ylostalo, R. H. Rosa, Jr., and D. J. Prockop. 2010. "Anti-inflammatory protein TSG-6 reduces inflammatory damage to the cornea following chemical and mechanical injury." *Proc Natl Acad Sci U S A* 107 (39):16875-80. doi: 10.1073/pnas.1012451107.

Parkar, A. A., J. D. Kahmann, S. L. Howat, M. T. Bayliss, and A. J. Day. 1998. "TSG-6 interacts with hyaluronan and aggrecan in a pH-dependent manner via a common functional element: implications for its regulation in inflamed cartilage." *FEBS Lett* 428 (3):171-6.

Peate, W. F. 2007. "Work-related eye injuries and illnesses." *Am Fam Physician* 75 (7):1017-22.

Poon, M. W., L. Yan, D. Jiang, P. Qin, H. F. Tse, I. Y. Wong, D. S. Wong, V. Tergaonkar, and Q. Lian. 2015. "Inhibition of RAP1 enhances corneal recovery following alkali injury." *Invest Ophthalmol Vis Sci* 56 (2):711-21. doi: 10.1167/iovs.14-15268.

Rall, D.P., and C.M. Pechura. 1993. "History and analysis of mustard agent and lewisite research programs in the United States." In *Veterans at Risk: The Health Effects of Mustard Gas and Lewisite*, 21-60. National Academies Press.

Roddy, G. W., J. Y. Oh, R. H. Lee, T. J. Bartosh, J. Ylostalo, K. Coble, R. H. Rosa, Jr., and D. J. Prockop. 2011. "Action at a distance: systemically administered adult

stem/progenitor cells (MSCs) reduce inflammatory damage to the cornea without engraftment and primarily by secretion of TNF-alpha stimulated gene/protein 6." *Stem Cells* 29 (10):1572-9. doi: 10.1002/stem.708.

Roper-Hall, M. J. 1965. "Thermal and chemical burns." *Trans Ophthalmol Soc U K* 85:631-53.

Singh, P., M. Tyagi, Y. Kumar, K. K. Gupta, and P. D. Sharma. 2013. "Ocular chemical injuries and their management." *Oman J Ophthalmol* 6 (2):83-6. doi: 10.4103/0974-620X.116624.

Sugar, J., and J. W. Chandler. 1974. "Experimental corneal wound strength." *Arch Ophthalmol* 92 (3):248-9.

Thygeson, P., M. J. Hogan, and S. J. Kimura. 1953. "Cortisone and hydrocortisone in ocular infections." *Trans Am Acad Ophthalmol Otolaryngol* 57 (1):64-85.

Tuft, S. J., and A. J. Shortt. 2009. "Surgical rehabilitation following severe ocular burns." *Eye (Lond)* 23 (10):1966-71. doi: 10.1038/eye.2008.414.

Wagoner, M. D. 1997. "Chemical injuries of the eye: current concepts in pathophysiology and therapy." *Surv Ophthalmol* 41 (4):275-313.

Watanabe, J., A. K. Shetty, B. Hattiangady, D. K. Kim, J. E. Foraker, H. Nishida, and D. J. Prockop. 2013. "Administration of TSG-6 improves memory after traumatic brain injury in mice." *Neurobiol Dis* 59:86-99. doi: 10.1016/j.nbd.2013.06.017.

Willmann, D., and S. W. Melanson. 2018. "Corneal Injury." In *StatPearls*. Treasure Island (FL).

Wisniewski, H. G., and J. Vilcek. 2004. "Cytokine-induced gene expression at the crossroads of innate immunity, inflammation and fertility: TSG-6 and PTX3/TSG-

14." *Cytokine Growth Factor Rev* 15 (2-3):129-46. doi: 10.1016/j.cytogfr.2004.01.005.

Xiang, H., L. Stallones, G. Chen, and G. A. Smith. 2005. "Work-related eye injuries treated in hospital emergency departments in the US." *Am J Ind Med* 48 (1):57-62. doi: 10.1002/ajim.20179.

Figure Legends

Fig.1. Treatment protocol of corneal alkali burn with rhTSG-6.

Fig. 2. Alkali-injured rat cornea treated with intravenous (IV) injection of PBS or rhTSG-6 and harvested at one day after injury. (A) Opacity score with the use of a clinical grading standard system on a scale from 0 to 4. Quantification of the mRNA expression levels of (B) neutrophil Elastase (ELANE), (C) vascular endothelial growth factor-A (VEGF), (D) interleukin (IL)-1β, (E) IL-6, and (F) C-C motif ligand (CCL2) mRNA. mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SD, n=8. *$p<0.05$ compared with the control. Hematoxylin-eosin staining of injured cornea of (G) PBS treated and (H) TSG-6 treated animal. Neutrophil elastase immunohistochemistry of (I) PBS treated (J) and TSG-6 treated injured cornea. 20 x magnification, scale bar 100 μm.

Fig. 3. Alkali-injured rat cornea treated with intravenous (IV) injection of PBS or TSG-6, and harvested 7 days after injury. (A) Photographic representation from days 0 (prior to injury), 1, 3, 5, and 7 after injury for PBS injection group and TSG-6 treatment group. (B) Opacity score of PBS and TSG-6 treated cornea during the injury progression. The expression of (C) IL-1β, (D) EMR1, (E) thrombomodulin (CD141; Thbd), and (F) Collagen type I (Col1a1). mRNA expression levels were measured with real-time qRT-

PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SD, n=8.

Fig. 4. Alkali-injured rat cornea treated with anterior chamber (AC) injection of rhTSG-6 and harvested one day after injury. (A) Opacity score with the use of a clinical grading standard system on a scale from 0 to 4. Quantification of the mRNA expression levels of cornea inflammation related genes (B) ELANE, (C) VEGF, (D) IL-1β, (E) IL-6, and (F) CCL2 mRNA. mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SD, n=8. *$p<0.05$ compared with the control

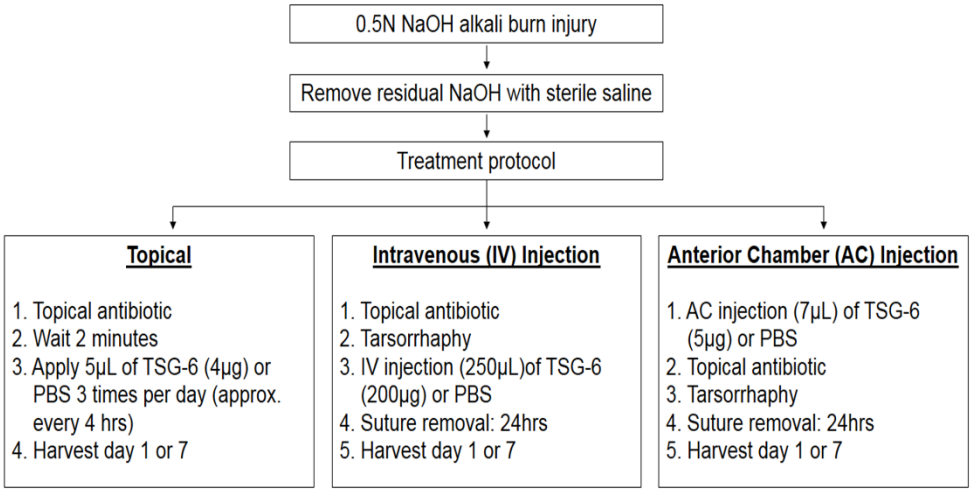
Fig. 5. Alkali-injured rat cornea treated with anterior chamber (AC) injection of PBS or rhTSG-6 and harvested at 7 days after injury. (A) Opacity score of PBS and TSG-6 treated corneas during the injury progression. (B) Col1a1, (C) IL-1β, (D) EMR1, (E) Thbd mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SD, n=8.

Fig. 6. Alkali-injured rat cornea treated with topical application of PBS or rhTSG-6 and harvested one day after injury. (A) Opacity score with the use of a clinical grading standard system on a scale from 0 to 4. Quantification of the mRNA expression levels of cornea inflammation related genes (B) ELANE, (C) VEGF, (D) IL-1β, (E) IL-6, and (F) CCL2 mRNA. mRNA expression levels were measured with real-time qRT-PCR and

were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SD, n=8.

Fig. 7. Alkali-injured rat cornea treated with topical application of PBS or rhTSG-6 and harvested at 7 days after injury. (A) Opacity score of PBS and TSG-6 treated cornea during the injury progression. (B) Col1a1, (C) IL-1β, (D) EMR1, (E) Thbd mRNA expression levels were measured with real-time qRT-PCR and were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results are presented as the mean ± SD, n=8.

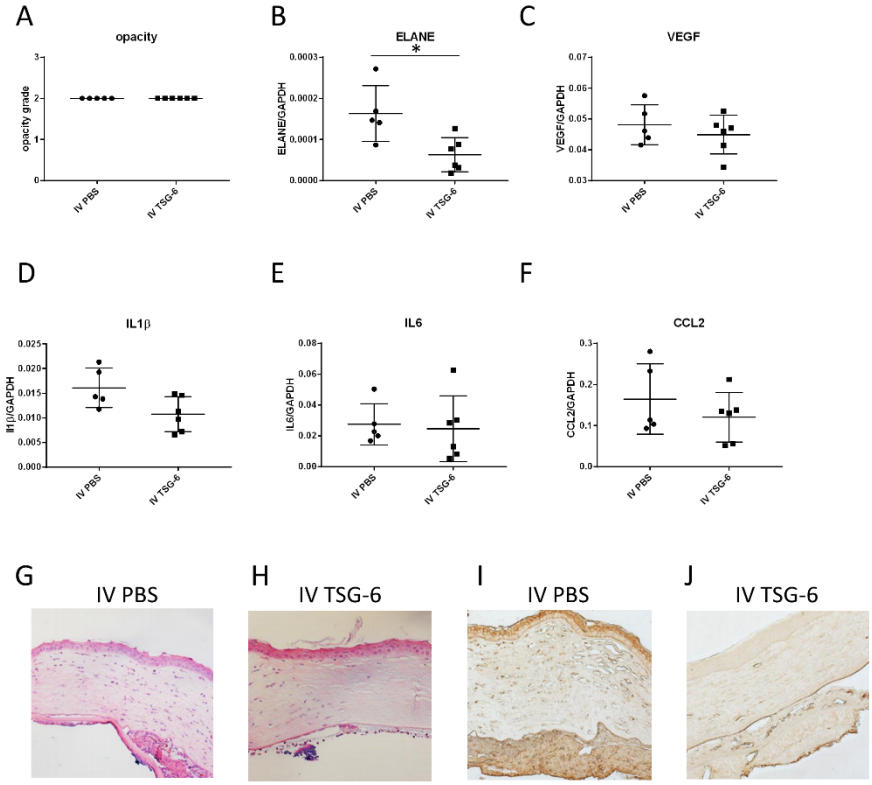
Figure 1



587

588

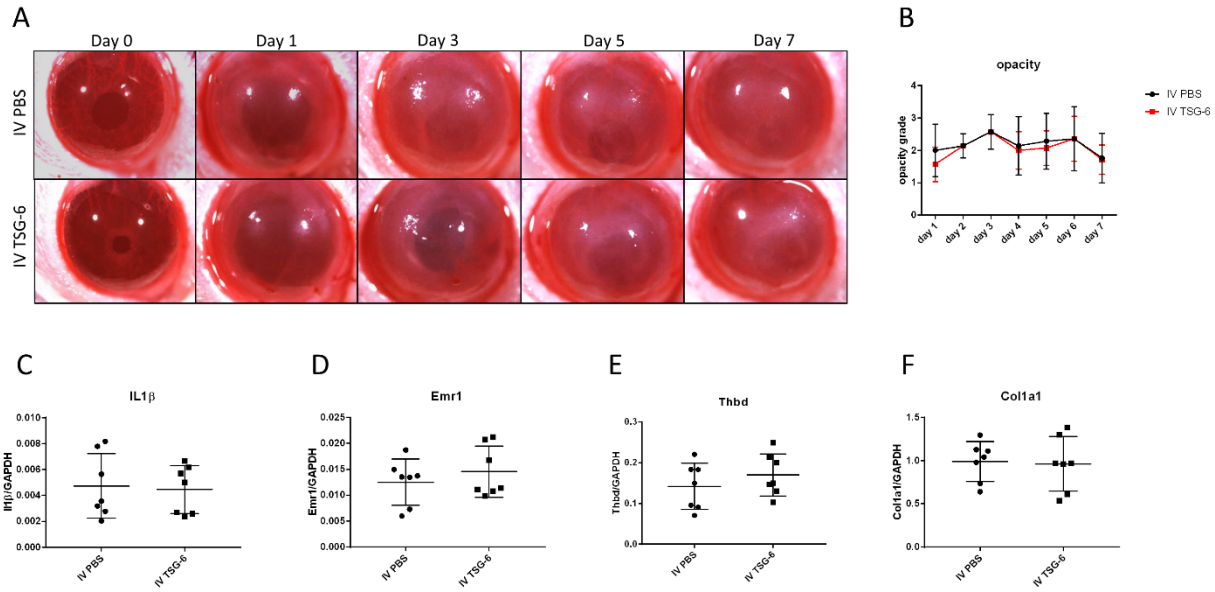
Figure 2



589

590

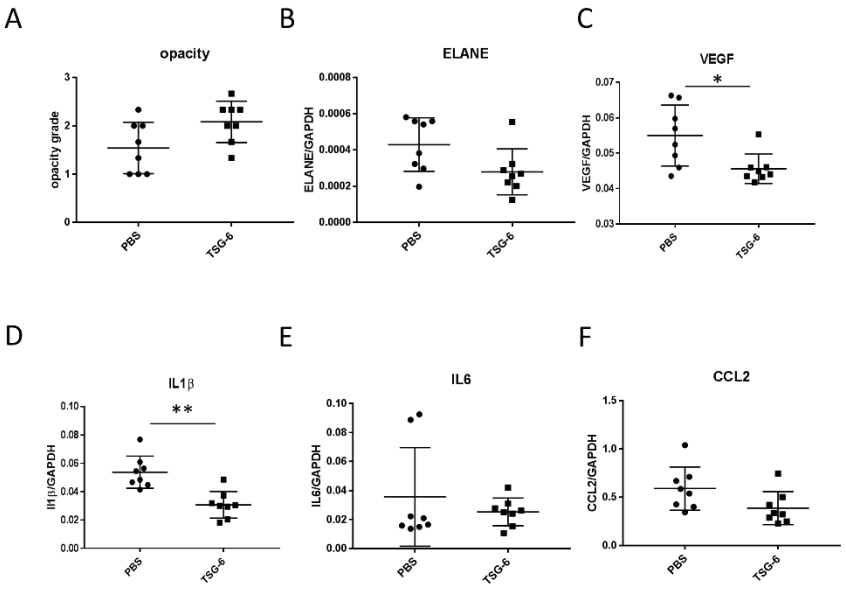
Figure 3



591

592

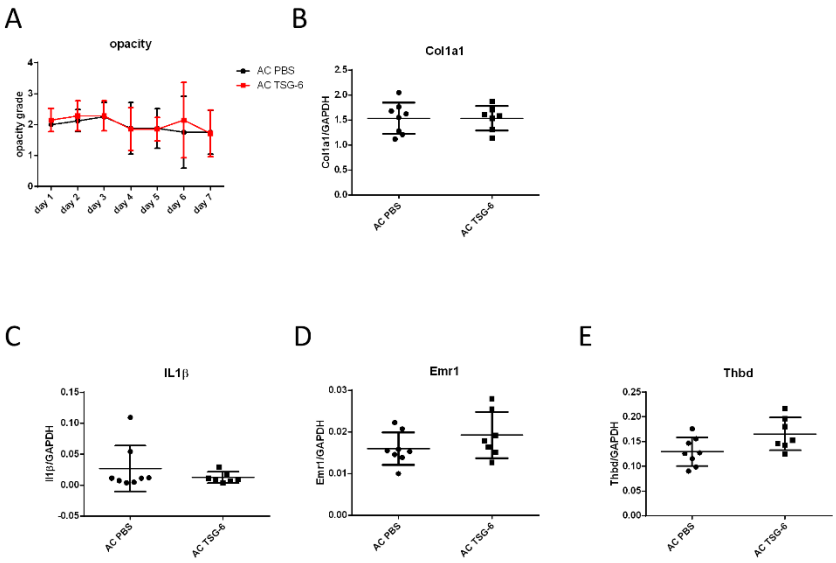
Figure 4



593

594

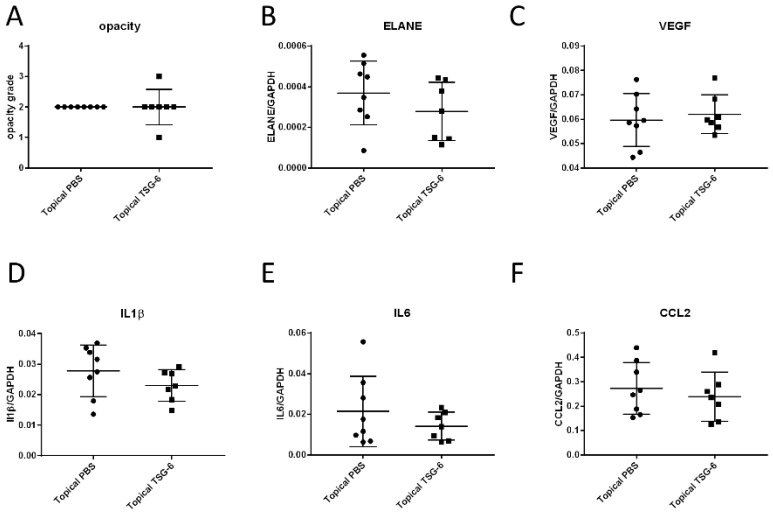
Figure 5



595

596

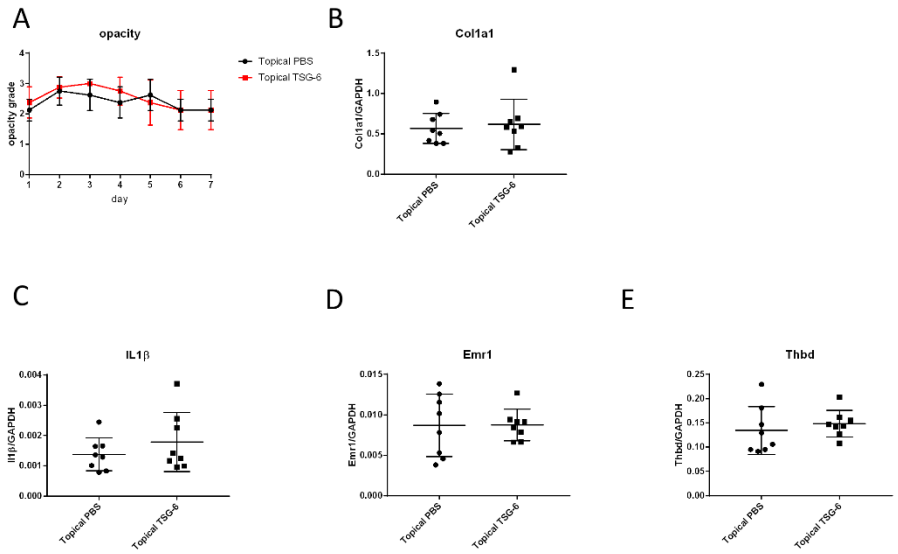
Figure 6



597

598

Figure 7


A
opacity
Topical PBS
Topical TSG-6
opacity grade
day
B
Col1a1
Col1a1/GAPDH
Topical PBS
Topical TSG-6
C
IL1β
Il1β/GAPDH
Topical PBS
Topical TSG-6
D
Emr1
Emr1/GAPDH
Topical PBS
Topical TSG-6
E
Thbd
Thbd/GAPDH
Topical PBS
Topical TSG-6

599

Appendix 10 Final Report

Corneal Injury Model

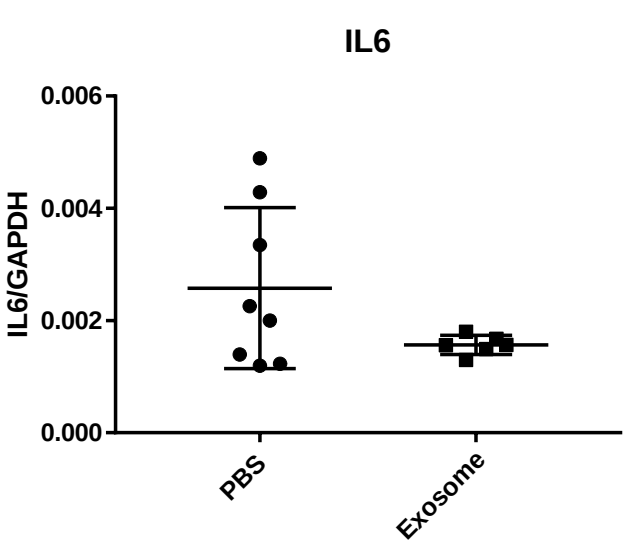
2014-013-R

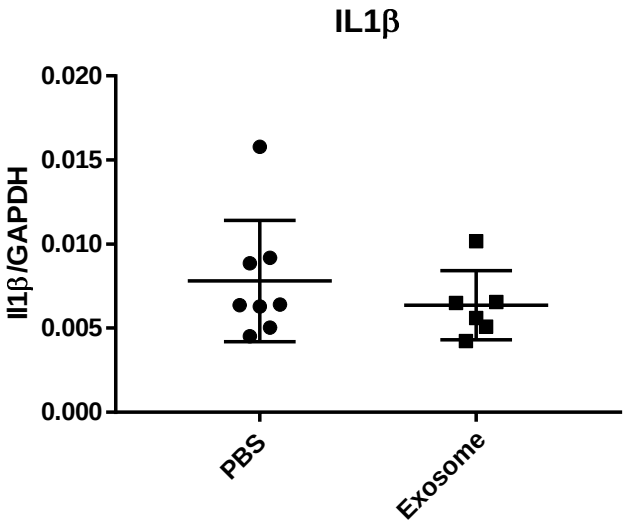
NaOH 0.5 N (30 sec); 50 mL saline flush + 50 mL saline rinse (15 minutes)

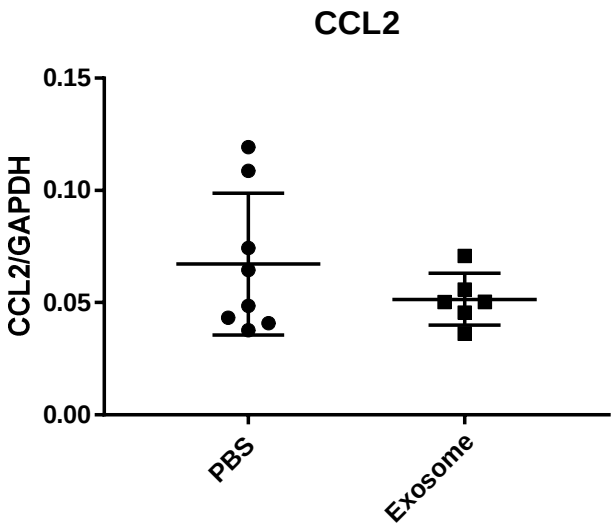
- **Number of animals used:**
  16 Lewis rats

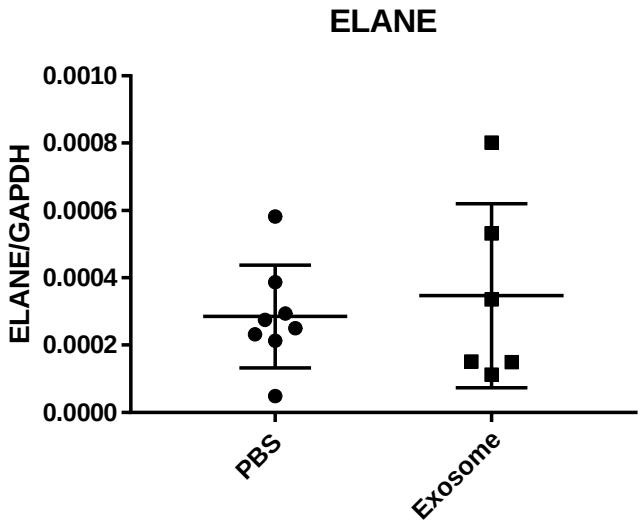
- **AC injection**
  8 rats / PBS (7µl/injection)
  8 rats / exosome (7µl/injection; 5 µg/injection)

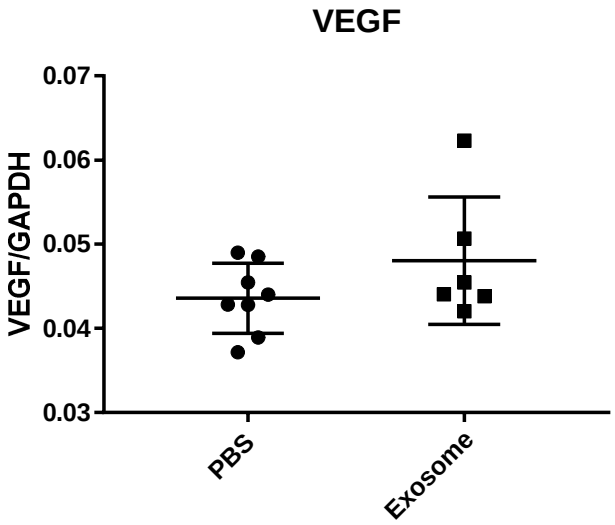
- **Harvest day / number of animals:**
  Day 1 harvest / 16 rats


IL6
IL6/GAPDH
0.006
0.004
0.002
0.000
PBS
Exosome


IL1β
Il1β/GAPDH
0.020
0.015
0.010
0.005
0.000
PBS
Exosome


CCL2
CCL2/GAPDH
0.15
0.10
0.05
0.00
PBS
Exosome


ELANE
ELANE/GAPDH
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
PBS
Exosome


VEGF
VEGF/GAPDH
0.07
0.06
0.05
0.04
0.03
PBS
Exosome

Corneal Injury Model

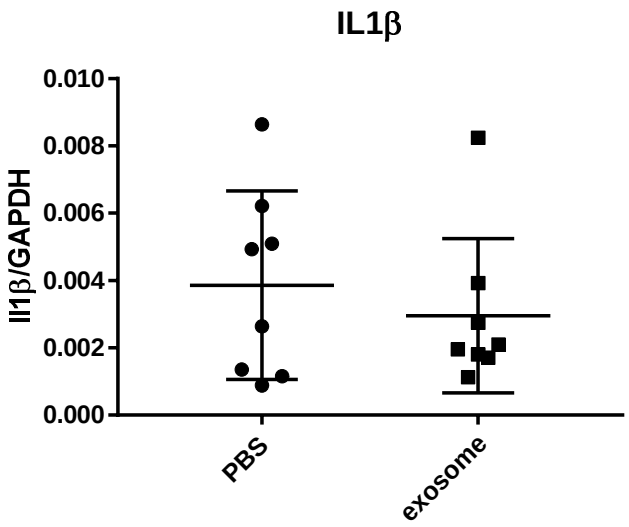
2014-013-R

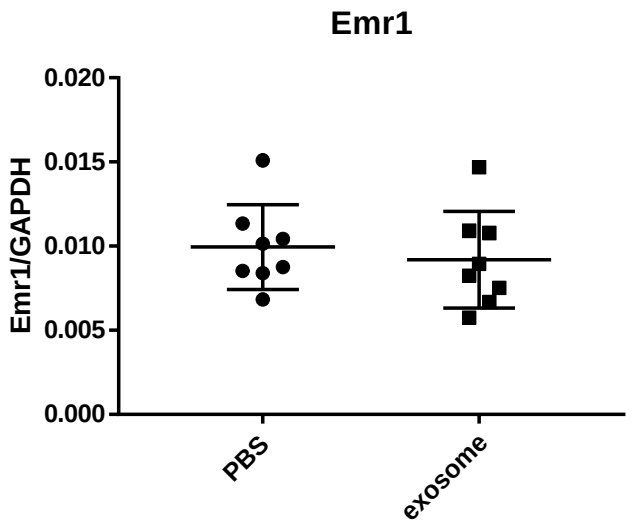
NaOH 0.5 N (30 sec); 50 mL saline flush + 50 mL saline rinse (15 minutes)

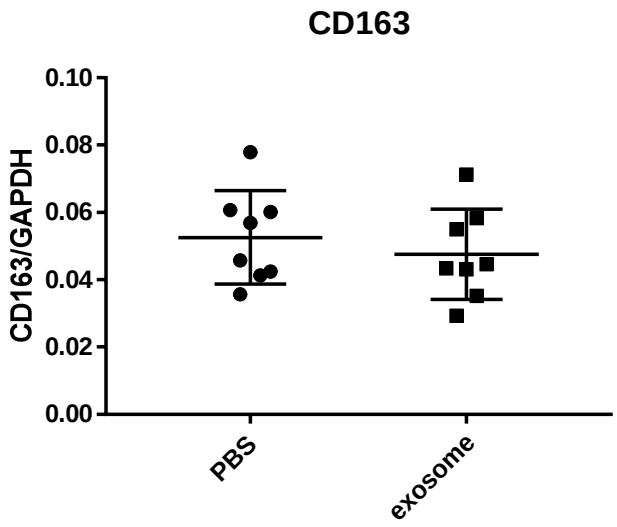
- **Number of animals used:**
  16 Lewis rats

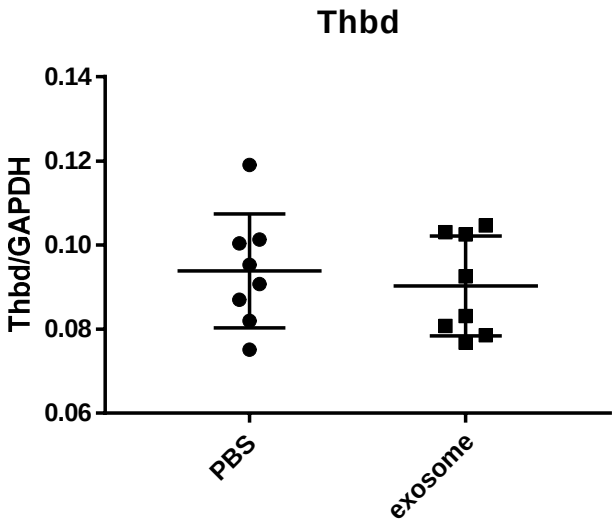
- **AC injection**
  8 rats / PBS (7µl/injection)
  8 rats / exosome (7µl/injection; 5 µg/injection)

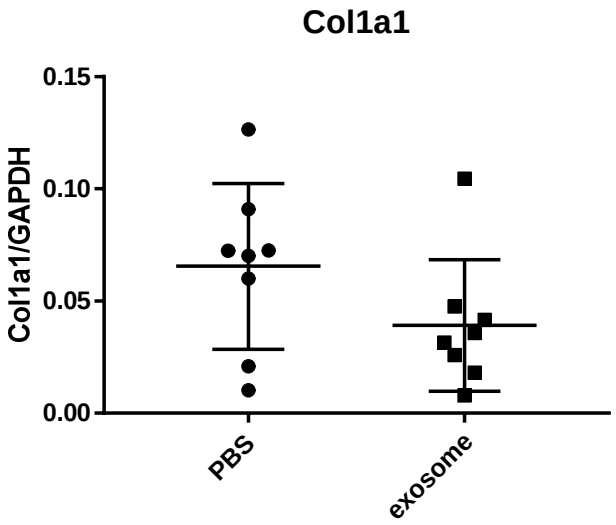
- **Harvest day / number of animals:**
  Day 7 harvest / 16 rats

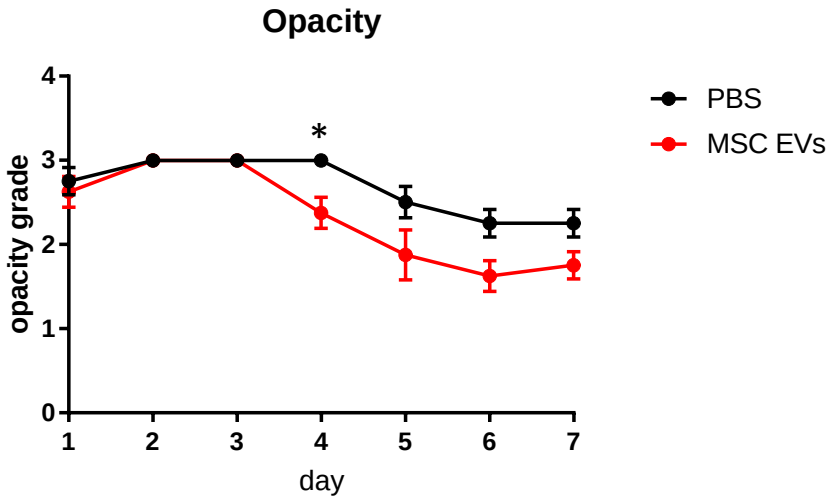

IL1β
Il1β/GAPDH
0.010
0.008
0.006
0.004
0.002
0.000
PBS
exosome


Emr1
Emr1/GAPDH
0.020
0.015
0.010
0.005
0.000
PBS
exosome


CD163
CD163/GAPDH
0.10
0.08
0.06
0.04
0.02
0.00
PBS
exosome


Thbd
Thbd/GAPDH
0.14
0.12
0.10
0.08
0.06
PBS
exosome


Col1a1
Col1a1/GAPDH
0.15
0.10
0.05
0.00
PBS
exosome


Opacity
opacity grade
0
1
2
3
4
1
2
3
4
5
6
7
day
*
PBS
MSC EVs

Appendix 11 Final Report

Corneal Injury Model

2014-013-R

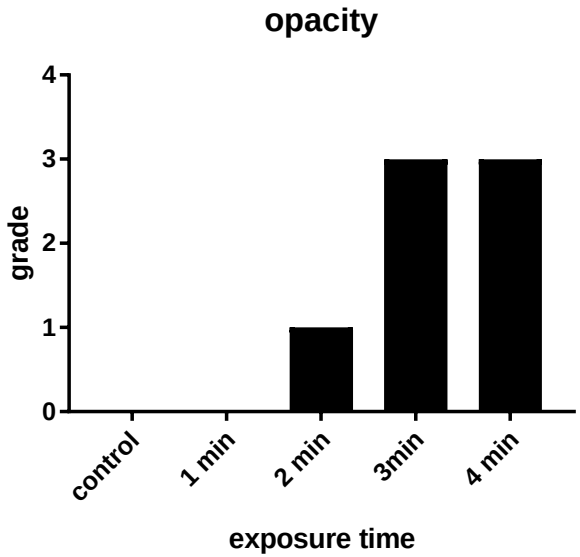
1% Nitrogen Mustard (NM)(90µl/ring)

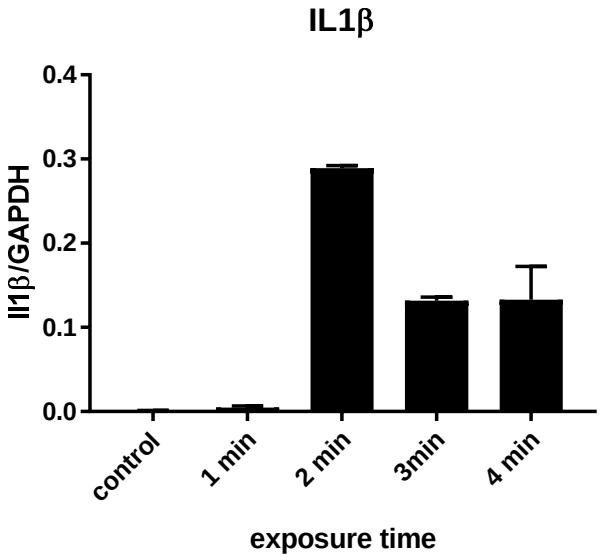
50 mL saline flush + 50 mL saline rinse (15 minutes)

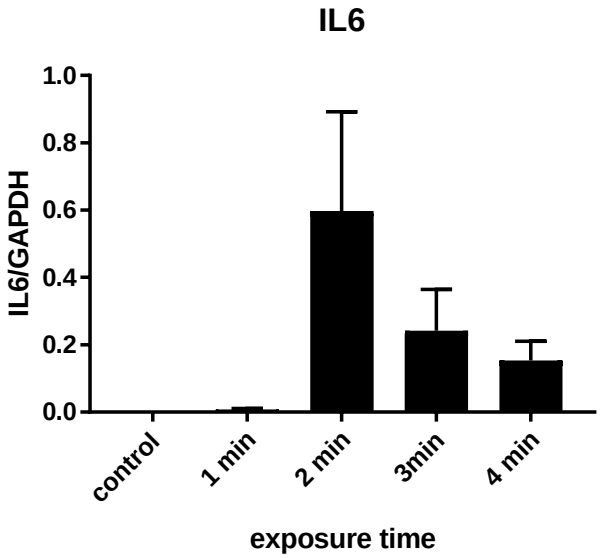
- **Date of study:**
  1-7-18 through 1-9-18

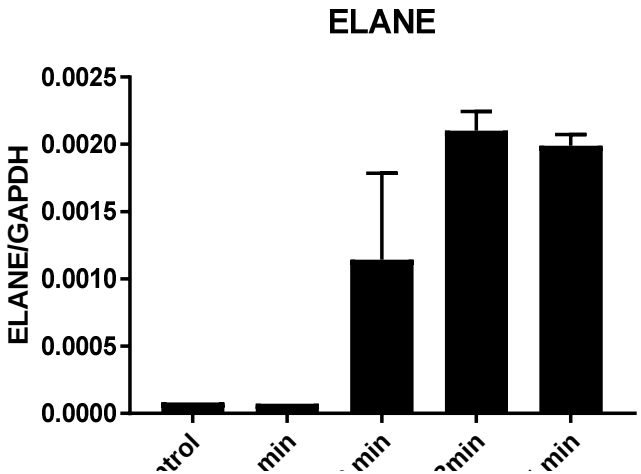
- **Number of animals used:**
  8 Lewis rats

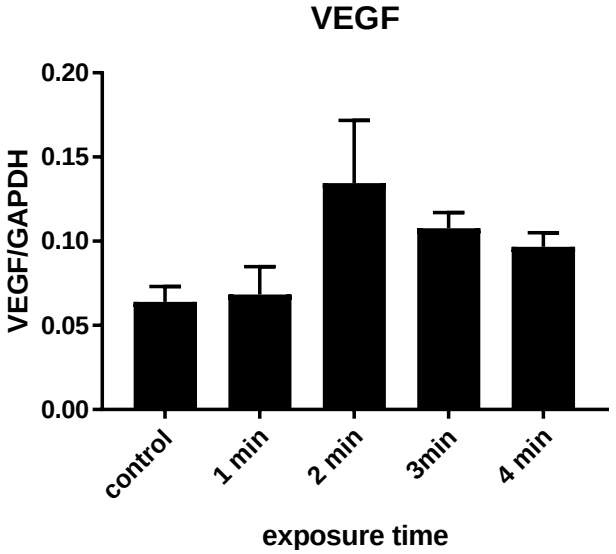
- **NM exposure time:**
  2 rats / 1 min
  2 rats / 2 min
  2 rats / 3 min
  2 rats / 4 min
- **Harvest day / number of animals:**
  Day 2 harvest / 8 rats


opacity
grade
4
3
2
1
0
control
1 min
2 min
3min
4 min
exposure time


IL1β
Il1β/GAPDH
0.4
0.3
0.2
0.1
0.0
control
1 min
2 min
3min
4 min
exposure time


IL6
IL6/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
control
1 min
2 min
3min
4 min
exposure time


ELANE
ELANE/GAPDH
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000


VEGF
VEGF/GAPDH
0.20
0.15
0.10
0.05
0.00
control
1 min
2 min
3min
4 min
exposure time

Corneal Injury Model

2014-013-R

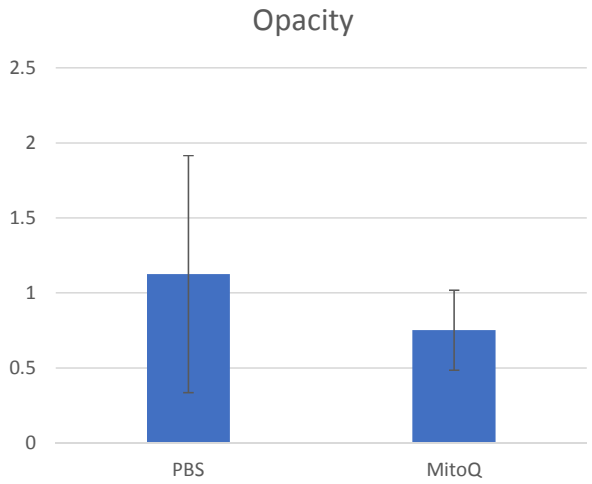
1% Nitrogen Mustard (NM)(90µl/ring); 2 min exposure
50 mL saline flush + 50 mL saline rinse (15 minutes)

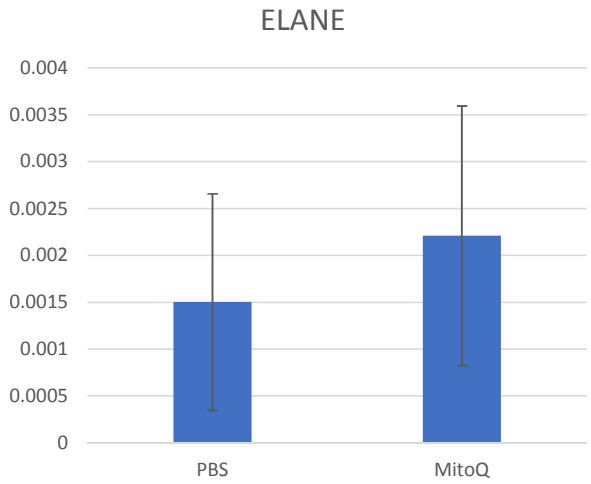
- **Number of animals used:**
  16 Lewis rats

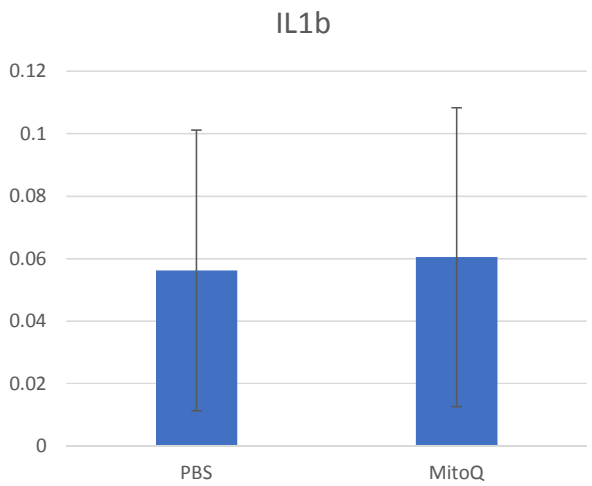
- **Topical (3x/day)**
  8 rats / PBS
  8 rats / MitoQ

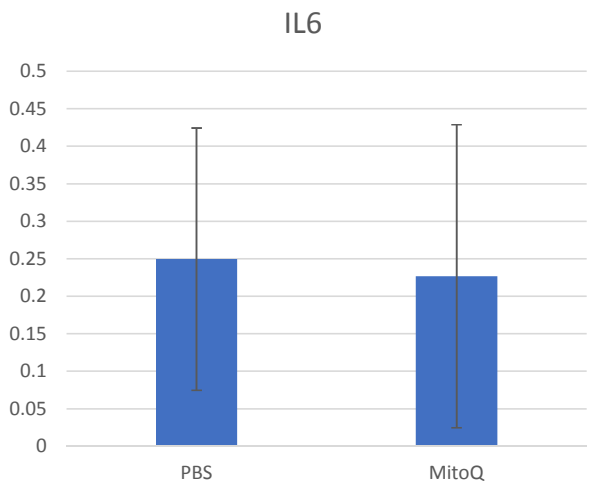
- **Harvest day / number of animals:**
  Day 1 harvest / 16 rats

1% nitrogen mustard 2 min exposure
5 uL of mitoQ solution (0.45 µM)
1 day harvest









Corneal Injury Model

2014-013-R

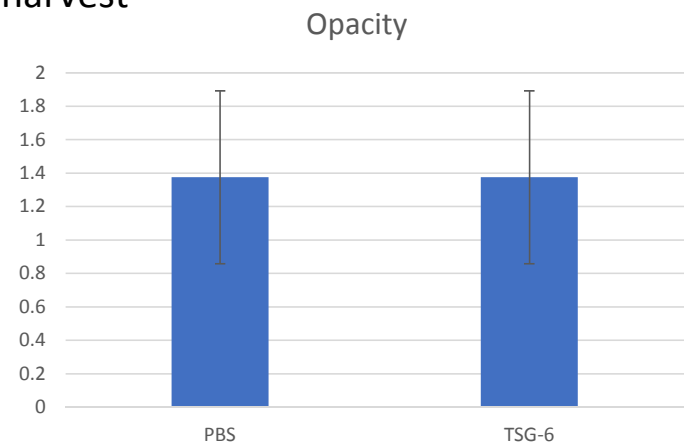
1% Nitrogen Mustard (NM)(90µl/ring); 2 min exposure
50 mL saline flush + 50 mL saline rinse (15 minutes)

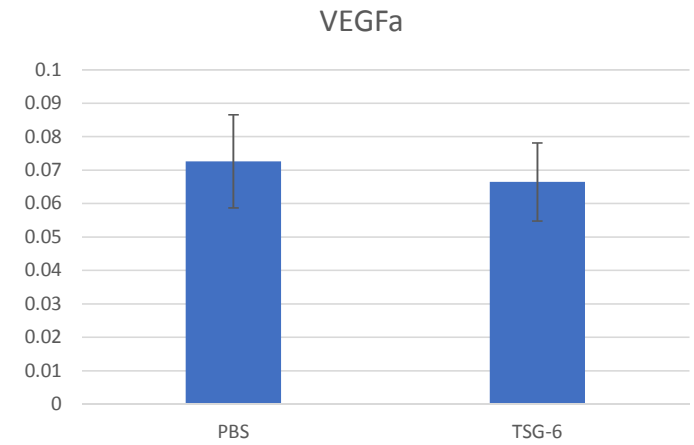
- **Number of animals used:**
  16 Lewis rats

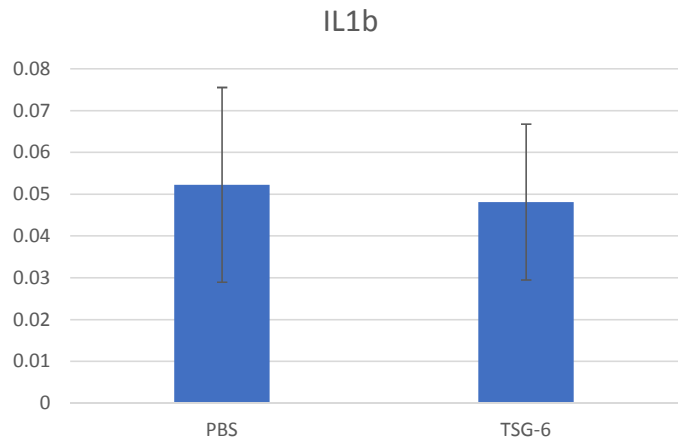
- **Topical (3x/day)**
  8 rats / PBS
  8 rats / TSG-6

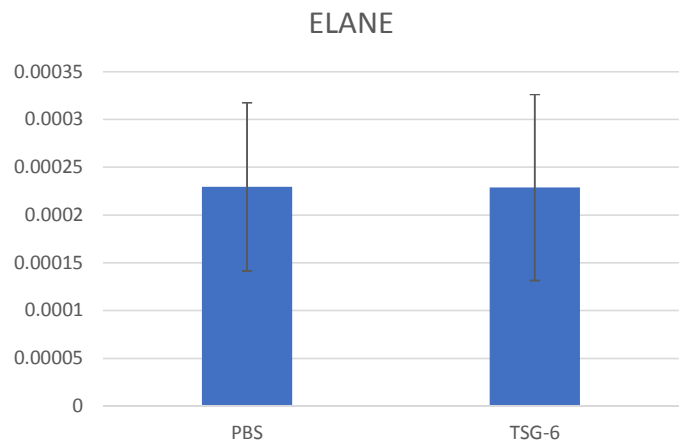
- **Harvest day / number of animals:**
  Day 2 harvest / 16 rats

1% nitrogen mustard 2 min exposure
5 uL of TSG-6 solution (0.795 mg/mL)
2 day harvest





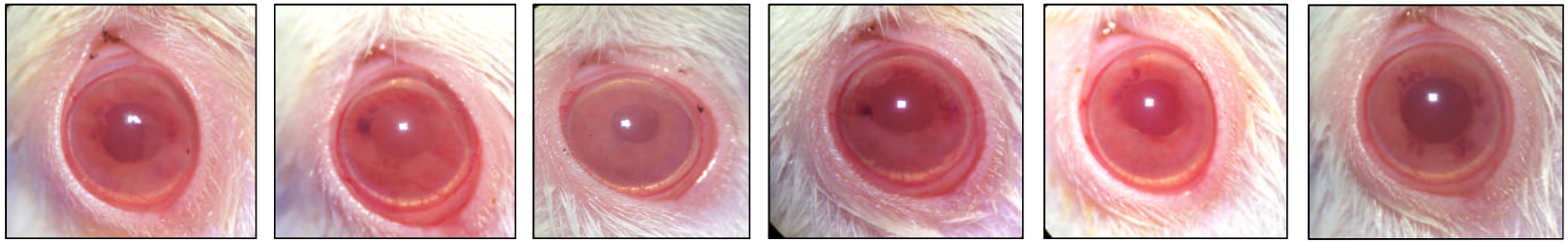




Appendix 12 Final Report

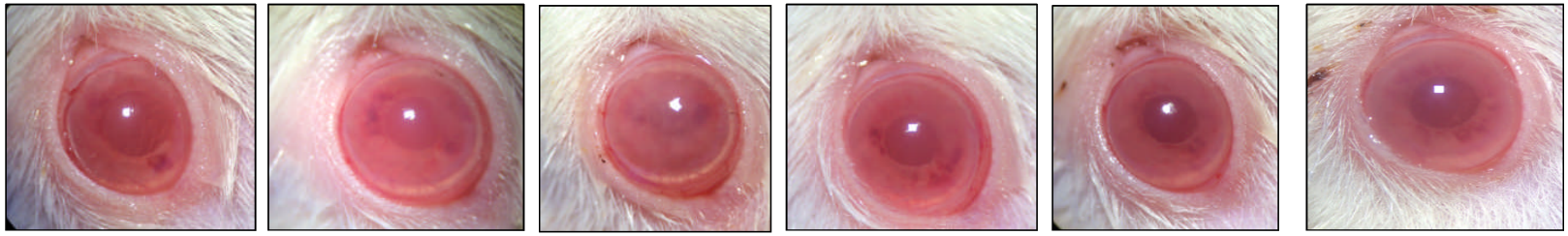
1% Nitrogen Mustard
2 min exposure
Topical PBS/Exosomes
Day 2 harvest

Day 1

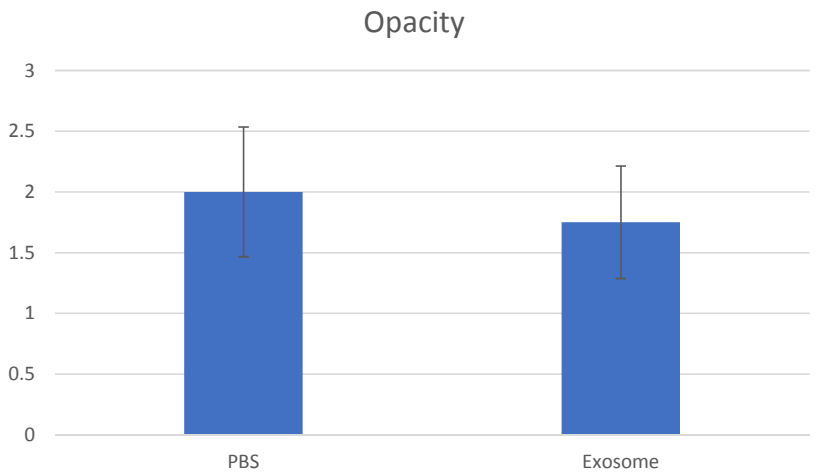


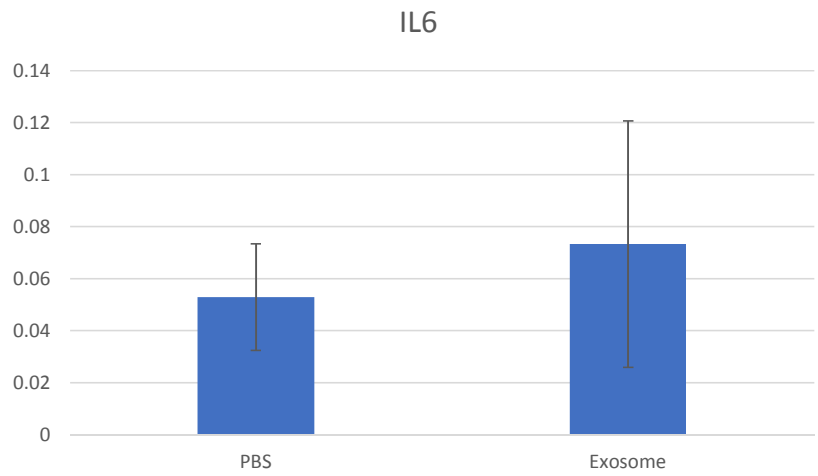
Rat 2 Rat 5 Rat 6 Rat 8 Rat 11 Rat 13

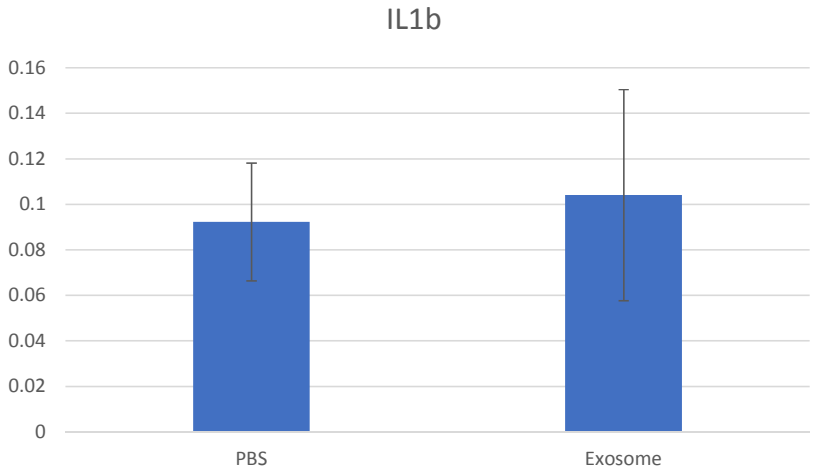
Day 2

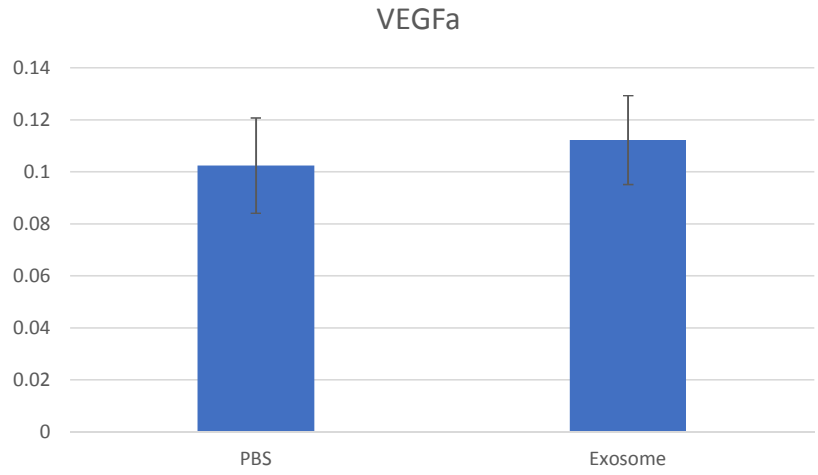


Rat 2 Rat 5 Rat 6 Rat 8 Rat 11 Rat 13


Opacity
3
2.5
2
1.5
1
0.5
0
PBS
Exosome


IL6
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
PBS
Exosome


IL1b
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
PBS
Exosome


VEGFa
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
PBS
Exosome

Appendix 13 Final Report

Inflammation Modulatory Protein TSG-6 for Chemical Injuries to the Cornea

MR130174
W81XWH-14-1-0495

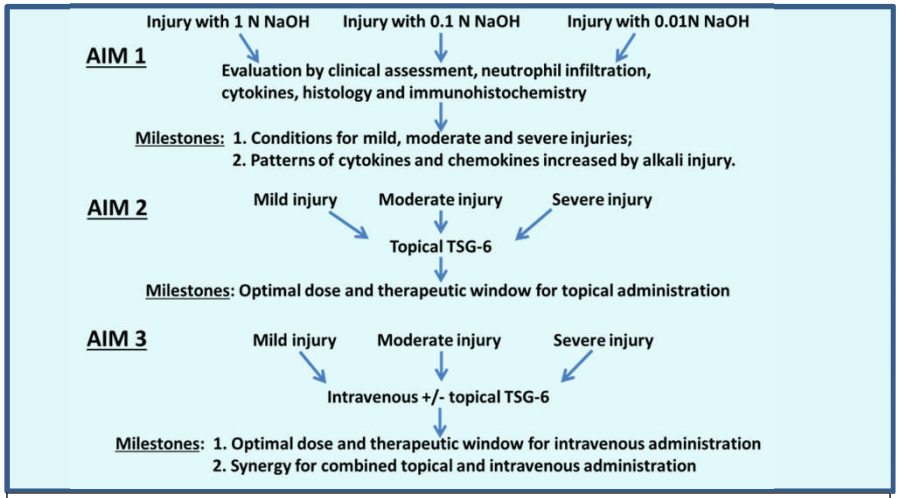
PI: Samuel F.A. Fulcher MD **Org: Central Texas Veterans Foundation** **Award Amount: $985,149**

Study/Product Aim(s)

- **Aim 1.** Determine the timing and patterns of inflammation and other cellular and molecular changes in response to the severity of alkali injury. The data will allow us to select the optimal conditions for evaluating the effectiveness of TSG-6 therapy in Aims 2 and 3.
- **Aim 2.** Establish the optimal dose and the time window for effective topical and anterior chamber administration of TSG-6 therapy as a function of the severity of the alkali injury.
- **Aim 3**. Establish the optimal dose and the time window for effective intravenous administration of TSG-6 as a function of the severity of the alkali injury as well as combined topical and IV administration.

Approach

Expose the corneas of rats to varying concentrations of alkali. Assays of inflammatory markers and clinical grading of injury and healing will be used to assess effectiveness of treatment. The results will establish the limits under which the limbal epithelial stem cells can still be rescued by modulating inflammation with TSG-6.



Outline of Proposed Project

Timeline and Cost

Activities CY	14	15	16	17
Aim 1.Establish appropriate conditions for testing TSG-6.				
Aim 2. Optimal dose and time window for topical, AC TSG-6.				
Aim 3. Optimal dose and time window for intravenous and topical + IV TSG-6.				
Estimated Budget ($K)	**$298**	**$356**	**$331**	**$985**

Updated: (09/18/2018)

Goals/Milestones (Example)

CY14 Goal – Establish the appropriate conditions for testing TSG-6

☑Determine timing and patterns of cellular and cytokine inflammatory responses as a function of alkali injury severity.

CY15 Goals – Optimize treatment parameters for topical TSG-6.

☑ Optimize topical dose of TSG-6

☑ Determine time window for topical therapy

CY16 Goal – Optimize treatment parameters for intravenous TSG-6.

☑ Optimize intravenous dose of TSG-6

☑ Determine time window for intravenous therapy

☑ Determine synergistic effects of combined topical and intravenous TSG-6.

Comments/Challenges/Issues/Concerns

- Aims 1,2,3 completed. Amended Aims 4,5 completed 30/6/2018.

Budget Expenditure to Date

Projected Expenditure: $985,184

Actual Expenditure: $985,184 (as of 30/6/2018)