UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO

Cíntia S. De Paiva

Dry eye worsens the outcome of corneal alkali burns

RIBEIRÃO PRETO

2015

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Tese apresentada à Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo para obtenção do título de Doutor em Ciências Médicas.
Área de Concentração: Mecanismos Fisiopatológicos nos Sistemas Visual e Áudio-Vestibular.

Orientador: Prof. Dr. Eduardo Melani Rocha

RIBEIRÃO PRETO
2015

Catalogação da Publicação
Faculdade de Medicina de Ribeirão Preto - USP

FICHA CATALOGRÁFICA

De Paiva, Cintia Sade

Dry eye worsens the outcome of corneal alkali burns [Olho seco agrava o desfecho da queimadura alcalina da córnea]./Cintia S. De Paiva; orientador Eduardo M Rocha- Ribeirão Preto, 2015.

116p.

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Titulo em Português: Olho seco agrava o desfecho da queimadura alcalina da córnea

1.Alkali burn. 2. Dry eye. 3. Matrix metalloproteinases 4. Neutrophils. 5. Cytokines 6.Animal models 7. Keratitis

Nome: De Paiva, Cintia S.

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Dedicatórias/Special acknowledgements

A Faculdade de Medicina de Ribeirão Preto, pela oportunidade de cursar o doutorado;

Ao Departamento de Oftalmologia, Otorrinolaringologia e Cirurgia de Cabeça e Pescoço da FMRP for ter me dado esta oportunidade de avançar na minha carreira;

A Maria Cecília, pela imensa paciência e múltiplas orientações como todos os formulários, inclusive por ter me ajudado a achar o meu CPF que andava sumido há 11 anos;

A minha primeira professora de iniciação científica Profa. Dra. Maria do Carmo Alberta, por ter me ensinado a ter paciência com metodologia, a me ensinar a pensar como cientista e por me iniciar nesta carreira que hoje sigo e adoro;

A Baltazar Pereira de Paula, por ter sido o primeiro a me ensinar o cuidado com nossos animais;

A minha amiga Flávia Pelegrino que veio ficar comigo nos Estados Unidos por dois anos e que inicialmente trabalhou neste projeto;

Aos meus amigos Lara Dias, Luis Fernando Nominato por dividirem comigo as aulas obrigatórias mas também muitas risadas e dicas como navegar a pós-graduação;

A Carolina Maria Módulo, Larissa Ferreira e Ana Carolina Dias Siqueira pelas inúmeras trocas de protocolos e dicas de trabalho;

To Bill Farley, who has worked with me for the past 14 years, for teaching me to understand a Texan accent and for trying really hard to understand a Brazilian accent; you have been here for me since day one;

To Ruthie McNeill, who has made my life easier by providing a loving and caring environment and teaching me what friendship is really about;

To Mahira Zaheer and Kevin, for all the mouse work and technical assistance, but also for the friendship and laughs throughout the years;

To Fang Bian who is tireless worker and wonderful post-doctoral fellow;

To De-Quan Li, a wonderful scientist and mentor, who taught me that the only way of not repeating an experiment is to get it right at the first time; and to get it right the first time takes experience and planning;

To Stephen Pflugfelder, my dearest mentor and now colleague, who took me in when I had no experience at all but was willing to work hard and learn fast; who has taught me how much fun science can be and there are still a million of questions left unanswered;

Ao meu orientador Eduardo Melani Rocha, a quem eu conheço desde a época da faculdade; por ter aceito ser meu orientador nesta jornada e pela paciência com as minhas eternas perguntas; pelas trocas científicas e tudo mais;

Aos meus pais Celina e Guilherme, por estarem sempre do meu lado, mesmo quando tomo decisões dificíes e imprevisíveis; pelo apoio diário e irrestrito, pela companhia constante nas minhas alegrias e tristezas e também alegrias e sucessos;

Aos meus irmãos Karen e William, que estão comigo desde que eu entendo que o mundo é mundo e que fazem parte da pessoa que sou hoje;

Ao meu marido Ricardo Fujisaki, que caminha comigo diariamente, que me apoia e divide comigo a criação de nosso filhos e que está sempre presente mesmo quando eu não me sinto muito animada, que não me deixa desistir de nada e que sempre tem um sorriso pra mim; sem seu amor eu sou imperfeita;

Aos meus filhos, Lucas e Eric, por me ensinarem a verdadeira expressão do amor e me ajudar a enxergar o mundo com olhos confiantes e inocentes e otimistas com o futuro; sem vocês eu continuo imperfeita;

To everyone else that I cannot remember now but has contributed to my success and to what I am now.

A big, enormous, Thank you!

“Learning never exhausts the mind.”

Leonardo da Vinci

A person who never made a mistake never tried anything new.”

Albert Einstein

Resumo

de PAIVA, C.S. **Olho seco agrava o desfecho da queimadura alcalina da córnea.** 116f. 2015. Tese (Doutorado). Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo. Ribeirão Preto. 2015.

Introdução: As queimaduras alcalinas da córnea fazem parte dos ferimentos mais devastadores para o olho. **Objetivo:** Os objetivos foram investigar o efeito aditivo do olho seco na atividade de proteases na superfície ocular e complicações corneanas apos lesão ocular alcalina e também investigar a eficácia da terapia anti-inflamatória controlar este processo. **Métodos:** Um modelo combinado (CM) de olho seco e queimadura alcalina unilateral foi usada. Resumidamente, camundongos C57BL /6 foram submetidos à queimadura alcalina unilateral (AB) com ou sem olho seco concomitante por 2 ou 5 dias. Um grupo separado de animais foram submetidos a ambos modelos (AB e olho seco) foram tratados topicamente com a Dexametasona, ou Doxiciclina ou colírio controle de solução salina balanceada (BSS). Os camundongos foram observados diariamente para verificar o aparecimento de perfuração da córnea. Córneas inteiras foram colhidas e homogeneizadas para extração de RNA. PCR quantitativo em tempo real foi realizada para medir a expressão de citocinas inflamatórias, metaloproteinases de matriz (MMP). Ativdade da MMP-9, atividade da gelatinase e da atividade da mieloperoxidase (MPO) foram avaliados em córneas homogeneizadas. A presença da infiltração de neutrófilos foi avaliada por imunohistoquímica e citometria de fluxo. **Resultados**: Os olhos submetidos ao modelo combinado de AB e olho seco (CM) tiveram 20% de taxa de perfuração estéril da córnea 1 dia após a lesão inicial; que aumentou para 35% em 5 dias. Houve um atraso no fechamento da ferida e aumento de opacidade residual da córnea. Aumento dos níveis de IL-1β, IL-6, e as MMPs 1, -3, -8, -9, 13 e CXCL1, foram encontrados após 2 dias no CM comparando com córneas AB. Um aumento da imunorreatividade da MMP-1, -3, -9 e -13 e atividade gelatinolítica da MMP-9 foram observadas em comparação com córneas do grupo CM comparado com AB. O aumento da infiltração de neutrófilos e a atividade da mieloperoxidase foi observado no grupo CM comparando-se com córneas do grupo AB após dois dias da lesão inicial. Não foram observadas perfurações nas córneas tratadas com Dexametasona. Nos olhos tratados com Doxiciclina, 100% do fechamento da ferida pós-lesão no dia 2 e pontuação significantemente menor na escala de opacidade da córnea em relação ao BSS também foram observadas nos dias 4 e 5. Córneas tratadas com Dexametasona apresentaram a menor pontuação de opacidade da córnea. Tratamento com Dexametasona diminuiu significativamente os níveis de mRNA da IL-1β, IL-6, e MMP-1, -9, -13, e o TIMP-1 depois de 2 dias, e aumentou os níveis de MMP-8, enquanto que o tratamento com Doxiciclina diminuiu significativamente IL-1β, IL-6, MMP-8, -9 e, em comparação com córneas tratadas com BSS. A diminuição da imunorreatividade da MMP-1, -9 e -13 e atividade gelatinolítica foram vistos em córneas tratadas com Doxiciclina e Dexametasona em comparação com o veículo BSS. O aumento da infiltração de neutrófilos e a atividade da mieloperoxidase foi observado no grupo BSS comparação com o grupo Dexametasona 2 dias pós-lesão. **Conclusões:** O olho seco ambiental piora o resultado da queimadura ocular alcalina, criando uma tempestade de citocinas e proteases, aumentando o risco de perfuração corneana. Entretanto, o tratamento inicial com terapia anti-inflamatória é muito eficaz na preservação da transparência corneana e facilita a cicatrização de feridas, enquanto controla a produção de MMP e a migração de neutrófilos.

Palavras-chave: Queimadura alcalina, Olho seco, Metaloproteinases de matriz, Neutrófilos, Citocinas, Modelo animal, Ceratite.

Abstract

de PAIVA, C.S. **Dry eye worsens the outcome of corneal alkali burns.** 116f. 2015. Thesis (Doctoral). Ribeirão Preto Medical School, University of São Paulo. Ribeirão Preto. 2015.

Introduction: Alkali burns to the cornea are among the most devastating injuries to the eye. **Purpose:** To evaluate the effects of dry eye on ocular surface protease activity and sight threatening corneal complications following ocular surface chemical injury and also to investigate the efficacy of anti-inflammatory therapy controlling this. **Methods:** A combined model (CM) of unilateral alkali burn and dry eye was used. Briefly, C57BL/6 mice were subjected to unilateral alkali burn (AB) with or without concomitant dry eye for 2 or 5 days. A separate group of mice subjected to both AB and dry eye were topically treated with Dexamethasone (Dex), Doxycycline (Doxy) or saline control (BSS). Mice were observed daily for appearance of corneal perforation. Whole corneas were harvested and lysed for RNA extraction. Quantitative real time PCR was performed to measure expression of inflammation cytokines, matrix metalloproteinases (MMP). MMP-9 activity, gelatinase activity and myeloperoxidase (MPO) activity were evaluated in corneal lysates. Presence of infiltrating neutrophils was evaluated by immunohistochemistry and flow cytometry. **Results:** Eyes subjected to the combined model of AB and dry eye (CM) had 20% sterile corneal perforation rate as soon as 1 day after the initial injury, which increased to 35% by 5 days, delayed wound closure and increased corneal opacity. Increased levels of IL-1β, IL-6, and MMPs 1,-3,-8,-9, 13, and CXCL1 transcripts were found after 2 days in CM compared to AB corneas. Increased MMP-1, -3,-9 and -13 immunoreactivity and gelatinolytic activity were seen in CM corneas compared to AB. Increased neutrophil infiltration and MPO activity was noted in the CM group compared to AB 2 days post injury. No perforations were observed in the Dex treated corneas. Doxy treated eyes had 100% of wound closure 2D post-injury, and significant lower corneal opacity scores at days 4 and 5 compared to BSS. Dex-treated corneas showed the lowest corneal opacity score. Dex treatment significantly decreased mRNA levels of IL-1β, IL-6, and MMPs -1,-9, -13, and TIMP-1 after 2 days with increased levels of MMP-8, while Doxy treatment significantly decreased IL-1β, IL-6, MMP-8, and -9, compared to BSS-treated corneas. Decreased MMPs -1,-9 and -13 immunoreactivity and gelatinolytic activity were seen in corneas treated with Doxy and Dex compared to BSS vehicle. Increased neutrophil infiltration and MPO activity was noted in the BSS group compared to Dex 2 D post-injury. **Conclusions:** Desiccating stress worsens outcome of ocular alkali burn, creating a cytokine and protease storm with greater neutrophil infiltration, increasing the risk of corneal perforation. However, early treatment with anti-inflammatory therapy is very efficacious in preserving corneal clarity and facilitating wound healing, while controlling MMP production and migration of neutrophils.

Keywords: Alkali burn, Dry eye, Matrix metalloproteinases, Neutrophils, Cytokines, Animal models, Keratitis.

Support

Support: Supported by W81XWH-12-1-0616 (CSDP), NIH Training Grant T32-AI053831 (FB), NEI/NIH Core Grant EY-002520, RPB Research to Prevent Blindness, NIH funding to Cytometry and Cell Sorting Core at Baylor College of Medicine (NIAID P30AI036211, NCI P30CA125123, and NCRR S10RR024574), The Oshman Foundation, William Stamps Farish Fund and The Hamill Foundation.

Presented in part as abstracts at the annual meeting of the Association for Research in Vision and Ophthalmology, 2011, 2013 and 2014.

Abbreviations and symbols

^{O} C	Grau Celsius.
%	por cento.
Et al.	*Et alia* (e outros).
AB	alkali burn
APC	Allophycocyanin dye
BID	bis in die, i.e., twice a day
BSS	balanced salt solution
CM	combined model (AB+DS)
D	days
Dex	Dexamethasone
Doxy	Doxycycline
DS	Desiccating stress
FITC	Fluorescein isothiocyanate dye
IL	Interleukin
ICU	intensive care unit
MMP	matrix metalloproteinase
M-MuLV	Moloney murine leukemia virus
MPO	myeloperoxidase
NGAL	neutrophil gelatinase associated lipocalin
PE	R-phycoerythrin dye
PMN	polymorphonuclear
QID	quater in die, i.e., 4 times a day
UT	untreated

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SUMÁRIO

Introduction

Chemical/thermal injuries to the eye are potentially blinding conditions. Barely detected in an unwounded cornea, matrix metalloproteinases (MMPs) are strongly induced during wound healing and chemical burns. The MMP family includes more than 25 members that can be divided into collagenases (MMPs-1,-8,-13, that degrades collagen types I, II, and III); gelatinases (MMP-2 and MMP-9 that degrade collagen types IV , V, VII and X as well as decorin, fibronectin, and laminin); stromelysins (MMP-3 and MMP-10); matrilysins (MMP-7 and MMP-26 that degrade proteoglycans, laminin, and glycoproteins substrates); and the membrane-type MMPs that are bound to epithelial cell membranes, and can activate MMPs, according to their structure and substrate specificity (MMP-14, to-17 and -24) (Johnson *et al.*, 1998; Nagase & Woessner, 1999; Visse & Nagase, 2003; Brejchova *et al.*, 2009). Collectively, these MMPs are able to degrade the entire extracellular matrix and basement membranes components.

Both chemical and thermal injuries to the surface of the eye have a high potential to cause blindness. These injuries often damage the epithelium covering the cornea, conjunctiva, and eyelid margins and in more severe cases destroy the stem cells that renew these epithelia. In many cases, the supporting stromal cells and matrix are damaged and chronic inflammation is induced. Furthermore, most patients with severe ocular surface injuries develop a secondary dry eye due to destruction of tear producing cells, which has the potential to worsen the outcome. In addition to lubricating the ocular surface, the tears contain numerous growth and anti-inflammatory factors that are essential for wound repair and suppressing inflammation and tissue destruction.

The controlled desiccating environment many of us live in is under recognized as an initiator of dry eye-induced ocular surface inflammation. Virtually all modern office buildings are air-conditioned, often with high flow, low humidity air. Low humidity (in offices, aircraft cabins, deserts and winter season) impacts tear film and ocular health (Alex *et al.*, 2013). While it is currently known that low humidity exposure increases tear evaporation rate and increases frequency of eye irritation, (Wolkoff & Kjaergaard, 2007; Wolkoff, 2008), specific inflammatory pathways/mediators that are stimulated are poorly understood. Some authors have proposed exposure to pollution as a cause of dry eye based on clinical studies with individuals exposed to pollution (Alves *et al.*, 2014)

Eye injuries in the military and civilian population are frequent events (Ward & Gorie, 1991; Lau *et al.*, 2000; Ari, 2006; Buglisi *et al.*, 2007). The main causes of eye injuries in the military have changed with changes in strategy and weaponry. These include sharp, blast fragmentation, penetrating and perforating injuries of the globe, occasionally with intraocular foreign bodies and ocular surface chemical, thermal, nuclear and laser burns (Wong *et al.*,

1997; Seet & Wong, 2001). Sulfur mustard gas can potentially cause severe ocular surface injury, including blepharospasm, lid edema and corneal ulceration. This chemical warfare agent has been in several military conflicts, including the recent the Iran-Iraq War (Safarinejad *et al.*, 2001; Kehe *et al.*, 2009). Another frequent cause of chemical burns are accidents related to domestic and commercial use of cleaning products, products used during civil construction and chemical industry.

Therapeutic strategies for ocular surface alkali injuries have been directed towards promoting epithelial healing and suppressing inflammation and tissue destruction, during the acute phase (Reim *et al.*, 2001). Treatment of the chronic phases, including the anatomical sequelae, sometimes require a multi-disciplinary approach and very often, surgical procedures, such as amniotic transplantation or stem cell transplantation (Luengo *et al.*, 2007). Although these approaches have slightly improved visual outcomes, it still remain poor in large part due to inadequate control of inflammatory and proteolytic components of the wound healing response. Among the treatments proposed, several inhibitors of MMPs and inflammation have been used in the past with some success (Fini *et al.*, 1991; Sosne *et al.*, 2002; Kato *et al.*, 2006).

Previous studies have demonstrated a marked anti-collagenolytic effect for the family of tetracycline antibiotics in rat, rabbit, and human corneas, both in vivo and in vitro (Seedor *et al.*, 1987; Li *et al.*, 2001; Li, 2003; Li *et al.*, 2004). Doxycycline (Doxy) was discovered in the early 1960s as a semisynthetic long-acting tetracycline derivative useful as a bacterial ribosome inhibitor in a wide variety of microbes. It is also proven to be effective primary treatment for common ocular surface diseases such as acne rosacea, recurrent corneal erosions, and sterile corneal ulceration (Dursun *et al.*, 2001). Sub-antimicrobial doses have been reported to be effective for treating chronic meibomian gland dysfunction (Yoo *et al.*, 2005), as well as an effective adjunctive treatment for adult periodontitis (Akpek *et al.*, 1997; Caton *et al.*, 2000). Doxy, administered by subconjunctival injections or drops, successfully prevented neutrophil infiltration and promoted healing in corneas subjected to alkali burn from half mustard in an animal model (DeSantis *et al.*, 2008; Lee *et al.*, 2008).

It has been suggested that early systemic and topical administration of corticosteroids after alkali injury may be of benefit by decreasing inflammation, scarring, and neovascularization in the cornea after alkali burn injuries, improving success rates for patients for whom corneal transplantation is their only alternative (Donshik *et al.*, 1978; Mattax & McCulley, 1988; Brodovsky *et al.*, 2000; Saud *et al.*, 2012). However, early reports showing decreased efficacy in deep alkali burns in rabbits has limited its use (Donshik *et al.*, 1978; Kenyon *et al.*, 1979).

Dry eye is comorbidity that markedly worsens the outcome of ocular surface chemical burns by increasing the risk of sterile corneal ulceration. We have previously reported that combining alkali burn with dry eye leads to delayed wound healing, greater corneal opacity, greater production and activity of MMPs, and increased neutrophil infiltration (de Paiva *et al.*, 2011).

Objectives

Objectives

The purpose of this study was twofold.

First, we created a double-hit injury model by subjecting a mouse with unilateral corneal burn to environmental dry eye as soon as anesthetic recovery. We created this model because 1) we wanted to delay corneal re-epithelization, which is very fast in rodents and 2) we wanted a more severe model to simulate the most severe ocular burns that happens in patients with extensive facial burns and in intensive care units. To achieve both objectives, we found that addition to environmental dry eye with is very deleterious to the cornea integrity. We used this combined model to evaluate the effects of experimental dry eye on inflammatory cytokines, chemokines and MMPs (-1, -2, -3, -8, -9, -12 -13) after ocular surface chemical injury and the consequences of increase protease activity on corneal epithelial healing. We also investigated neutrophil infiltration as neutrophils are involved in chemical burns.

Second, we investigated the efficacy of early topical anti-inflammatory therapy in this new model. Separate groups of mice, subjected to CM for 2 and 5 days, were treated bilaterally with Doxy, Dexamethasone (Dex) or vehicle QID. Topical anti-inflammatory therapy was greatly superior to vehicle treatment without causing corneal perforations.

Material and Methods

Material and Methods

Animals

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the standards in the ARVO Statement for the use of animals in Ophthalmic and Vision Research.

Combined model of alkali burn and desiccating stress

After systemic anesthesia with isoflurane using a vaporizer (SomnoSuite, Kent Scientific, Torrington, Connecticut), a unilateral alkali burn (AB) was created on the right eye of 6-8 week old C57BL/6 mice. This was achieved by placing one 2.0 mm diameter filter paper disc that had been pre-soaked with 1N NaOH on the central cornea for 10 seconds, followed by extensive rinsing with balanced salt solution (Alcon, Fort Worth, TX, USA), as previously described (Takahashi *et al.*, 2007). Precautions were taken to avoid damage to the peripheral cornea, conjunctiva, and lids. AB was created at day 0 and animals were sacrificed after 2 or 5 days. A separate group of animals (n=6) were followed for up to 21 days. The contralateral eyes served as untreated controls (UT). A separate group of mice that received unilateral AB were also subjected to desiccating stress (DS) after anesthetic recovery for 2 or 5 days (AB+DS, referred to as the combined model = CM). The contralateral eyes served as desiccating stress controls (not alkali burned, but in a low humidity environment). A schematic is provided in Figure 1.

Desiccating stress was induced in female C57BL/6 mice aged 6-8 weeks by sterile subcutaneous injection of 0.5 mg/1 mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) QID into alternating flanks and exposure to a drafty low humidity (<30% relative humidity) environment for 2 or 5 days (DS, DS2, and DS5 respectively) as previously described (de Paiva *et al.*, 2009). Mice subjected to AB or CM received no eyedrops or antibiotics.

At least forty-five animals without corneal perforations were used per group (AB and CM) and per time point: 12 for histology [6 for frozen sections and 6 for paraffin], 12 for corneal opacity and wound closure, 15 for real-time PCR, 4 for myeloperoxidase assay, 4 for MMP-9 assay, and 10 for flow cytometry. Corneal opacity and wound closure rate were evaluated in 12 live mice that were used for either histology, PCR or flow cytometry. Contralateral eyes in the AB group were used as untreated controls; while, contralateral eyes

in CM group were used as DS controls. Whenever an ocular perforation was observed, mice were euthanized and removed from the study with the exception of the histology.

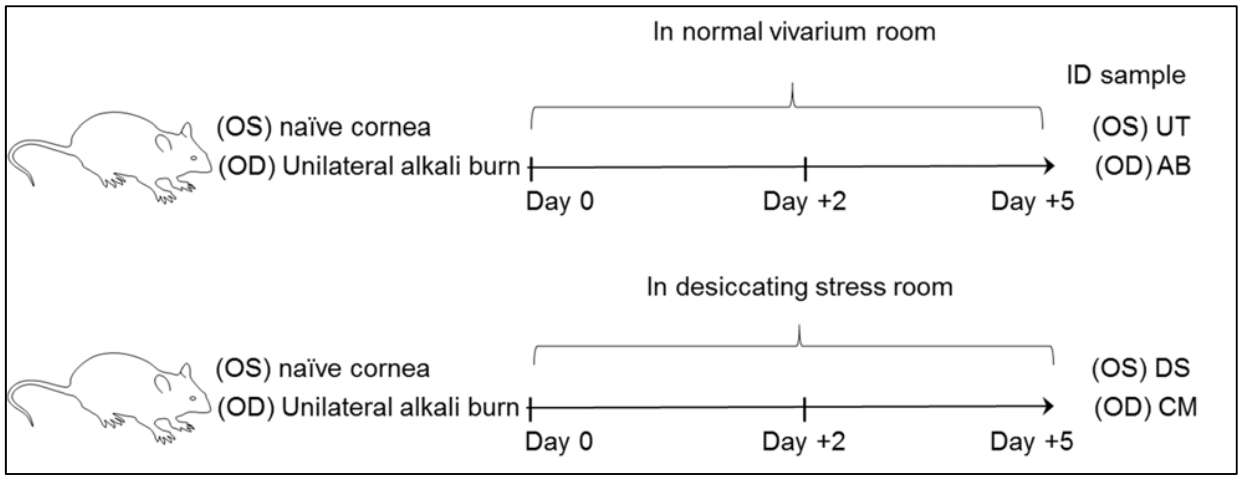

Fig. 1. Schematic of experimental design. A unilateral alkali burn (AB) in the right cornea was performed. Mice were then kept in a normal vivarium room (resulting in AB and untreated (UT) corneas) or subjected to desiccating stress (DS) in a specially designed room (resulting in DS or combined model [CM=AB+DS] corneas).

Topical treatment

Mice subjected to the combined model for 2 and 5 days were topically treated either with 2μL sodium phosphate Dexamethasone (Dex, 0.1%, Spectrum Laboratory, Gardena, CA), 2μL of freshly prepared Doxycycline USP (Doxy, 0.025%,Sigma-Aldrich, St. Louis, MO) or vehicle (balanced salt solution, BSS, Alcon, Fort Worth, TX) QID and compared to naïve control corneas. A schematic of treatment regimen is provided in Figure 2.

At least 32 animals without corneal perforations were used per group (BSS, Doxy, Dex) and per time point: six for histology, fifteen for real-time PCR, four for myeloperoxidase assay, four for MMP-9 assay, three for gelatinase zymography. Corneal opacity and wound closure rate were evaluated in twelve live mice that were used for either histology or PCR. Thirty-two naïve control animals were used.

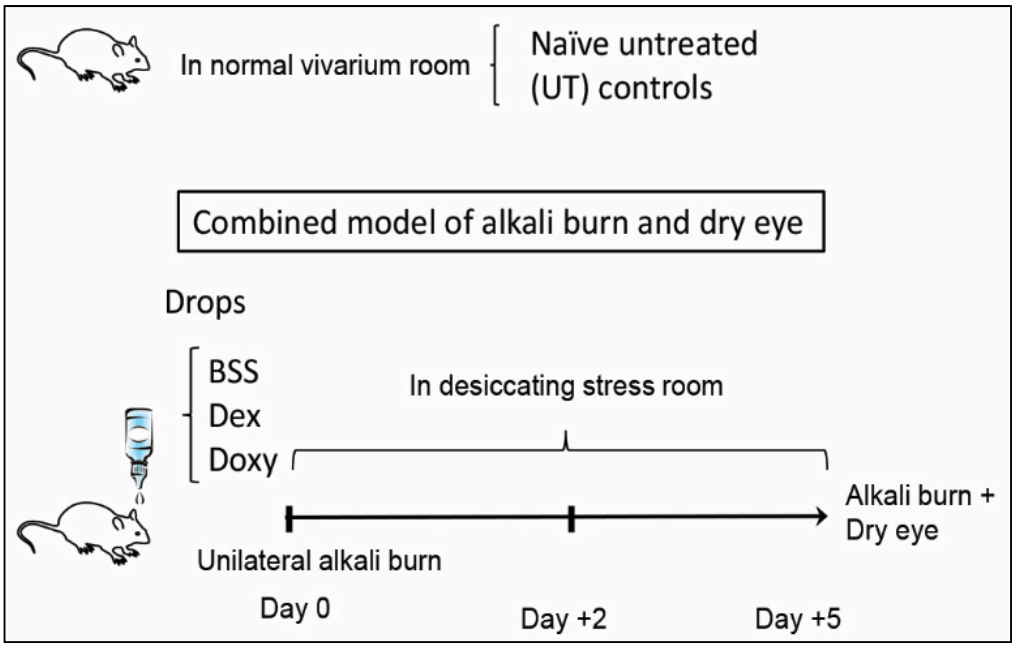

Fig. 2. Schematic of experimental design of the treatment in the combined model of alkali burn and dry eye. A unilateral alkali burn (AB) was created on the right cornea as described in materials and methods. Mice were then subjected to desiccating stress and topically treated with either balanced salt solution (BSS), Dexamethasone (Dex) or Doxycycline (Doxy). Control mice were kept in a normal vivarium room (untreated animals, UT).

Extended dosing after short-term CM

To evaluate if prolonged anti-inflammatory therapy promoted corneal melting, a distinct group of animals (n=6/group) were subjected to the combined model and dosed with either BSS, Doxy or Dex QID for 5 days. On the 6th day, mice were placed in a normal vivarium room with normal environment (Relative humidity >40-60%) and topical treatment continued for another 16 days, for a total of 21 days of topical regimen.

Clinical Findings: Ocular Perforation and Opacity Score

All eyes in each treatment group were examined daily under a microscope (SMZ 1500, Nikon, Melville, New York) for the presence of any corneal perforation. Once corneal perforation was observed, mice were euthanized. Perforated corneas were not used for any experiments other than the histology. The number of corneal perforations occurring each day was recorded and survival curves were calculated using Graph Pad Prism 6.0 software (GraphPad Software Incorporation, San Diego, CA).

Corneal edema and opacity were graded by two masked observers in images taken with a microscope equipped with a color digital camera (DS-Fi1, Nikon, Melville, NY) by the method described by Yoeruek.(Yoeruek *et al.*, 2008) Corneal opacity was scored using a grading scale of 0-4: grade 0=completely clear; grade 1=slightly hazy, iris and pupils easily visible; grade 2= slightly opaque, iris and pupils still detectable; grade 3=opaque, pupils hardly detectable; and grade 4= completely opaque with no view of the pupils.

Measurement of corneal epithelial defect

Corneal epithelial healing was assessed daily in the experimental groups (4 individual right corneas/group/experiment; 3 sets of experiments). Briefly, 1 µL of 0.1% liquid sodium fluorescein was instilled onto the ocular surface. Corneas were rinsed with phosphate-buffered saline and photographed with a stereoscopic zoom microscope (SMZ 1500; Nikon, Melville, NY) under fluorescence excitation at 470 nm (DS-Qi1Mc, Nikon, Melville, NY). Corneal epithelial defects were graded in digital images by two masked observers in a categorical manner (present/absent) to generate a survival curve. Biological replicate scores were transferred to excel database where the results were analyzed.

Ocular cultures

Cornea and conjunctiva of mice that developed corneal perforations were swabbed using sterile cotton swabs and plated on sheep blood agar and transported to the microbiology laboratory at the Methodist Hospital, Houston, Texas, for routine bacterial and fungal cultures to identify any potential microbial infection as cause for the corneal perforation. Culture plates were read each day for the first 2 days and then again at the 7th day after seeding.

Histology and immunostaining

Mice were euthanized either 2 or 5 days after initial injury. Eyes and adnexae (n=6/experimental group/time point) were surgically excised, fixed in 10% formalin, paraffin embedded, and 8-µm tissue sections were cut. These sections were stained with haematoxylin and eosin (H&E) for evaluating morphology and inflammatory signs. They were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon, Melville, NY).

For immunohistochemistry, additional eyes and adnexae from each group/time point (n=6/experimental group/time point) were excised, embedded in optimal cutting temperature compound (VWR, Suwanee, GA), and flash frozen in liquid nitrogen. Sagittal 8μm tissue sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at -80°C. Immunohistochemistry was performed to detect neutrophils using rat anti-Gr-1 antibody (Ly6G; 1:250, clone 1A8, BD Pharmingen). Cryosections were stained with the primary antibody and appropriate biotinylated secondary antibody (1:100 biotin goat α-rat, BD Pharmingen, San Diego, CA) using a Vectastain Elite ABC kit and Nova Red reagent (Vector, Burlingame, CA). Six sections from each animal/group/time point were examined and photographed with microscope equipped with a digital camera (Eclipse E400 with a DS-Qi1Mc; Nikon, Melville, NY, USA). The numbers of Gr-1 positive (+) cells were counted in cornea sections from each animal at 20X magnification and results were averaged and expressed as the number of positive cells per cornea.

Immunofluorescent staining for IL-1β and MMPs was performed in frozen tissue sections with rabbit polyclonal antibody anti-MMP-1 (1:50, NBP1-77209; Novus Biologicals), anti-MMP-9 (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX), anti-IL-1β (1:100, Upstate-Millipore Corp., Bedford, MA), goat anti-MMP-3 and anti-MMP-13 (1:100, SC6839 and SC-123630, respectively, both from Santa Cruz Biotechnology) anti-neutrophil-gelatinase associated lipocalin (1:50, ab63929, Abcam, Cambridge, MA). Secondary goat-anti rabbit or donkey anti-goat Alexa-Fluor 488 conjugated IgG antibodies were used, as previously described.(Corrales *et al.*, 2006) The images were captured and photographed by a Nikon fluorescence microscope (Eclipse E400 equipped with a DS–F1 digital camera; Nikon, Melville, NY).

RNA isolation and quantitative PCR

Four to five whole corneas/group/time point/per experiment (total of 3 independent experiments) were excised, minced and total RNA was extracted using a Qiagen MicroPlus RNeasy isolation® Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, quantified by a NanoDrop® ND-2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at -80°C. First-strand cDNA was synthesized with random hexamers by M-MuLV reverse transcription (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Inc., Arlington Heights, NJ), as previously described (de Paiva *et al.*, 2009).

Real-time polymerase chain reaction (PCR) was performed with specific Taqman MGB probes (Applied Biosystems, Inc., Foster City, CA) and PCR master mix (Taqman

Gene Expression Master Mix), in a commercial thermocycling system (StepOnePlus™ Real-Time PCR System, Applied Biosystems), according to the manufacturer's recommendations. Quantitative real time PCR was performed using gene expression assay primers and MGB probes specific for murine targets as described in Table 1. The hypoxanthine guanine phosphoribosyl transferase (HPRT-1) gene was used as an endogenous reference for each reaction to correct for differences in the amount of total RNA added. The results of quantitative PCR were analyzed by the comparative cycle threshold (C_T) method where target change = $2^{-\Delta\Delta C_T}$. The results were normalized by the C_T value of HPRT-1 and the relative mRNA level in the untreated group was used as the calibrator.

Table 1. Oligonucleotide primers used for real-time PCR.

Gene Name	Symbol	Assay ID*
Interleukin 1 beta	IL1-β	Mm00434228
Interleukin 6	IL-6	Mm99999064
Chemokine (C-X-C motif) ligand 1	CXCL1	Mm04207460
Matrix metalloproteinase 1	MMP-1	Mm00473493
Matrix metalloproteinase 2	MMP-2	Mm00439506
Matrix metalloproteinase 3	MMP-3	Mm00440295
Matrix metalloproteinase 8	MMP-8	Mm00439509
Matrix metalloproteinase 9	MMP-9	Mm00442991
Matrix metalloproteinase 13	MMP-13	Mm00439491
Tissue inhibitor of metalloproteinase-1	TIMP1	Mm00441818
Tissue inhibitor of metalloproteinase-2	TIMP2	Mm00441825
Tissue inhibitor of metalloproteinase-3	TIMP3	Mm00441826
Tissue inhibitor of metalloproteinase-4	TIMP4	Mm00446568
Hypoxanthine guanine phosphoribosyl transferase 1	HPRT-1	Mm00446968

* Identification number from Life Technologies (www.lifetechnologies.com).

Myeloperoxidase assay

Myeloperoxidase (Samtani *et al.*) activity has been used as a parameter of neutrophil infiltration. (Paterson *et al.*, 1984) MPO activity was measured using a myeloperoxidase colorimetric activity assay kit as described by the manufacturer (Sigma-Aldrich, CA). Briefly, whole cornea lysates from AB, CM or control corneas (n=4 samples/group/time point) were homogenized in MPO assay buffer and the homogenate was centrifuged at 14000 ×g for 20 min at 4°C. Total protein concentration was measured by the BCA protein assay as previously described.(de Paiva *et al.*, 2009) A 50 µg/sample was mixed with MPO assay buffer and MPO substrate, incubated at room temperature for 2 hours, and then mixed with tetramethylbenzidine probe. Fluorescence was measured at 412 nm using a Tecan Infinite

M200 plate reader equipped with Magellan V6.55 software. Biologic replicate samples were averaged. Results are presented as mean ± SEM (milliunits).

MMP-9 activity assay

Whole corneas were excised, rinsed and homogenized in RIPA buffer and the homogenate was centrifuged at 14000 ×g for 20 min at 4°C.Total protein concentration of the whole cornea cell lysate (n=4/group/time point) was measured by the BCA protein assay as previously described (de Paiva *et al.*, 2009). Total MMP-9 enzyme activity was measured with a MMP activity assay kit (Biotrak; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol as previously published.(Chotikavanich *et al.*, 2009). In brief, 100 µL of each pro-MMP-9 standard (0.125–4 ng/mL), 50µg of cornea extract and assay buffer (for blanks) were incubated at 4°C overnight in wells of a microtiter plate precoated with anti-mouse-MMP-9 antibodies and washed. Total MMP-9 activity was measured by activating bound pro-MMP-9 with 50 µL of 0.5 mM p-amino phenylmercuric acetate (APMA) in assay buffer at 37°C for 1.5 hours, followed by incubation with a detection reagent at 37°C for two hours. Active MMP-9 was detected through its ability to activate a modified prodetection enzyme that subsequently cleaved to its chromogenic peptide substrate. Absorbance was read at 405 nm using a Tecan Infinite M200 plate reader equipped with Magellan V6.55 software. The activity of MMP-9 in a sample was determined by interpolation from a standard curve. Biological replicates were averaged and the results were presented as mean ± SEM (pg/ml).

Gelatin zymography

MMP-9 and MMP-2 are gelatinases that can digest gelatin. Gelatin zymography has been used to evaluate gelatinase activity in tears and lysates as gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

The relative amount of MMP-9 in whole cornea lysates (n=4/group) was measured by gelatin zymography, using a previously reported method (Sobrin *et al.*, 2000; Li *et al.*, 2001). Whole cornea lysates prepared for MMP-9 activity (20 µg/sample) were fractionated in an 8% polyacrylamide gel containing gelatin (0.5 mg/mL). The gels were soaked in 0.25% Triton X-100 for 30 minutes at room temperature, then incubated in a digestion buffer containing 5 mmol/L phenylmethyl sulfonyl fluoride at 37°C overnight. They were stained with 0.25%

Coomassie brilliant blue R-250 in 40% methanol for 2 hours, then destained overnight in 10% acetic acid.

Flow-cytometry analysis of infiltrating cells in cornea

Single-cell suspensions of corneas (n=5/group/time point/experiment, for a total of 2 experiments) were excised, rinsed, and prepared by treatment of minced tissue fragments with 0.1% collagenase D (60 minutes at 37°C, Invitrogen-Gibco, Carlsbad, California) and sequentially neutralized with media and filtered, then resuspended and stained with anti-CD16/32 (BD Pharmingen, San Diego, CA), followed by cell-surface staining with fluorescein isothiocyanate (FITC)-F4/80 (macrophage marker, clone BM8, Biolegend, San Diego, CA), R-phycoerythrin (PE)-Gr-1 conjugated (neutrophil marker, clone 1A8, BD Pharmingen) and Allophycocyanin (APC)-CD45 (pan-bone marrow derived cell marker, clone 30-F11, BD Pharmingen) conjugated antibodies. Single-cell preparations of bone marrow cells obtained from control mice were stained with the same antibodies and served as positive controls. Cells were kept on ice until flow cytometry analysis was performed. A BD LSRII Benchtop cytometer was used. The gating strategy was as follows: lymphocytes were individually identified on the basis of forward scatter and side scatter properties, subsequently gated on the basis of forward scatter height versus forward scatter area (singlets 1), then gated on side scatter height versus side scatter area (singlets 2) followed by CD45 histogram gating. Propidium iodide exclusion was used to discriminate live cells. At least 50,000 events were collected. Results were analyzed with BD Diva software (version 2.1, BD Pharmingen) and FlowJo software (version 10.07, Tree Star Inc., Ashland, OR).

Statistical Analysis

Results are presented as the mean ± SEM. Two- way analysis of variance (ANOVA) with Bonferroni post hoc testing was used for statistical comparisons of gene expression since there were two variables (time and different groups of treatment). $P \leq 0.05$ was considered statistical significant. These tests were performed using GraphPad Prism 6.0 software (GraphPad Incorporation, San Diego, CA).

Results

Concomitant alkali burn and desiccating stress leads to ocular perforation

Because environmental dry eye is an under recognized variable in the management of severe burned patients, including the ones treated in intensive care units, we have developed a combined model (CM) of alkali burn and dry eye by subjecting mice to desiccating stress immediately after anesthetic recovery following creation of alkali burn.

We observed that eyes subjected to CM perforated as early as 2 days post-initial injury (20%) and the rate of perforation in some experiments was up to 40% by day 5 (Fig. 3A, B), while eyes subjected to alkali burn alone did not perforate. Naïve and DS corneas did not perforate at all (data not shown). Corneas that did not perforate in the initial 5 days post-injury did not perforate at later time point (even up to 21 days), showing that the initial inflammatory has the greatest effect on corneal integrity (Fig. 3B).

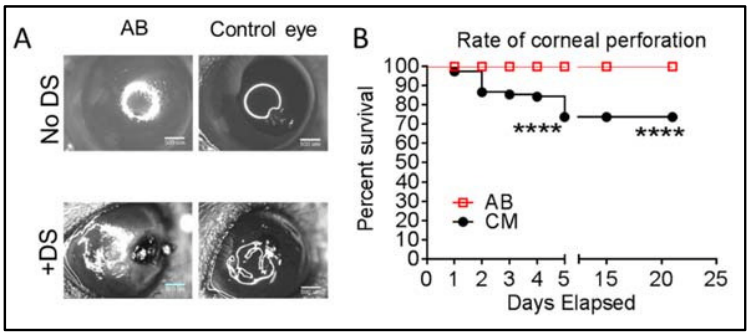

Fig. 3. Greater incidence of ocular perforations in the CM model. A. Digital images of bright field pictures from control, alkali burn (AB), combined model (CM) and desiccating stress (DS) 5 days post-injury. Note perforation in the combined group. **B**. Rate of ocular perforation in eyes subjected to AB and CM.

Histologically, perforated corneas subjected to the CM had collapsed anterior chamber, iris and lens tamponade, total loss of corneal epithelium and massive infiltration of corneal stroma by inflammatory cells that dissected into the cornea from the limbus (Fig. 4). Microbial cultures of perforated corneas and conjunctivas (n=6) showed no microorganism growth, confirming the sterile nature of the perforation. Eyes subjected to AB had a central corneal epithelial defect and moderate inflammatory infiltration in the corneal stroma, but no perforation.

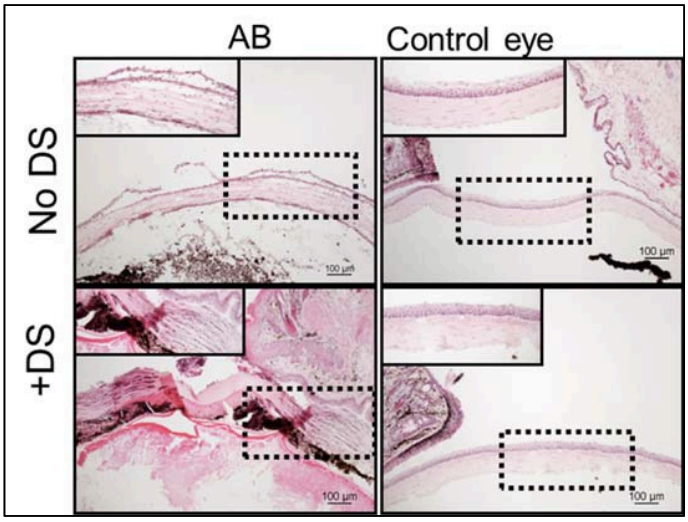

Fig. 4. Representative HE staining of cryosections from control, AB, CM and 5 days post-injury. Perforated CM corneas developed massive inflammatory infiltration forming waves of inflammatory cells within the cornea stroma. Cornea perforation is sealed by lens and iris. AB corneas had loose epithelium and moderate inflammatory cells in the cornea stroma and anterior chamber. Original magnification 10x.Insets are high magnification of dotted areas.

We evaluate wound closure by staining corneas with 0.1% fluorescein daily and generated a survival curve of wound closure based on presence/absence of epithelial wound closure, independent of the area left to be closed (Fig. 5A, 4B). CM corneas had delayed reepithelization compared to AB alone (40% vs. 100% by day 5, respectively, Fig 5B). We also graded daily corneal opacification in digital images captured daily by masked examiners using a scale from zero (transparent cornea) to 5 (complete opaque cornea, Fig. 5C). We observed higher residual cornea opacification in the CM group compared to corneas subjected to AB alone (Fig. 5D).

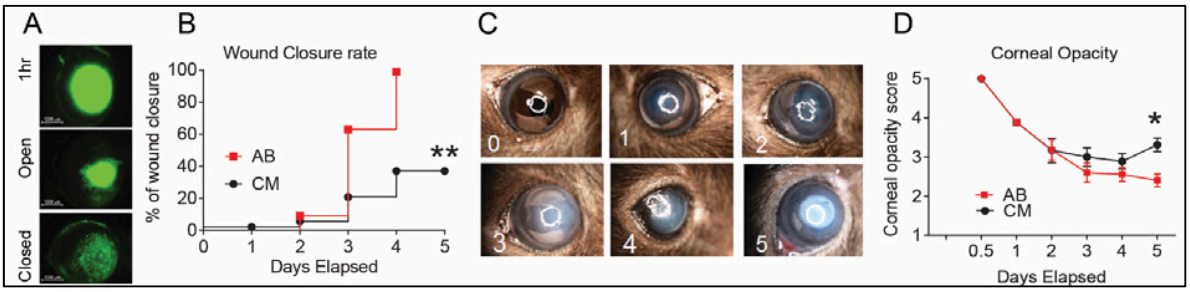

Fig. 5. Wound closure and residual opacification. A Representative digital images of corneas 1 hour after creation of alkali burn lesion and representative pictures of open and closed wounds 2 and 5 days after initial lesion used to generate the survival curve of corneal reepithelization seen in B.
B. Survival rate of wound closure in AB and CM groups

C. Representative bright field digital images of corneas showing opacification scale used to generate the graph seen in D (corneal opacity score).
D. Corneal opacity score in AB and CM groups.
**p<0.01; ****p<0.0001

Taken together, these results show that combining dry eye with alkali burn induces sterile corneal perforation in in up to 40% of the cases, delays wound healing and increases corneal opacity.

Cytokine and MMPs storm in the combined model

It has been recognized for decades that ocular surface chemical/thermal injury stimulates production of tissue degrading enzymes as part of the wound healing cascade (Afonso *et al.*, 1999; Li *et al.*, 2001; Sosne *et al.*, 2005; Herretes *et al.*, 2006; Takahashi *et al.*, 2007). Matrix degrading enzymes, including metalloproteinases (MMPs) have been identified as important factors in the inflammatory and wound healing response of the ocular surface, particularly in dry eye and ocular burns. Their induction during wound healing is thought to play a role in extracellular matrix remodeling, cytokine activation, and regulation of angiogenesis.

Because most of cornea perforations and greatest differences in cornea wound healing were seen early (at 2 and 5 days post-injury), we evaluated the levels of RNA transcripts encoding IL-1α, IL-6 and MMPs -1,-2,-3,-8 -9, -12, -13, by real-time PCR using whole corneas harvested from all groups at these same time points. Our results are presented in Fig. 6.

Two days post-injury, both AB and CM groups had significantly elevated expression of IL-6 and MMPs compared to untreated corneas; however, CM significantly increased IL-6 (83 vs. 17 fold), MMP-1 (75 vs.63 fold), MMP-3 (4 vs. 0.7 fold), MMP-8 (75 vs. 26 fold), MMP-9 (95 vs. 4 fold), MMP-13 (14 vs. 9 fold) mRNA transcripts compared to AB alone. DS corneas had upregulation of IL-6, MMPs -1, -3, -8, -9 compared to naïve corneas, however, this increase was significantly lower than the one observed in the AB and CM corneas. Five days post-injury, AB corneas had significantly elevated IL-6 and MMPs -1,-3,-8,-9, mRNA transcripts compared to the CM group (Fig. 2). IL-1α was equally elevated in both CM and AB groups after 2 and 5 days post-injury (2.7 vs. 1.7 fold and 2.5 vs. 3 fold, respectively) compared to control corneas (data not shown). DS corneas had significantly elevated

expression of MMP-3 and 9 after 5 days of desiccating stress, as we previously reported (Corrales *et al.*, 2006; de Paiva *et al.*, 2006b; de Paiva *et al.*, 2009).

MMP-12, also known as macrophage elastase, and produced by macrophages, fibroblasts and epithelial cells (Ye *et al.*, 2000; Lavigne *et al.*, 2004; Iwanami *et al.*, 2009; Chan *et al.*, 2013) was equally elevated in both AB and CM corneas 2 days after injury (15.85±1.66 vs. 12.97±0.65 fold) but at 5 days its expression significantly increased in the CM group (42.03±0.23 vs. 67.30±0.18 fold, P<0.001).

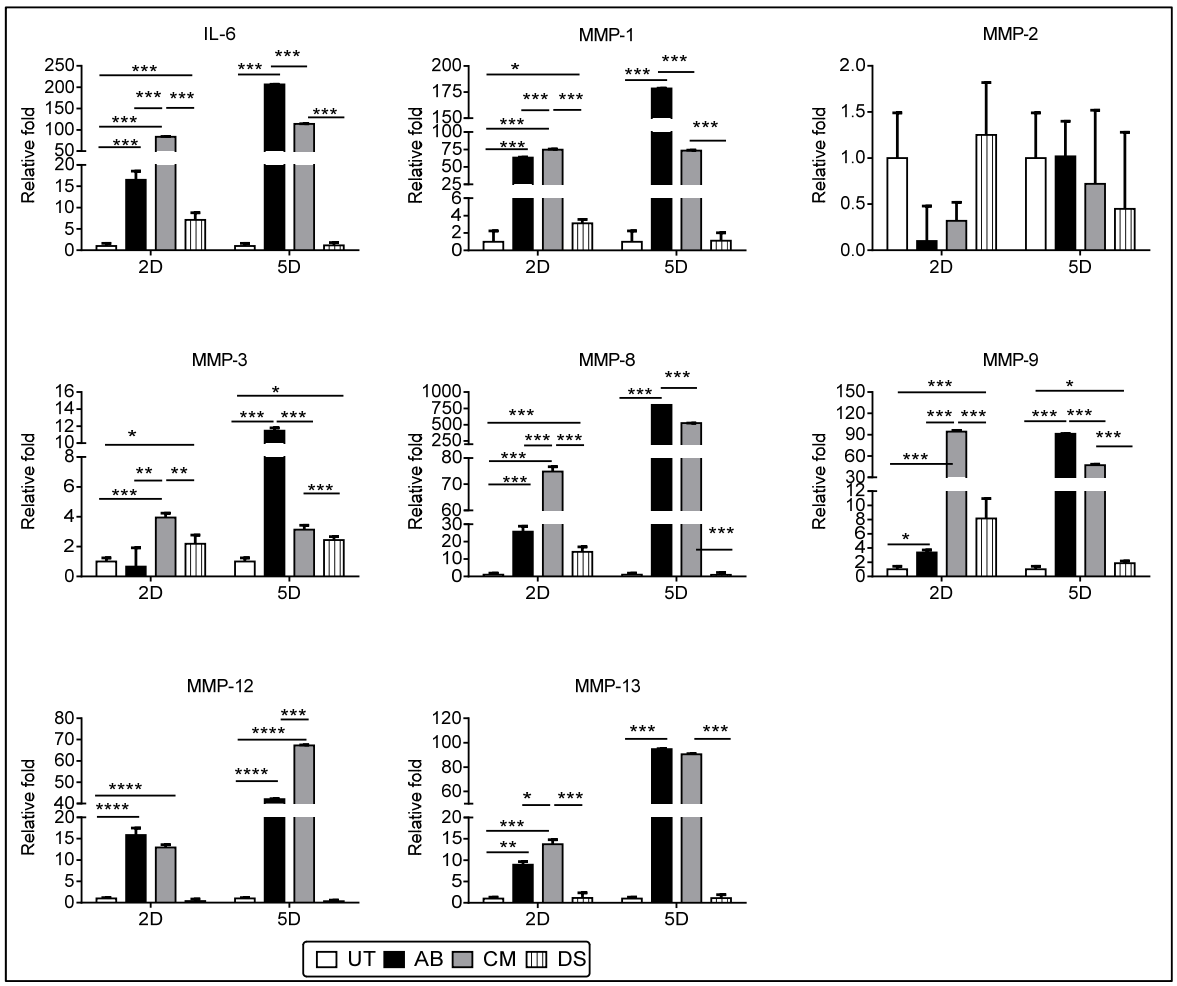

Fig. 6. Early increase cytokine and MMP storm in combined model corneas.

Relative fold of expression of IL-6 and MMPs -1,-2,-3,-8,-9, -12, -13, in whole corneas subjected to alkali burn (AB) or combined model (CM). Bar graphs show means ± SD of one representative experiment with five samples per group/time point (experiment was repeated three times with similar results). UT=untreated cornea
*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001

The immunoreactivity of corneas to collagenases (MMP-1 and MMP-13 (Dumin *et al.*, 2001)), MMP-3 (a physiological activator of MMP-9 (Johnson *et al.*, 2011), and MMP-9 were evaluated by immunostaining (Fig. 7). Minimal staining of MMPs -1, -3,-9 and -13 was noted in the control corneas, while increased immunoreactivity was observed in the corneal epithelium of CM, AB and DS corneas (CM <AB<DS).

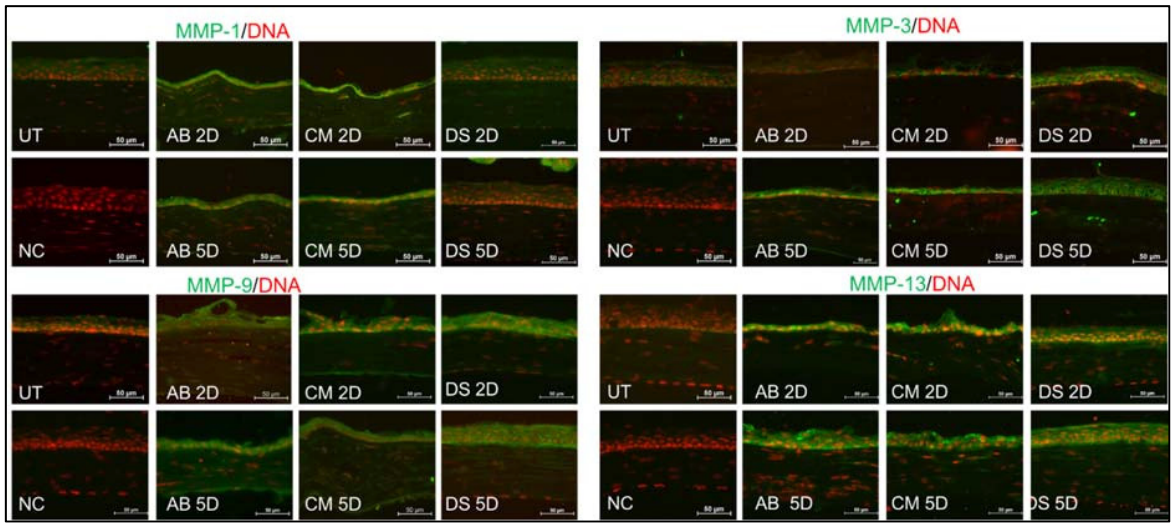

Fig. 7. Increased MMP protein expression in combined model of alkali burn and dry eye. Representative merged digital images of corneas cryosections immunostained for MMP-1, MMP-3, MMP-13 and MMP-9 (all in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in corneas subjected to alkali burn (AB), desiccating stress (DS) or combined model (CM). Scale bar=50μm.

Among the MMPs, MMP–9 plays a prominent role being produced by stressed cornea and conjunctival epithelial cells and has both matrix degrading and pro-inflammatory activities (Matsubara *et al.*, 1991; Fini *et al.*, 1992). MMP-9 has been reported to delay corneal wound healing (Mohan *et al.*, 2002). We evaluated MMP-9 activity in whole cornea lysates using an MMP-9 activity assay and observed that the CM group had significantly higher levels of active MMP-9 than AB both at day 2 and day 5 post-injury (Fig 8A). Gelatin zymography showed increased amounts of both pro and active MMP-9 bands in the CM and AB groups compared to control corneas (Fig. 8B); however, as suspected from the MMP-9 activity assay, greater amounts of pro and activated MMP-9 bands were present in the CM group. Negligible amounts of MMP-2 were present in AB and CM groups and they did not differ from untreated corneas.

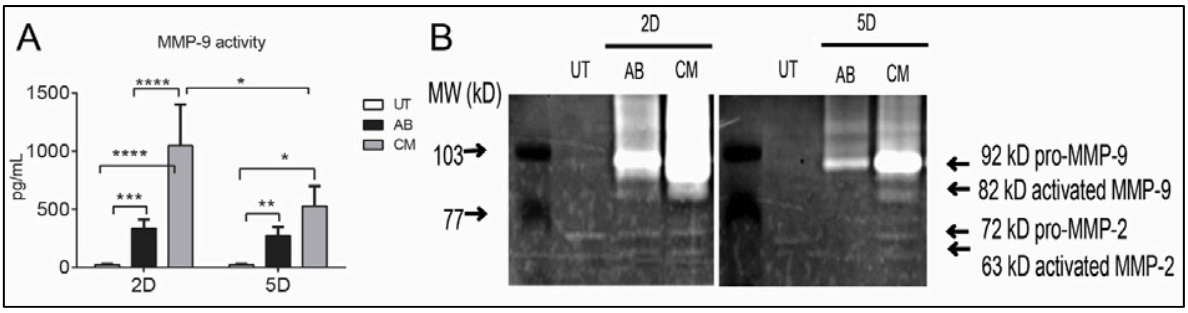

Fig. 8. CM leads to increased MMP-9 activity. A MMP-9 activity in whole cornea lysates in AB, CM and untreated group (n=4/group). **B.** Representative zymogram showing MMP-9 bands whole cornea lysates in the treatment groups (n=4/group). UT=untreated cornea, AB=alkali burn, CM=combined model,
*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001

These results indicated that the early peak of inflammatory cytokines and MMPs in the CM group coincides with the time period where most of the corneal perforations occur, indicating that the early additive effect of dry eye with alkali burn jeopardizes corneal integrity

Increased infiltration of neutrophils in CM group

Polymorphonuclear leukocytes (neutrophils) are the first inflammatory cells to migrate into sites of tissue injury. The pathogenic role of neutrophils in alkali burn can be appreciated as neutrophil-depleted C57BL/6 corneas subjected to alkali burn healed faster than non-depleted controls (Ueno M, 2005). IL-1 is a cytokine produced by epithelium, fibroblasts and neutrophils (Tiku *et al.*, 1986; Wilson *et al.*, 1992) and participates in collagen degradation by stimulating neutrophils (Li, 2003). The chemokine CXCL1 is crucial for the recruitment of neutrophils to inflammatory sites (De Filippo *et al.*, 2013).

We evaluated expression of IL-1β and CXCL1 in corneas subjected to AB and CM and compared to controls. CM significantly increased IL-1β (72 vs. 11 fold) and CXCL1 (27 vs. 5 fold) two days after injury compared to AB alone. At 5 days, IL-1β levels maintained elevated in CM (393 vs. 267 fold) and similar levels of CXCL1 were seen 5 days post-injury in both models (~20 fold; Fig. 9).

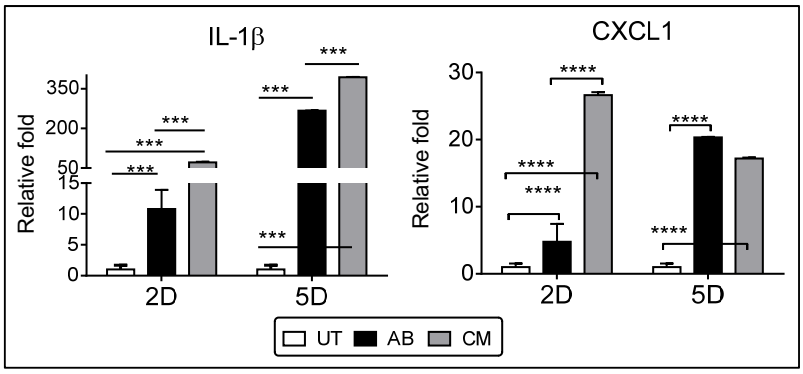

Fig. 9. Increased neutrophil chemoattractant. Relative fold of expression of IL-1β and CXCL1 in whole corneas subjected to alkali burn (AB), or combined model (CM, alkali burn+ dry eye). Bar graphs show means ± SD of one representative experiment with five samples per group/time point (experiment was repeated twice with similar results). UT=untreated cornea; *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001

Minimal expression of IL-1β was seen in untreated corneas; however, AB and CM increased immunoreactivity in corneal epithelium to IL-1β, which peaked at day 5 post injury (Fig. 10).

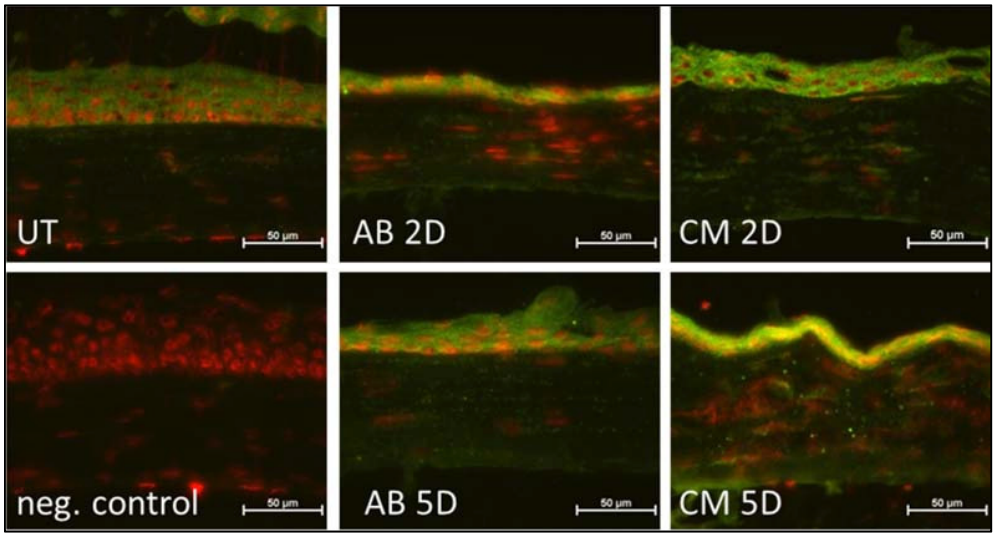

Fig.10. Increased immunoreactivity of IL-1 β in the CM. Representative merged digital images of corneas cryosections immunostained for IL-1β (in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in corneas subjected to alkali burn (AB) or combined model (CM). Scale bar=50μm.

We evaluated presence of neutrophils in injured corneas by immunohistochemistry using the Gr-1 antibody, by flow cytometry and also by myeloperoxidase activity assay in whole cornea lysates. In a naïve cornea, a few resident neutrophils can be found at the limbal area (data not shown). A significant influx of Gr-1+ cells was observed in AB and CM groups

2 and 5 days post-injury; the infiltration was not restricted to the limbal area (Fig. 11 insert), but extended to the central cornea (Fig. 11A).

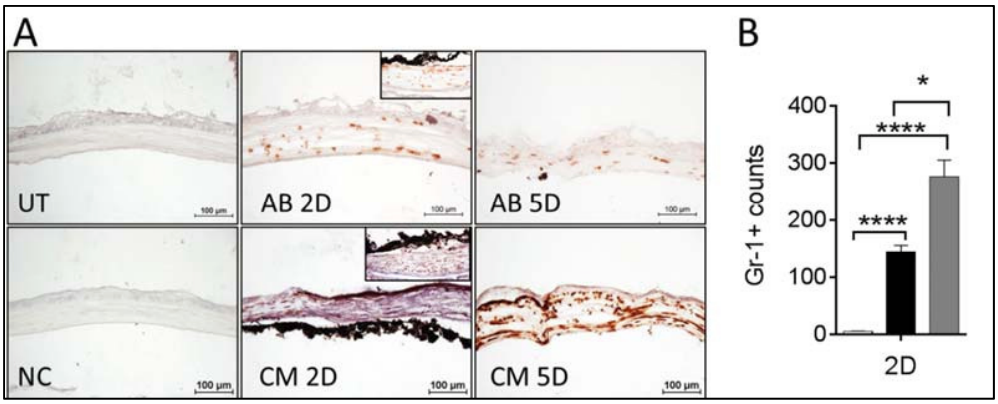

Fig. 11. Neutrophil infiltration in the CM of alkali burn and dry eye. Representative pictures of Gr-1$^+$ cells (Reim *et al.*) of central cornea cryosections among the AB, CM and control groups used to generate the bar graph showing counts in B. Insets are representative pictures of limbal area. B. Bar graphs (mean±SEM) of Gr-1$^+$ cell counts in whole cornea/groups (n=5/group).

CM corneas had the highest Gr-1+ cell counts (Fig. 11B) compared to AB and UT corneas 2 days after injury. These results were confirmed by flow cytometry as we observed a significant increase in CD45+ cells in corneas subjected to AB and CM (Fig. 12); the majority of CD45+ infiltrating cells were Gr-1+ cells.

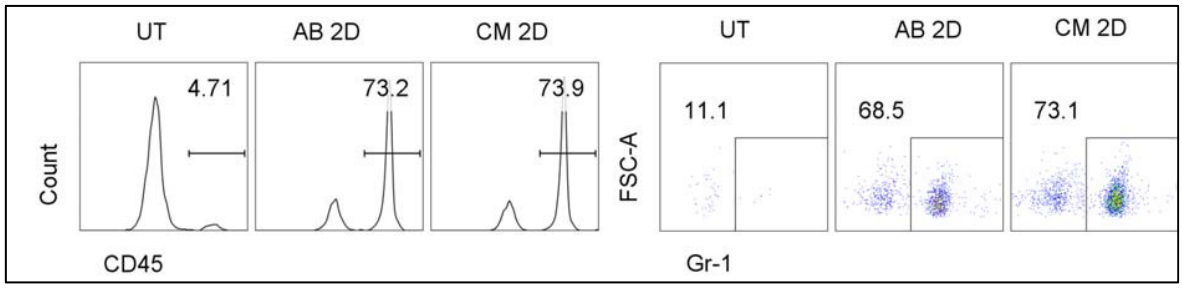

Fig.12. Representative flow cytometry plots. Flow cytometry in corneas stained for bone-marrow derived cells (CD45) and neutrophils (Gr-1). Representative histogram (top panel) and dot plot (bottom panel) of CD45$^+$ and Gr-1$^+$ cells evaluated by flow cytometry. Numbers in the squares indicate percentage of positive cells.

There was a significant increase in the frequency of CD45+Gr-1+ (neutrophils) cells in CM group compared to AB alone 2 days post-injury (Fig.13A), while there was a lower frequency of CD45+F4/80+ cells (macrophages) compared to naïve corneas. The pattern of Gr-1+ infiltration was similar to expression pattern of CXCL1 in cornea. These results are in agreement with the literature that showed that neutrophils can be found in central cornea as soon as 8 hours post-injury (Hanlon *et al.*, 2014).

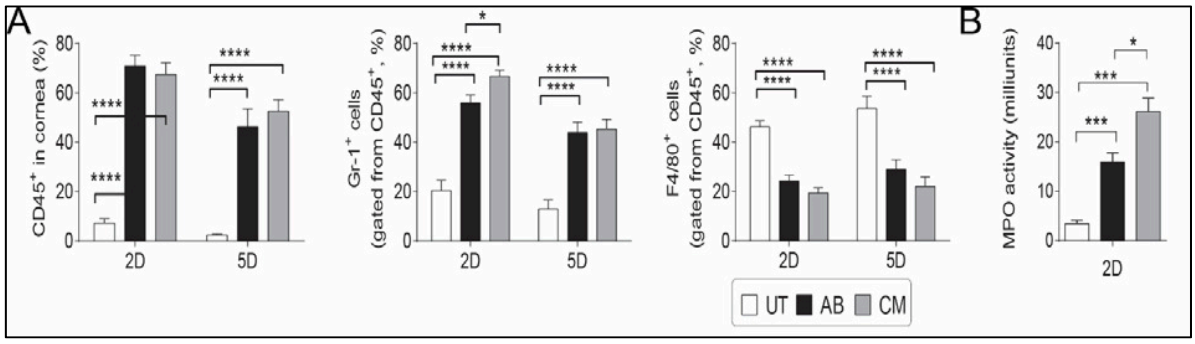

Fig. 13. Frequency of bone marrow derived cells, neutrophils and macrophages.
Bar graphs (Mean ±SEM) of frequency of $CD45^+$ (total bone marrow derived cells) and $CD45^+Gr\text{-}1^+$ (neutrophils) and $CD45^+F4/80^+$ cells (macrophages) quantified by flow cytometry in whole cornea single cell suspension (n=5/group/time point).
B. Myeloperoxidase (Samtani *et al.*) activity in whole corneas lysates 2 days after injury in untreated, AB and CM groups (mean±SEM of 4 samples). *p<0.05; ***p<0.001; ****p<0.0001. UT=untreated cornea, AB=alkali burn, CM=combined model, DS=desiccating stress, D=days. FSC-A=forward side scatter area.

Because neutrophil infiltration peaked 2 days post-injury, we measured MPO activity at the same time point, since MPO is the most abundant protein found in neutrophils. Our results, presented in Fig. 12B, demonstrate that significantly higher MPO activity than control was seen in CM and AB groups, and MPO activity in CM was higher than AB. MPO activity in naïve control corneas was minimal, confirming the immunohistochemistry and flow cytometry results.

These results indicated that combining dry eye with AB worsens the severity of AB, by increasing neutrophil infiltration and activity.

Clinical effects of anti-inflammatory therapy on a combined model of alkali burn and dry eye

It is well recognized that alkali burns to the cornea induce a significant amount of inflammation and MMPs (Brown *et al.*, 1969; Donshik *et al.*, 1978). Corneal scarring and opacification are sight-threatening sequelae of severe chemical burns. In these cases, corneal transplantation may be required to corneal clarity (Mattax & McCulley, 1988), however, these eyes have high risk of graft failure. Herein we subjected mice to unilateral alkali burn and concomitant desiccating stress and topically treated them with either Dex or Doxy and compared the effects to BSS treated corneas.

We evaluated corneal opacification in digital images by masked observers using a scale from zero (transparent cornea) to 5 (complete opaque cornea). We also evaluated wound closure daily for the duration of the experiment. Representative images for corneal opacity and wound closure are shown on Fig. 14 A, B.

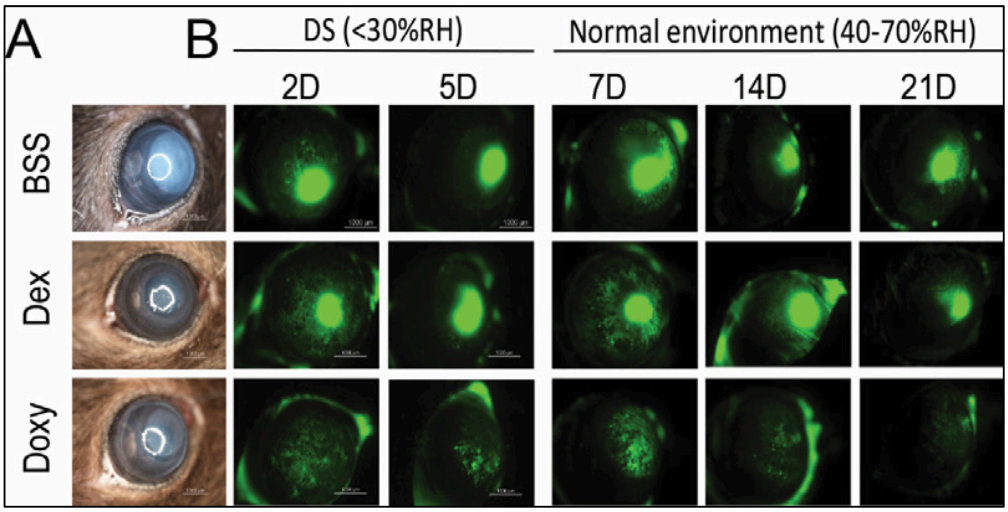

Fig. 14. Representative clinical pictures. A. Digital images of bright field pictures alkali burn and dry eye combined model treated with either balanced salt solution (BSS), Dexamethasone (Dex) or Doxycycline (Doxy) 5 days (5D) post-injury. Scale bar=1000μm **B**. Representative digital images of corneas 5 days stained with 0.1% sodium fluorescein after creation of alkali burn lesion and induction of dry eye topically treated with BSS, Dex or Doxy for 5 days and then topically treated for 16 days in normal environment. Scale bar=1000μm

Dex-treated corneas had the lowest corneal opacity score among all treatment groups (Fig. 15A). Doxy-treated corneas healed significantly faster than BSS-treated corneas; with 100% wound closure 2 days post-injury (Fig 15B, $p<0.001$). Dex-treated corneas showed delayed cornea wound healing, with 60% of corneas showing areas of epithelial defect, while 75% of BSS treated corneas had epithelial defects 5 days post-injury.

BSS- and Doxy-treated corneas subjected to the combined model showed early corneal perforations as early as day 1 after the initial insult; however, more BSS-treated corneas perforated than Doxy-treated (27 vs. 14%, $p=0.03$, Fig. 15C). Dex-treated corneas did not perforate for the duration of the experiment.

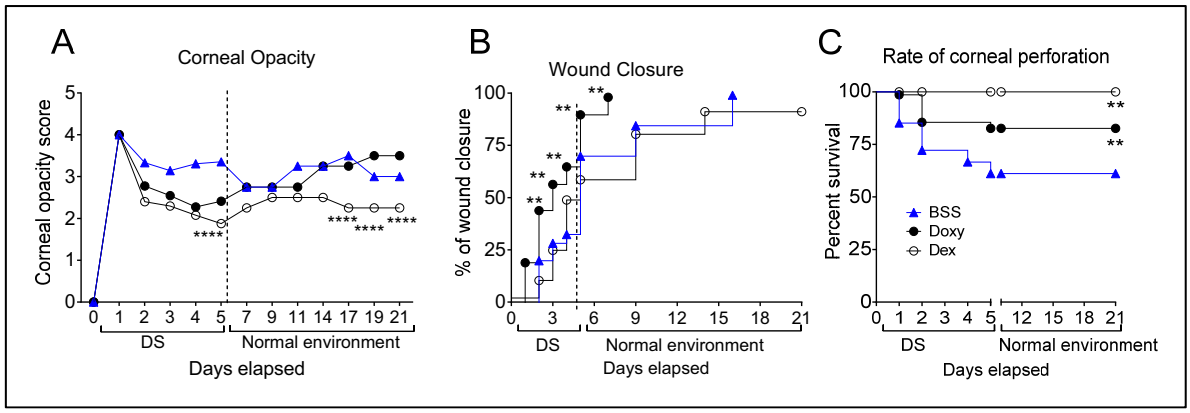

Fig. 15. Rate of corneal opacity, wound closure and corneal perforation. A. Corneal opacity score in CM corneas topically treated with BSS, Dex or Doxy (n=12/group).**B.** Survival rate of wound closure in CM corneas topically treated with BSS, Dex or Doxy.**C**. Rate of ocular perforation in eyes subjected to CM topically treated with BSS, Dex, Doxy. (n=12/group). *p<0.05, **p<0.01; ***p<0.001, ****p<0.0001

Our results confirm that alkali injury in the context of a dry eye dramatically exacerbates the inflammatory response and increases the rate of corneal perforation, while treatment with Doxycycline or Dexamethasone is efficacious in preventing this sight-threatening complication.

Extended anti-inflammatory therapy for up to 21 days does not lead to corneal perforations

Conflicting results of prolonged use of glucorticoids promoting corneal perforation after alkali burn in animal models have been reported (Brown *et al.*, 1969; Newsome & Gross, 1977; Donshik *et al.*, 1978; Saud *et al.*, 2012). To investigate if prolonged anti-inflammatory treatment in mice after this combined model would also lead to corneal perforation, we subjected a separate group of mice to alkali burn and dry eye for 5 days; and subsequently placed them in a normal environment (RH 40-70%) for 16 days. Mice were continuously treated with either BSS, Doxy or Dex for a total of 21 days and corneal opacity, wound closure and rate of corneal perforation were evaluated. A representative corneal fluorescein staining can be found in Fig. 14.

We observed that if BSS- or Doxy-treated corneas did not perforate in the first 5 days, they did not perforate at all (Fig. 15C), indicating that this is the critical period for corneal ulceration leading to perforation. Dex-treated corneas did not perforate even after continuous

treatment for up to 21 days, indicating that prolonged therapy for up to 21 days does not cause corneal melting.

Doxy-treated corneas showed the fastest wound healing, but no difference in corneal opacity scores compared to vehicle treated eyes (Fig.15A-C). While prolonged treatment with Dex showed incomplete wound closure similar to BSS controls even after 21 days, it yielded significantly lower corneal opacity scores (Fig. 15A). These results indicate that in mice subjected to alkali burn and dry eye develop more corneal disease than alkali burn alone and that topical anti-inflammatory therapy does not promote corneal perforation.

Controlling host response with anti-inflammatory therapy in a combined model of dry eye and alkali burn

Since our results showed that combining alkali burn and dry eye led to significantly increased levels of IL- and MMPs-1, -3, -8, -9, and -13 transcripts in the cornea compared to alkali burn alone, we evaluated the efficacy of anti-inflammatory therapy with Doxy or Dex at 2 and 5 days post initial injury. Corneas subjected to alkali burn and dry eye and topically treated with BSS showed a significant increase in IL-6, MMPs -1, -3, -8, -9, -13 and TIMP-1 compared to naïve corneas. MMPs -1, -3, -8, -9,-13 and TIMP-1 transcripts peaked at 2 days and decreased by 5 days, while IL-6 continued to increase up to day 5 (Fig. 16). No changes in MMP-2, TIMP-2, TIMP-3 or TIMP-4 were observed (data not shown).

Doxy treatment corneas significantly decreased MMPs -8, -9, -13 two days post-injury, while Dex treatment significantly decreased IL-6, MMPs -1, -3, -9, -13 and TIMP-1 but significantly increased MMP-8 RNA transcripts (up to a 1000 fold). Doxy treatment showed greater suppression of IL-6, and MMPs -8, -9, and MMP-13 five days after the initial injury than BSS (Fig. 16).

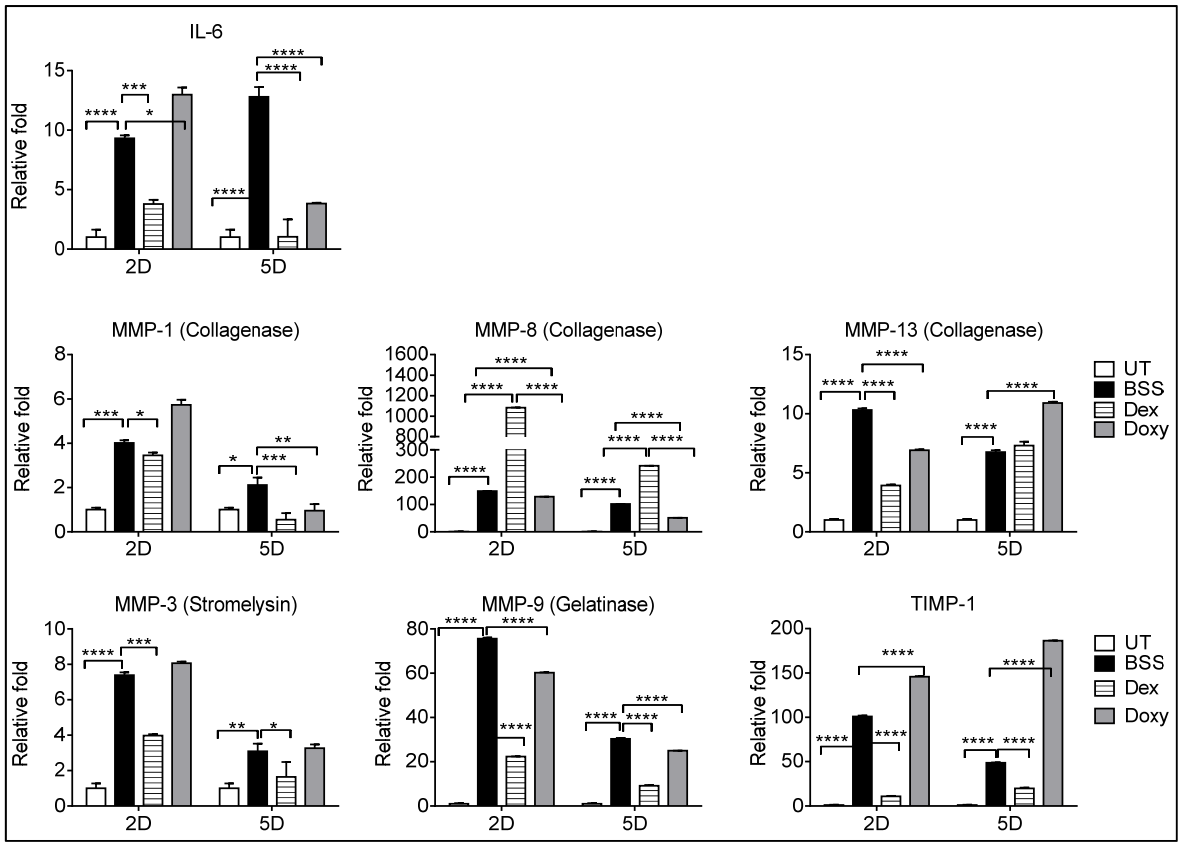

Figure 16. **Inflammatory cytokines and MMPs decrease after anti-inflammatory therapy in a combined model (CM) of alkali burn and dry eye.** Relative fold of expression of IL-6, collagenases (MMPs -1, -8 and 13), the stromelysin MMP-3, the gelatinase MMP-9 and TIMP-1 in whole corneas subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy. Bar graphs show means ± SEM of one representative experiment with four samples per group/time point (experiment was repeated three times with similar results). UT = untreated cornea, BSS = corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex = corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy = corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, D=days.

The immunoreactivity of corneas to collagenases (MMPs -1, -13) and MMP-9 was evaluated by immunostaining (Fig. 17A). Minimal levels of MMP-1, -9 and -13 were present in the control corneas. Increased reactivity against these MMPs in the corneal epithelium was seen BSS treated corneas. Both Doxy and Dex-treated corneas showed decreased immunoreactivity of corneal epithelium to MMP-1, MMP-9 and MMP-13 compared to BSS treatment (Fig. 17A).

MMP-9KO mice have been noted to heal epithelial defects faster than wild-type mice, indicating that MMP-9 can delay corneal wound healing (Mohan *et al.*, 2002). MMP-9 activity was evaluated by gelatin zymography, which showed increased amounts of both pro and active MMP-9 bands in the BSS group compared to control corneas (day 2 > day 5). Dex and

Doxy groups showed no activated MMP-9 bands and had reduced pro-MMP-9 bands, in both day 2 and 5 post injury samples. Negligible amounts of MMP-2 were present in all groups (Fig. 17B).

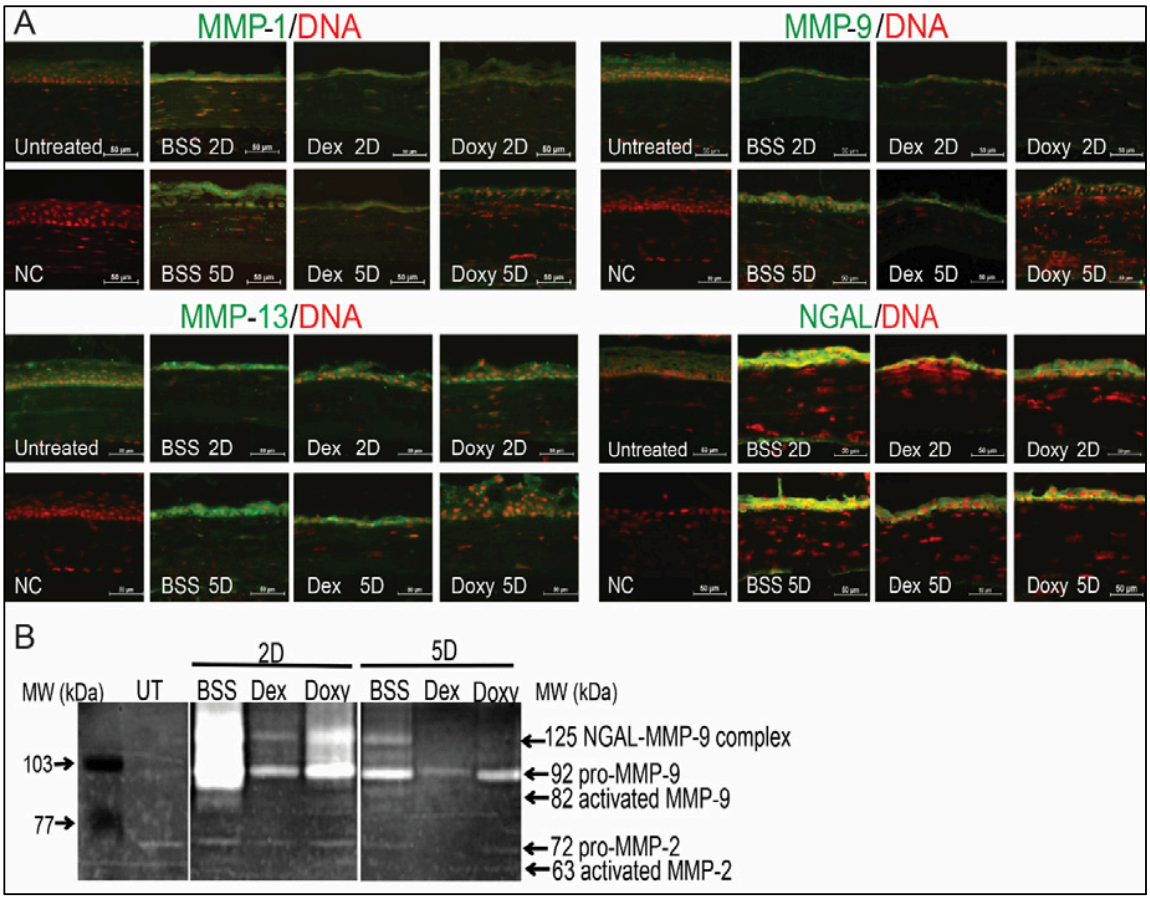

Figure 17. Anti-inflammatory therapy decreases MMP protein expression and gelatinolytic activity. A Representative merged digital images of cornea cryosections immunostained for MMP-1, MMP-9, MMP-13 and NGAL (all in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in mice subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy for 2 or 5 days (2D or 5D, respectively). Scale bar=50μm.
B Representative zymogram showing MMP-9 and NGAL-MMP-9 bands of whole corneal lysates in the treatment groups (n=4/group).UT=untreated cornea, BSS= corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex= corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy= corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, D= days, NGAL= Neutrophil-gelatinase associated lipocalin, NC=negative control

These results confirm the efficacy of broad anti-inflammatory therapy in controlling MMP production and level of gelatinases in the cornea after alkali burn and dry eye. The corticosteroid Dexamethasone showed greater anti-inflammatory effect than Doxycycline.

Dexamethasone decreases lipocalin-2-MMP-9 complex formation

Neutrophil-gelatinase associated lipocalin (NGAL) belongs to the lipocalin family. It is involved in cell growth, migration, differentiation and has anti-microbial properties (Kjeldsen *et al.*, 1993; Gupta *et al.*, 2007; Kubben *et al.*, 2007). It is released by the kidney tubular cells under stressful conditions (Sen *et al.*, 2015) and expressed in the colonic epithelia during cancer and colitis (Nielsen *et al.*, 1996). NGAL exists as a monomer (25kDa), a homodimer (46kDa) or as a heterodimer (125kDa) (Yan *et al.*, 2001). The heterodimer form is composed of NGAL and MMP-9 and it is known to protect MMP-9 from degradation in a concentration dependent manner (Kjeldsen *et al.*, 1993).

We investigated the expression of NGAL in the combined model by immunostaining frozen sections. While barely detected in naïve corneas, increased immunoreactivity to NGAL was observed in the epithelium of BSS treated corneas. Treatment with Dex or Doxy decreased NGAL immunoreactivity (Fig. 17A). We also observed that a 125-kDa band in our zymography gels, corresponding to the NGAL-MMP-9 complex, was markedly decreased in Dex-treated corneas compared to BSS 2 days after injury. Doxy had a moderate effect decreasing it (Fig. 17B).

Anti-inflammatory therapy decreases neutrophil infiltration in CM

Neutrophils are the first cells to respond to a site of injury. Several enzymes and proteases, including collagenases, are store within the cytoplasmic granules of neutrophils. Elegant experiments showed that depletion of neutrophils dramatically improved the fate of alkali burn injuries (Kenyon *et al.*, 1979; Foster *et al.*, 1982).Doxycycline and Dexamethasone have been shown to decrease migration of polymorphonuclear leukocytes (PMN) (Martin *et al.*, 1974; Kenyon *et al.*, 1979; Seedor *et al.*, 1987; Zentay *et al.*, 1999).

We have previously reported that combining alkali burn with dry eye significantly increases neutrophil infiltration in wounded corneas (de Paiva *et al.*, 2011; de Paiva *et al.*, 2014). We observed increased immunoreactivity of MMP-8 in the corneal epithelium, stroma, and endothelium after 2 and 5 days of the combined model (Fig. 18A). In addition, we investigated the expression of the cytokine IL-1 and the chemokine CXCL1 since they have been implicated in the migration of neutrophils (Oliveira *et al.*, 2008). Dex treatment in the combined model significantly decreased the early peak of IL-1β and CXCL1 2 days after initial injury as well as at day 5. Doxycycline was more efficacious than vehicle decreasing both mediators by day 5 (Fig. 18B).

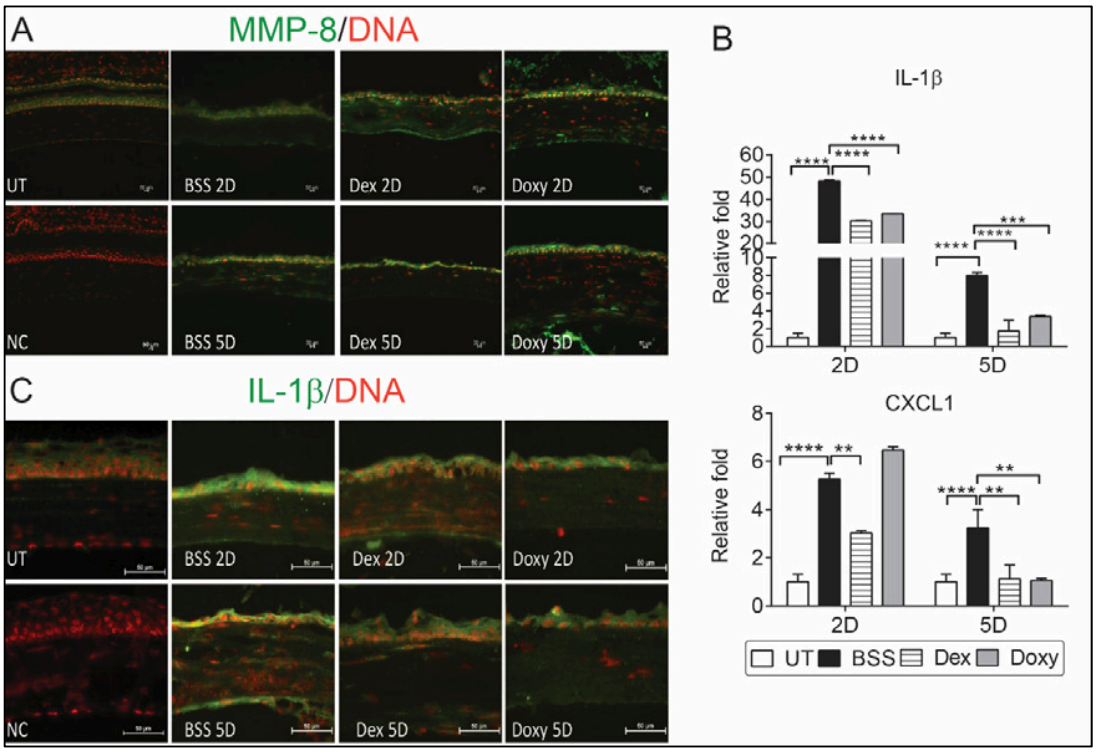

Fig. 18. Expression of MMP8, IL-1β and chemokines. A. Representative merged digital images of corneal cryosections immunostained for MMP-8 (in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in corneas subjected to the combined model topically treated with BSS, Dex or Doxy. NC= negative control. Scale bar=100μm.
B. Relative fold of expression of IL-1β and CXCL1 in whole corneas subjected to alkali burn and dry eye topically treated with BSS, Dex or Doxy. Bar graphs show means ± SEM of one representative experiment with five samples per group/time point (experiment was repeated three times with similar results). UT=untreated cornea, NC=negative control
*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001

To investigate if topical treatment would decrease neutrophil infiltration, we performed immunohistochemistry in cornea cryosections using the Gr-1 marker, and investigated myeloperoxidase activity assay in whole corneal lysates. In naïve corneas, a few resident neutrophils were found at the limbal area (data not shown). A significant influx of Gr-1$^+$ cells was observed in BSS-treated corneas 2 and 5 days post-injury; the infiltration was not restricted to the limbal area, but extended to the central cornea (Fig. 19A). Dex-treated corneas had a significant decrease in Gr-1$^+$ cell counts compared to BSS corneas both days 2 and 5 after injury (Fig. 19A, B).

We measured MPO activity in corneal lysates in treated groups 2 days after the injury. Significantly higher MPO activity was seen in BSS treated corneas, and significantly lower MPO activity was present in Dex groups, confirming the IHC results (Fig. 19C).

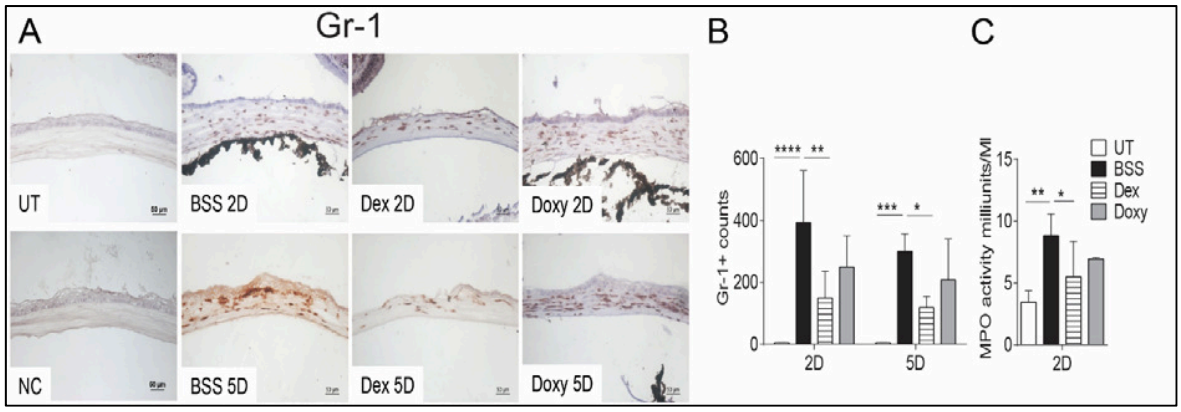

Fig. 19. Neutrophil infiltration decreases after anti-inflammatory therapy. Representative merged digital images of central cornea cryosections immunostained for IL-1β (in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in corneas subjected to combined model topically treated with BSS, Dex or Doxy. NC=negative control. Scale bar=50μm.
D. Representative images of Gr-1$^+$ cells (Reim *et al.*) of central cornea cryosections from animals subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy used to generate the bar graph showing counts in E.
E. Bar graphs (mean±SEM) of Gr-1$^+$ cell counts in whole cornea/groups (n=12/group)
F. Myeloperoxidase (Samtani *et al.*) activity in whole corneas lysates from animals subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy 2 days after injury (mean±SEM) (n=12/group).
*p<0.05; ***p<0.001; ****p<0.0001.
UT = untreated cornea, BSS = corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex = corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy = corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, D = days. NC=negative control

These results confirm that Dex treatment increases production of MMP-8 while at the same time decreases influx of neutrophils and production of myeloperoxidase that is associated with decreased CXCL1 expression.

Discussion

Alkali burns of the cornea are potentially blinding conditions. Alkali can penetrate rapidly into the eye and cause intense inflammation. There is a consensus that the prognosis of an alkali burn depends on the area of the insult, the penetration of the agent and early treatment controlling host response and inflammation. The visual outcomes from these injuries still remain poor in large part due to inadequate control of inflammatory and proteolytic components of the wound healing response, leading to corneal perforation, corneal opacification and corneal neovascularization (Kenyon, 1969; Kenyon *et al.*, 1979; Kenyon, 1985, 1989).

Dry eye can contribute to the severity of chemical and thermal injuries to the cornea, either during the initial healing phase following the burn or during the late phases. Dry eye can worsen the injury through a variety of events: reduced tear clearance and wash out, decrease provision of growth factors, decrease corneal sensation and corneal exposure (Pflugfelder *et al.*, 2008). Having all this in mind, we created a combined model where experimental dry eye is induced immediately after creation of alkali burn and anesthetic recovery.

Our results showed that inhibition of tear production and exposure to desiccating environment markedly worsened corneal inflammation and matrix degradation, leading to perforation in nearly 40% of eyes. This was accompanied by increased production of IL-1β, IL-6 and MMPs mRNA transcripts as well as increased MMP-9 activity and MMP-3, MMP-13 and MMP-9 immunoreactivity in corneal epithelium and increased frequency and function of neutrophils.

We observed that perforated corneas in the CM group showed detached epithelium, disrupted Bowman layer, and stromal ulceration. Although the basement membrane is not directly subjected to alkaline agents placed on the surface of the eye, its degradation by matrix metalloproteinases (collagenases and gelatinases) secreted by damaged or infiltrating cells contributes to the pathogenic ulceration and perforation of the stroma (Saika *et al.*, 2005). Stromal ulceration did not develop following alkali injury, in a standardized alkali burn rabbit model using sodium hydroxide concentrations equal to or below 1N (Ormerod *et al.*, 1989). Healing after alkali burn is characterized by extended inflammatory cell infiltration of the stroma, persistent epithelial defect, and degradation of the basement membrane and corneal neovascularization in late phases. In tissue sections from humans, different cell types can produce MMPs; neutrophils, stromal fibroblasts, and epithelial cells. It is important to note, however, that the cellular sources of collagenase appear to be somewhat species-dependent (Fini *et al.*, 1998). Studies in animal models of chemical or thermal injury reveal that there are very few fibroblasts in newly ulcerating corneas, due to cell death from the

initiating agent. In the alkali-burned cornea, the epithelium surrounding the wound has long been thought to participate in stromal destruction.

We observed a significant increase in MMPs -1,-8,-9 mRNA levels in the CM group compared to AB and DS. The activation of p38 MAPK alone has been shown to induce expression of MMP-1 and MMP-3 in an activator protein 1-independent manner by stabilizing the corresponding mRNAs in human skin fibroblasts (Capoulade *et al.*, 2001). We have previously reported that DS upregulates MMPs, in particular MMP-3, and MMP-9 transcripts in the corneal epithelium (Corrales *et al.*, 2006). The precise regulatory mechanisms inducing MMP production in wound healing have not yet been fully elucidated despite extensive investigations, but are thought to be stimulated by cytokines and growth factors, and cell-matrix or cell-cell interactions. During the repair period of cornea remodeling, the rate of collagen turnover is much higher than it is in the normal cornea (Fini *et al.*, 1998), suggesting the involvement of MMPs in the remodeling process. In a rabbit model of superficial or penetrating injury to the stroma, increased expression of gelatinase (MMP-2), collagenases (MMP-1, and -13), and stromelysins MMP-3 was found.

We found a significant increase in MMP-9 (both RNA and protein level, including activity) in corneas subjected to the combined model, peaking at 2 days post injury. MMP-9 has been implicated in both favoring and decreasing reepithelization: MMP-9KO has been shown to accelerate healing of corneal wounds (Mohan *et al.*, 2002), while delaying reepithelization in large cutaneous wound (Hattori *et al.*, 2009; Reiss *et al.*, 2010). In human cornea wounds, MMP-9 is expressed in basal epithelial cells at the leading edge of the migrating epithelial front closing the corneal wound.(Matsubara *et al.*, 1991) We have previously reported that human dry eye and experimental desiccating stress stimulates production of MMP–9, as well as other MMPs by the ocular surface epithelia (Luo *et al.*, 2005; Pflugfelder *et al.*, 2005; Corrales *et al.*, 2006; de Paiva *et al.*, 2006a). MMP-9 was found to degrade the tight-junction protein occludin and to decrease apical epithelial barrier function in the cornea (Pflugfelder *et al.*, 2005). MMP-9 in human tears has also been found to increase in a variety of ocular surface diseases, including sterile corneal ulceration (Afonso *et al.*, 1999; Gabison *et al.*, 2003; Leonardi *et al.*, 2003; Reviglio *et al.*, 2003; Sakimoto *et al.*, 2003). In a group of dry eye patients, we observed a strong positive correlation between tear MMP-9 activity and severity of corneal epithelial disease. Tear MMP-9 activity levels also correlated positively with reduced contrast visual acuity (Chotikavanich *et al.*, 2009). The significant increase in MMP-9 in the combined model (~100 fold) compared to AB alone may suggest that MMP-9 could be delaying wound healing and facilitating cornea melting. Increased expression of MMP-9 and gelatinase activity has been reported in melted corneas

from severe primary Sjögren Syndrome patients. (Brejchova *et al.*, 2009) Interestingly, other MMPs were also present in these melted specimens, (intense MMP-1, -7, and moderate MMP-3, -8 immunoreactivity was noted) (Brejchova *et al.*, 2009). The concentrations of IL-1β, IL-6, MMP-8 and MMP-9 were significantly up-regulated in the tear fluid of the ulcer patients, corroborating our findings (Sakimoto *et al.*, 2014).

Neutrophils are the first inflammatory cell responders to migrate towards the site of inflammation. Our results showed a significant influx of PMNs in the CM group, which coincides with a significant increase in CXCL1 in cornea. During the acute phase in corneal alkali burn, the PMNs began to infiltrate the stroma, exerting their phagocytic functions to clear cellular debris, but at the same time, neutrophils release their proteolytic contents, including MMPs 1,-8,-9 (Tiku *et al.*, 1986) that may cause collateral damage. They enter the cornea from the limbus and move centrally in the superficial stroma somewhat behind the regenerating epithelium (Li *et al.*, 1991). Collagenase breakdown products have been shown to be chemotactic for PMN (Seedor *et al.*, 1987). Strategies developed to decrease neutrophil infiltration have been shown to improve wound healing following corneal alkali injury (Kenyon *et al.*, 1979; Foster *et al.*, 1982). Neutrophil depleted C57BL/6 subjected to alkali burn healed faster than non-depleted controls, demonstrating bystander damage to host tissue by PMN (Ueno *et al.*, 2005).

Eye injuries can be very costly. In a 10 year review of eye injuries in the Army, Navy and Air Force, Buckingham and colleagues showed that the average military eye injury misfortune results in 4.7 to 6.1 days lost from work and has a cost ranging from $4,222 to $9,724. They also estimated that there was an underreporting of eye trauma data by at least 250% (Buckingham *et al.*, 2001, 2005). Another underestimated factor is the cost for ambulatory visits, which is estimated to vary from $8.9 million to $14 million among the Army, Navy and Air Force (Buckingham *et al.*, 2005). Therapeutic strategies for ocular surface chemical injuries have attempted to favorably modify one or more aspects of this process, during the acute phase. The visual outcomes from these injuries still remain poor in large part due to inadequate control of inflammatory and proteolytic components of the wound healing response (Fini & Girard, 1990; Solomon *et al.*, 2000; Kato *et al.*, 2006).

Therapeutic strategies for ocular surface chemical injuries have attempted to favorably modify the immune host response by decreasing inflammation during the acute phase. Treatments have shown some efficacy include corticosteroids, citrate, and oral doxycycline (Brown *et al.*, 1969; Newsome & Gross, 1977; Donshik *et al.*, 1978; Kenyon *et al.*, 1979; Pfister *et al.*, 1981; Pfister & Haddox, 1996; Davis *et al.*, 1997; Kato *et al.*, 2006; Saud *et al.*, 2012). The management of alkali burns is often lengthy. Corneal transplants may be needed

to preserve the globe or improve vision in eyes with corneal perforation or scarring and depending on the residual scarring and opacity (Brodovsky *et al.*, 2000; Bunker *et al.*, 2014).

Our study showed that corneas treated with Doxycycline healed faster than controls, had a small number of corneal perforations and decreased mRNA levels of MMP-9, MMP-13, TIMP-1, IL-1β and CXCL1 (at day 5) while decreasing immunoreactivity of MMPs-1, -9 and 13 and levels of gelatinases in the cornea. Doxycycline has been extensively used in vitro and in vivo to decrease MMPs after inflammatory stimuli (Gilbertson-Beadling *et al.*, 1995; Boyle *et al.*, 1998; Dursun *et al.*, 2001; de Paiva *et al.*, 2006a). Oral Doxycycline is a FDA-approved drug to treat periodontitis (Gu *et al.*, 2012), another disease where MMPs are involved. We have previously reported that doxycycline decreases levels of MMP-9 mRNA transcripts, and prevents DS-induced increase in inflammatory cytokines IL-1 and TNF-α (de Paiva *et al.*, 2006a). The inhibitory effect of doxycycline on MMP-9 was also confirmed on osmotically stressed human corneal epithelia cells (Pflugfelder *et al.*, 2005). Doxycycline functions as a noncompetitive inhibitor of MMPs by interacting with the zinc or calcium atoms within the structural center of these enzymes that is required for their stability (Samtani *et al.*, 2009). Chelation of these metal ions by Doxycycline could be responsible for the decreased activity of collagenolytic enzymes (Seedor *et al.*, 1987).

Dexamethasone significantly increased MMP-8 transcript levels while this was not observed in the Doxycycline and BSS treated groups. MMP-8 is rarely detected in corneal epithelium (Li, 2003), but reports have shown increased immunoreactivity in context of inflammation (O'Brien *et al.*, 2001; Reviglio *et al.*, 2003). MMP-8, MMP-9 and lipocalin-2 are some of the many proteins stored in peroxidase negative granules in neutrophils (Faurschou & Borregaard, 2003). MMP-8 facilitates neutrophil infiltration in many models, including LPS stimulation of the cornea (Quintero *et al.*, 2010) (Lin *et al.*, 2008; Lenglet *et al.*, 2013). However, MMP-8 deficient mice showed delayed healing of cutaneous skin wounds that was accompanied by increased inflammation and expression of MMP-1, MMP-13 and MMP-9 (Gutierrez-Fernandez *et al.*). Based on these findings, we are planning future experiments to investigate the role of MMP-8 during Dexamethasone treatment in the combined model of alkali burn and dry eye.

We observed an increase in NGAL immunoreactivity in corneal epithelium and a 125-kDa band in the zymography that was markedly inhibited by Dexamethasone and moderately by Doxycycline compared to BSS vehicle. This 125kDa corresponds to the NGAL-MMP-9 complex (Gupta *et al.*, 2007; Kubben *et al.*, 2007). NGAL binds covalently to MMP-9 and this complex has been reported to protect MMP-9 from degradation and preserve MMP-9 activity in a concentration-dependent manner (Yan *et al.*, 2001; Kubben *et al.*, 2007). Levels of serum

NGAL-MMP-9 complex were increased in patients with active ulcerative colitis, correlated with neutrophil infiltration and it has been proposed as a surrogate marker of mucosal healing (de Bruyn *et al.*, 2014). NGAL immunoreactivity increased in the colonic epithelium in inflammatory and neoplastic, colorectal diseases (Nielsen *et al.*, 1996). Increased levels of NGAL-MMP-9 have been reported in tears upon awakening (Markoulli *et al.*, 2012) and observed in tears of Sjögren Syndrome patients (Solomon *et al.*, 2001). Therefore, the presence of NGAL-MMP-9 complex in BSS treated corneas may indicate a mechanism to preserve MMP-9 activity. Dexamethasone has another mechanism of action in addition to decreasing the MMP-9 itself.

Dexamethasone treatment paradoxically decreased TIMP-1 while Doxycycline treatment did not. We did not found any increase in the other TIMPs. TIMP-1 binds MMP-9, while TIMP-2 binds MMP-2 and MMP-14. Once recognized as negative regulators of MMPs, TIMPs are now recognized as bi-functional regulators (Gordon *et al.*, 2011). TIMP-2 exogenous application resulted stimulated wound closure (Terasaki *et al.*, 2003), while overexpression of TIMP-1 in a transgenic mouse of cutaneous wound decreased re-reepithelization.

Our results showed that topical steroid therapy greatly decreases inflammatory cytokines (IL-1β and IL-6), chemokine (CXCL1) and expression and activity of MMPs. It is interesting to note that Dexamethasone was more potent than Doxycycline. Dexamethasone greatly preserved corneal clarity, decreased neutrophil infiltration and expression of the neutrophil chemoattractant, CXCL1. Our results are in agreement with Saud and colleagues who showed an improvement in corneal opacity with no corneal perforations in rabbits subjected to alkali burn and subconjunctival injections of the corticosteroid triamcinolone (Saud *et al.*, 2012).

Davis and colleagues showed that treatment of human alkali burns with topical steroids for longer than 10 days after the initial injury was safe and did not induce corneal melting (Davis *et al.*, 1997). Among the anti-inflammatory treatments after alkali burn, the use of a topical steroid is by far the most controversial, specifically its use after the 6th day post-injury. While several animal models (rabbits and more recently, mice) have been used to study alkali burns, there is no consensus regarding the strength of alkali used to induce the burn (0.5N, 1N, 2N of NaOH), mode of application (pre-wet discs vs. topical instillation of alkali as eyedrops) or the duration of the contact (in case of pre-wetted discs, 30 seconds to 2 minutes) and volume of irrigation agents after the injury (from “brief irrigation” to “copious”, to “2 liters”). The diversity in models makes generalization more difficult regarding steroid-induced corneal perforations. While Donshik and colleagues described that treatment with

Dexamethasone sodium phosphate was just partially efficacious in preventing deep corneal ulcers and descemetoceles in rabbits, they used a strong (2N) sodium hydroxide-embedded filter paper and very brief rinsing (Donshik *et al.*, 1978). Chung and colleagues showed that in a milder model in rabbits (0.1N NaOH filter discs), 0.1% Dexamethasone was able to control inflammation without inducing corneal perforation, although it retarded corneal reepithelization (Chung *et al.*, 1998).

The type of corneal injury is also important to take into consideration. Thermal burns treated early with either Medroxyprogesterone or Prednisolone showed a significant reduction on deep ulcers in albino rabbits compared to vehicle-treated corneas, but not if treatment was started 6 days post-injury (Phillips *et al.*, 1983). Another consideration is the animal itself. It seems that alkali burns in rabbits will evolve to perforation more frequently than in mice. We have not observed corneal perforations after alkali burns in mice unless these mice are subjected to a desiccating stress and cholinergic blockade (de Paiva *et al.*, 2011). In our experiment with prolonged dosing of Dexamethasone (up to 21 days), we did not observe any corneal perforations. Another interesting consideration when evaluating corneal wounds is the size of wound itself. In our preliminary results, we observed that large corneal burns (>3mm) induced greater production of MMPs than smaller burns (2mm; data not shown). So it is possible that efficacy of anti-inflammatory agents will also vary according to the size of initial lesion.

Conclusions

Taken together our results showed that the stroma ulceration following alkali burn is not simply the passive breakdown of alkali denatured collagen and proteoglycans, is a complex process involving interactions between different cell types, regulation of collagenases, cytokines and a chemokine. Moreover, they show that alkali injury in the context of a dry ocular surface dramatically exacerbates the inflammatory response and increases the rate of corneal perforation. This combined model is therefore more severe than regular alkali burn and can be used to study severe devastating ocular injuries and facial burns that reach the eye, ocular injuries secondary to warfare agents and the effects of prolonged stay in environmental controlled low humidity ICU on disease severity. Topical anti-inflammatory therapy appears to be a valuable addition to inhibit sight threatening corneal ulceration and perforation.

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Appendices

May 08, 2015

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AN-5076: REGULATION OF PROTEASE ACTIVITY ON THE OCULAR SURFACE AFTER CHEMICAL INJURY

APPROVAL VALID FROM September 03, 2014 thru September 02, 2017

Dear Dr. DE PAIVA

The animal care and use of your research protocol indicated above has been approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Baylor College of Medicine has an Animal Welfare Assurance on file with the Office for Protection from Research Risks of the National Institutes of Health. The Assurance number is 3823-01.

As principal investigators of this research, you are required to provide a copy of the approved protocol and all subsequently approved amendments to each individual working on this project for their reference.

You will be notified should any U.S.D.A., Baylor College of Medicine, VAMC (if applicable) or other policies come into effect that would require you to amend your project.

Respectfully,

FREDERICK A PEREIRA, Ph.D.
Institutional Animal Care and Use Committee

Published Manuscript

Desiccating Stress-Induced MMP Production and Activity Worsens Wound Healing in Alkali-Burned Corneas

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Submitted: February 6, 2015
Accepted: June 12, 2015

Citation: Bian F, Pelegrino FSA, Pflugfelder SC, Volpe EA, Li D-Q, de Paiva CS. Desiccating stress-induced MMP production and activity worsens wound healing in alkali-burned corneas. *Invest Ophthalmol Vis Sci*. 2015;56:XXX–XXX. DOI:10.1167/iovs.15-16631

Purpose. To evaluate the effects of dry eye on ocular surface protease activity and sight threatening corneal complications following ocular surface chemical injury.

Methods. C57BL/6 mice were subjected to unilateral alkali burn (AB) with or without concomitant dry eye for 2 or 5 days. Mice were observed daily for appearance of corneal perforation. Whole corneas were harvested and lysed for RNA extraction. Quantitative real-time PCR was performed to measure expression of inflammation cytokines, matrix metalloproteinases (MMP). Matrix metalloproteinase–9 activity, gelatinase activity, and myeloperoxidase (MPO) activity were evaluated in corneal lysates. Presence of infiltrating neutrophils was evaluated by immunohistochemistry and flow cytometry.

Results. Eyes subjected to the combined model of AB and dry eye (CM) had 20% sterile corneal perforation rate as soon as 1 day after the initial injury, which increased to 35% by 5 days, delayed wound closure and increased corneal opacity. Increased levels of IL-1β, -6, and MMPs-1, -3, -8, -9, and -13, and chemokine (C-X-C motif) ligand 1 (CSCL1) transcripts were found after 2 days in CM compared with AB corneas. Increased MMP-1, -3, -9, and -13 immunoreactivity and gelatinolytic activity were seen in CM corneas compared with AB. Increased neutrophil infiltration and MPO activity was noted in the CM group compared with AB 2 days post injury.

Conclusions. Desiccating stress worsens outcome of ocular AB, creating a cytokine and protease storm with greater neutrophil infiltration, increasing the risk of corneal perforation.

Keywords: alkali injury, dry eye, neutrophils, MMPs, ocular perforation

Eye injuries in the military and civilian population are frequent events.[1–4] The main causes of eye injuries in the military has changed with changes in strategy and weaponry. These include sharp, blast fragmentation, penetrating, and perforating injuries of the globe, occasionally with intraocular foreign bodies to ocular surface chemical, thermal, nuclear, and laser burns.[5,6] Sulfur mustard gas can potentially cause severe ocular surface injury, including blepharospasm, lid edema, and corneal ulceration. This chemical warfare agent has been in several military conflicts, including the recent Iran–Iraq War.[7,8]

Barely detected in an unwounded cornea, matrix metalloproteinases (MMPs) are strongly induced during wound healing and chemical burns. The MMP family includes more than 25 members that can be divided into collagenases (MMPs-1, -8, and -13, that degrades collagen types I, II, and III); gelatinases (MMP-2 and -9 that degrade collagen types IV, V, VII, and X as well as decorin, fibronectin, and laminin); stromelysins (MMP-3 and -10); matrilysins (MMP-7 and -26 that degrade proteoglycans, laminin, and glycoproteins substrates); and the membrane-type MMPs that are bound to epithelial cell membranes, and can activate MMPs, according to their structure and substrate specificity (MMP-14 to -17 and -24).[9–12] Collectively, these MMPs are able to degrade the entire extracellular matrix and basement membranes components.

Both chemical and thermal injuries to the surface of the eye have a high potential to cause blindness. These injuries often damage the epithelium covering the cornea, conjunctiva, and eyelid margins and in more severe cases destroy the stem cells that renew these epithelia. In many cases, the supporting stromal cells and matrix are damaged and chronic inflammation is induced. Furthermore, most patients with severe ocular surface injuries develop a secondary dry eye due to destruction of tear producing cells, which has the potential to worsen the outcome. In addition to lubricating the ocular surface, the tears contain numerous growth and anti-inflammatory factors that are essential for wound repair and suppressing inflammation and tissue destruction.

The controlled desiccating environment many of us live in is under recognized as an initiator of dry eye–induced ocular surface inflammation. Virtually all modern office buildings are air-conditioned, often with high flow, low humidity air. Low humidity (in offices, aircraft cabins, deserts, and winter season) impacts tear film and ocular health.[13] While it is currently known that low humidity exposure increases tear evaporation rate and increases frequency of eye irritation,[14,15] specific inflammatory pathways/mediators that are stimulated are poorly understood.

The purpose of this study was to study the effects of dry eye on ocular surface protease activity following corneal chemical injury. A combined model of alkali burn (AB) and dry eye was

used to evaluate the effects of experimental dry eye on inflammatory cytokines, chemokines, and MMPs (-1, -2, -3, -8, -9, -12, and -13) after ocular surface chemical injury and the consequences of increase protease activity on corneal epithelial healing.

Materials and Methods

Animals

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine (Houston, TX, USA), and it conformed to the standards in the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Unilateral Alkali Burn

After systemic anesthesia with isoflurane using a vaporizer (SomnoSuite; Kent Scientific, Torrington, CT, USA), a unilateral AB was created on the right eye of 6- to 8-week-old C57BL/6 mice. This was achieved by placing one 2-mm diameter filter paper disc that had been presoaked with 1N NaOH on the central cornea for 10 seconds, followed by extensive rinsing with balanced salt solution (Alcon, Fort Worth, TX, USA), as previously described.[16] Precautions were taken to avoid damage to the peripheral cornea, conjunctiva, and lids. Alkali burn was created at day 0 and animals were euthanized after 2 or 5 days. A separate group of animals (n = 6) were followed for up to 21 days. The contralateral eyes served as untreated (UT) controls. A separate group of mice that received unilateral AB were also subjected to desiccating stress (DS) after anesthetic recovery for 2 or 5 days (AB+DS, referred to as the combined model = CM). The contralateral eyes served as desiccating stress controls (not AB, but in a low humidity environment).

Desiccating stress was induced in female C57BL/6 mice aged 6 to 8 weeks by sterile subcutaneous injection of 0.5 mg/1 mL scopolamine hydrobromide (Sigma-Aldrich Corp., St. Louis, MO, USA) quater in die (QID) into alternating flanks and exposure to a drafty low humidity (<30% relative humidity) environment for 2 or 5 days (DS, DS2, and DS5, respectively) as previously described.[17] Mice subjected to AB or CM received no eyedrops or antibiotics.

At least 45 animals without corneal perforations were used per group (AB and CM) and per time point: 12 for histology (6 for frozen sections and 6 for paraffin), 12 for corneal opacity and wound closure, 15 for real-time PCR, 4 for myeloperoxidase assay, 4 for MMP-9 assay, and 10 for flow cytometry. Corneal opacity and wound closure rate were evaluated in 12 live mice that were used for either histology, PCR, or flow cytometry. Contralateral eyes in the AB group were used as untreated controls; while contralateral eyes in CM group were used as DS controls. Whenever an ocular perforation was observed, mice were euthanized, and removed from the study with the exception of the histology as shown in Figure 1.

Clinical Findings: Ocular Perforation and Opacity Score

All eyes in each treatment group were examined daily under a microscope (SMZ 1500; Nikon, Melville, NY, USA) for the presence of any corneal perforation. Once corneal perforation was observed, mice were euthanized. Perforated corneas were not used for any experiments other than the histology. The number of corneal perforations occurring each day was recorded and survival curves were calculated using Graph Pad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA).

Corneal edema and opacity were graded by two masked observers in images taken with a microscope equipped with a color digital camera (DS-Fi1; Nikon) by the method described by Yoeruek.[18] Corneal opacity was scored using a grading scale of 0 to 4: grade 0 = completely clear; grade 1 = slightly hazy, iris and pupils easily visible; grade 2 = slightly opaque, iris and pupils still detectable; grade 3 = opaque, pupils hardly detectable, and grade 4 = completely opaque with no view of the pupils.

Measurement of Corneal Epithelial Defect

Corneal epithelial healing was assessed daily in the experimental groups (four individual right corneas/group/experiment; three sets of experiments). Briefly, 1 μL 0.1% liquid sodium fluorescein was instilled onto the ocular surface. Corneas were rinsed with PBS and photographed with a stereoscopic zoom microscope (SMZ 1500; Nikon) under fluorescence excitation at 470 nm (DS-Qi1Mc; Nikon). Corneal epithelial defects were graded in digital images by two masked observers in a categorical manner (present/absent) to generate a survival curve. Biological replicate scores were transferred to excel database where the results were analyzed.

Ocular Cultures

Cornea and conjunctiva of mice that developed corneal perforations were swabbed using sterile cotton swabs and plated on sheep blood agar and transported to the microbiology laboratory at the Methodist Hospital (Houston, TX, USA) for routine bacterial and fungal cultures to identify any potential microbial infection as cause for the corneal perforation. Culture plates were read each day for the first 2 days and then again at the seventh day after seeding.

Histology and Immunostaining

Mice were euthanized either 2 or 5 days after initial injury. Eyes and adnexae (n = 6/experimental group/time point) were surgically excised, fixed in 10% formalin, paraffin embedded, and 8-μm tissue sections were cut. These sections were stained with hematoxylin and eosin (H&E) for evaluating morphology and inflammatory signs. They were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon).

For immunohistochemistry, additional eyes and adnexae from each group/time point (n = 6/experimental group/time point) were excised, embedded in optimal cutting temperature compound (VWR, Suwanee, GA, USA), and flash frozen in liquid nitrogen. Sagittal 8-μm tissue sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at −80°C. Immunohistochemistry was performed to detect neutrophils using rat anti-Gr-1 antibody (Ly6G, 1:250, clone 1A8; BD Pharmingen, San Diego, CA, USA). Cryosections were stained with the primary antibody and appropriate biotinylated secondary antibody (1:100 biotin goat anti-rat; BD Pharmingen) using a Vectastain Elite ABC kit and Nova Red reagent (Vector, Burlingame, CA, USA). Six sections from each animal/group/time point were examined and photographed with microscope equipped with a digital camera (Eclipse E400 with a DS-Qi1Mc; Nikon). The numbers of Gr-1 positive (+) cells were counted in cornea sections from each animal at ×20 magnification and results were averaged and expressed as the number of positive cells per cornea.

Immunofluorescent staining for IL-1β and MMPs was performed in frozen tissue sections with rabbit polyclonal antibody anti–MMP-1 (1:50, NBP1-77209; Novus Biologicals, (Littleton, CO, USA), anti–MMP-9 (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), anti–IL-1β (1:100; Upstate-Millipore Corp., Bedford, MA, USA), and goat anti–MMP-3 and

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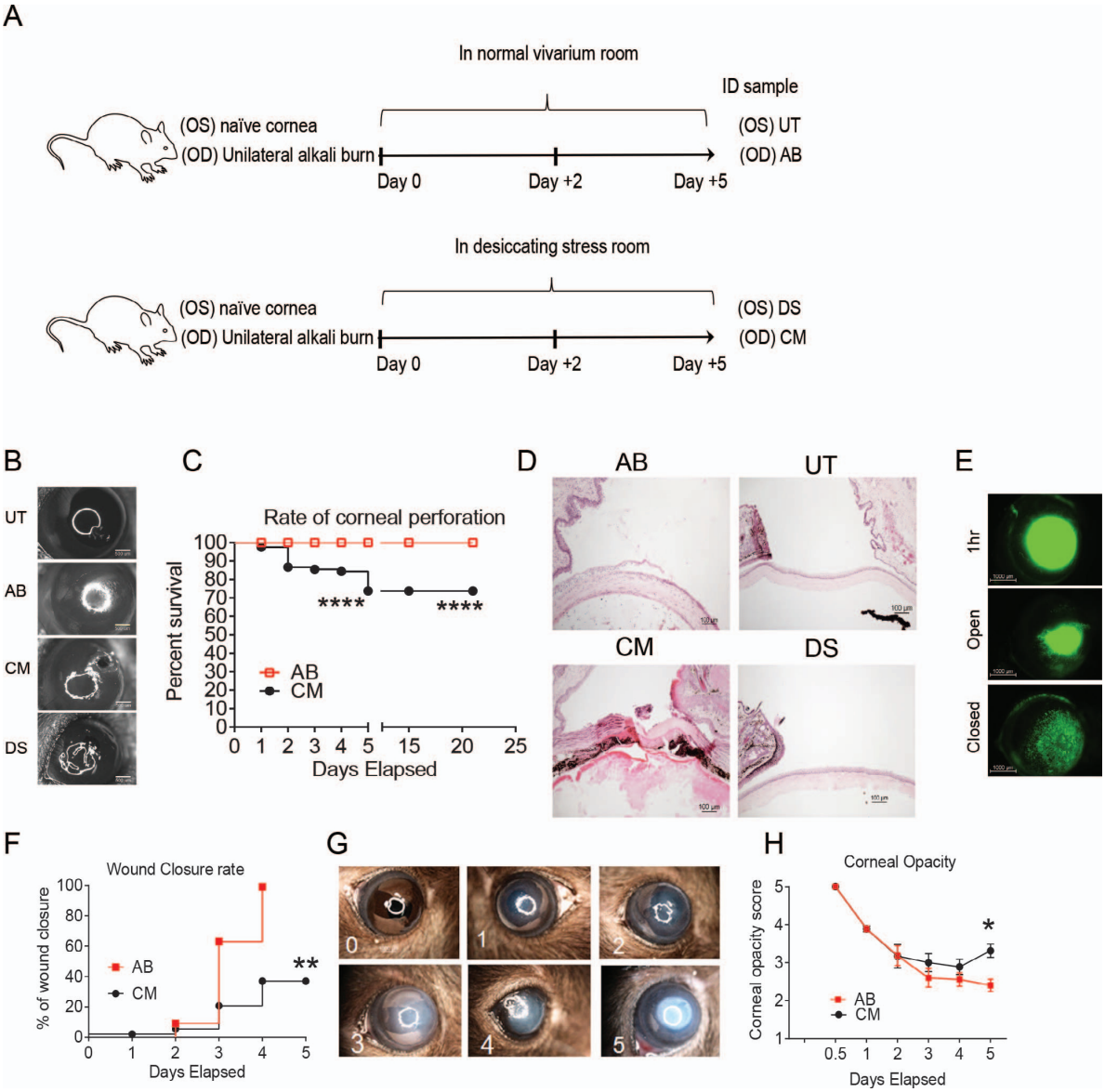

Figure 1. Dry eye jeopardized cornea integrity after AB injury. (**A**) Schematic of experimental design. A unilateral AB in the right cornea was performed as described in the Materials and Methods. Mice were then kept in a normal vivarium room (resulting in AB and UT corneas) or subjected to DS in a specially designed room (resulting in DS or combined model [CM = AB + DS] corneas). (**B**) Digital images of bright field pictures from control, AB, CM, and DS 5 days post injury. Note: perforation in the combined group. (**C**) Rate of ocular perforation in eyes subjected to AB and CM. (**D**) Representative H&E staining of cryosections from control, AB, CM, and 5 days post injury. Perforated CM corneas developed massive inflammatory infiltration forming waves of inflammatory cells within the cornea stroma. Cornea perforation is sealed by lens and iris. Alkali-burned corneas had loose epithelium and moderate inflammatory cells in the cornea stroma and anterior chamber. Original magnification: ×10. (**E**) Representative digital images of corneas 1 hour after creation of AB lesion and representative pictures of open and closed wounds 2 and 5 days after initial lesion used to generate the survival curve of corneal re-epithelization seen in (**F**). (**F**) Survival rate of wound closure in AB and CM groups. (**G**) Representative bright field digital images of corneas showing opacification scale used to generate the graph seen in ([**H**]; corneal opacity score). (**H**) Corneal opacity score in AB and CM groups. *P < 0.05; **$P < 0.01$; ****$P < 0.0001$.

anti–MMP-13 (1:100, SC6839 and SC-123630, respectively, both from Santa Cruz Biotechnology). Secondary goat-anti rabbit or donkey anti-goat Alexa-Fluor 488 conjugated IgG antibodies were used, as previously described.[19] The images were captured and photographed by a Nikon fluorescence microscope (Eclipse E400 equipped with a DS–F1 digital camera).

RNA Isolation and Quantitative PCR

Five whole corneas/group/time point/per experiment (total of three independent experiments) were excised, minced, and total RNA was extracted using a Qiagen MicroPlus RNeasy isolation Kit (Qiagen, Valencia, CA, USA) according to the

TABLE. Taqman Probes Used for Real-Time PCR

Gene Name	Symbol	Assay ID*
Interleukin 1 alpha	*IL1-α*	Mm00439620
Interleukin 1 beta	*IL1-β*	Mm00434228
Interleukin 6	*IL-6*	Mm99999064
Chemokine (C-X-C motif) ligand 1	*CXCL1*	Mm04207460
Matrix metalloproteinase 1	*MMP-1*	Mm00473493
Matrix metalloproteinase 2	*MMP-2*	Mm00439506
Matrix metalloproteinase 3	*MMP-3*	Mm00440295
Matrix metalloproteinase 8	*MMP-8*	Mm00439509
Matrix metalloproteinase 9	*MMP-9*	Mm00442991
Matrix metalloproteinase 12	*MMP-12*	Mm00500554
Matrix metalloproteinase 13	*MMP-13*	Mm00439491
Hypoxanthine guanine phosphoribosyl transferase 1	*Hprt-1*	Mm00446968

* Identification number from Life Technologies (in the public domain, www.lifetechnologies.com).

manufacturer's instructions, quantified by a NanoDrop ND-2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at −80°C. First-strand cDNA was synthesized with random hexamers by M-MuLV reverse transcription (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Inc., Arlington Heights, NJ, USA), as previously described.[20]

Real-time PCR was performed with specific Taqman minor groove binder (MGB) probes (Applied Biosystems, Inc., Foster City, CA, USA) and PCR master mix (Taqman Gene Expression Master Mix), in a commercial thermocycling system (StepOnePlus Real-Time PCR System; Applied Biosystems), according to the manufacturer's recommendations. Quantitative real-time PCR was performed using gene expression assay primers and MGB probes specific for murine targets as described in the Table. The hypoxanthine guanine phosphoribosyl transferase (*HPRT-1*) gene was used as an endogenous reference for each reaction to correct for differences in the amount of total RNA added. The results of quantitative PCR were analyzed by the comparative cycle threshold (C_T) method where target change $= 2^{-}(\triangle)(\triangle)^{C}{}_{T}$. The results were normalized by the C_T value of HPRT-1 and the relative mRNA level in the untreated group was used as the calibrator.

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity was measured using a myeloperoxidase colorimetric activity assay kit as described by the manufacturer (Sigma-Aldrich Corp.). Briefly, whole-cornea lysates from AB, CM, or control corneas ($n = 4$ samples/group/time point) were homogenized in MPO assay buffer and the homogenate was centrifuged at 14,000*g* for 20 minutes at 4°C. Total protein concentration was measured by the BCA protein assay as previously described.[17] A 50 μg/sample was mixed with MPO assay buffer and MPO substrate, incubated at room temperature for 2 hours, and then mixed with tetramethylbenzidine probe. Fluorescence was measured at 412 nm using a Tecan Infinite M200 plate reader (Tecan, Inc., San Jose, CA, USA) equipped with Magellan V6.55 software (Tecan, Inc.). Biological replicate samples were averaged. Results are presented as mean ± SEM (milliunits).

MMP-9 Activity Assay

Whole corneas were excised, rinsed, and homogenized in RIPA buffer and the homogenate was centrifuged at 14,000*g* for 20 minutes at 4°C. Total protein concentration of the whole-cornea cell lysate ($n = 4$/group/time point) was measured by the BCA protein assay as previously described.[17] Total MMP-9 enzyme activity was measured with a MMP activity assay kit (Biotrak; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol as previously published.[21] In brief, 100 μL each pro–MMP-9 standard (0.125–4 ng/mL), 50 μg cornea extract and assay buffer (for blanks) were incubated at 4°C overnight in wells of a microtiter plate precoated with anti–mouse-MMP-9 antibodies and washed. Total MMP-9 activity was measured by activating bound pro–MMP-9 with 50 μL 0.5 mM p-amino phenylmercuric acetate (APMA) in assay buffer at 37°C for 1.5 hours, followed by incubation with a detection reagent at 37°C for 2 hours. Active MMP-9 was detected through its ability to activate a modified prodetection enzyme that subsequently cleaved to its chromogenic peptide substrate. Absorbance was read at 405 nm using a Tecan Infinite M200 plate reader equipped with Magellan V6.55 software. The activity of MMP-9 in a sample was determined by interpolation from a standard curve. Biological replicates were averaged and the results were presented as mean ± SEM (pg/mL).

Gelatin Zymography

The relative amount of MMP-9 in whole-cornea lysates ($n = 4$/group) was measured by gelatin zymography, using a previously reported method.[22,23] Whole-cornea lysates prepared for MMP-9 activity (20 μg/sample) were fractionated in an 8% polyacrylamide gel containing gelatin (0.5 mg/mL). The gels were soaked in 0.25% Triton X-100 for 30 minutes at room temperature, then incubated in a digestion buffer containing 5 mM phenylmethyl sulfonyl fluoride at 37°C overnight. They were stained with 0.25% Coomassie brilliant blue R-250 in 40% methanol for 2 hours, then destained overnight in 10% acetic acid. Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

Flow-Cytometry Analysis of Infiltrating Cells in Cornea

Single-cell suspensions of corneas ($n = 5$/group/time point/experiment, for a total of two experiments) were excised, rinsed, and prepared by treatment of minced tissue fragments with 0.1% collagenase D (60 minutes at 37°C; Invitrogen-Gibco, Carlsbad, CA, USA) and sequentially neutralized with media and filtered, then resuspended and stained with anti-CD16/32 (BD Pharmingen), followed by cell-surface staining with FITC-F4/80 (clone BM8; Biolegend, San Diego, CA, USA), PE-Gr-1 (clone 1A8; BD Pharmingen), and APC-CD45 (clone 30-F11; BD Pharmingen). Single-cell preparations of bone marrow cells obtained from control mice were stained with the same antibodies and served as positive controls. Cells were kept on ice until flow cytometry analysis was performed. A BD LSRII Benchtop cytometer was used. The gating strategy was as follows: lymphocytes were individually identified on the basis of forward scatter and side scatter properties, subsequently gated on the basis of forward scatter height versus forward scatter area (singlets 1), then gated on side scatter height versus side scatter area (singlets 2), followed by CD45 histogram gating. Propidium iodide exclusion was used to discriminate live cells. At least 50,000 events were collected. Results were analyzed with BD Diva software (version 2.1; BD Pharmingen) and FlowJo software (version 10.07; Tree Star, Inc., Ashland, OR, USA).

Statistical Analysis

Results are presented as the mean ± SEM. Two-way ANOVA with Bonferroni post hoc testing was used for statistical comparisons of gene expression. *P* less than or equal to 0.05

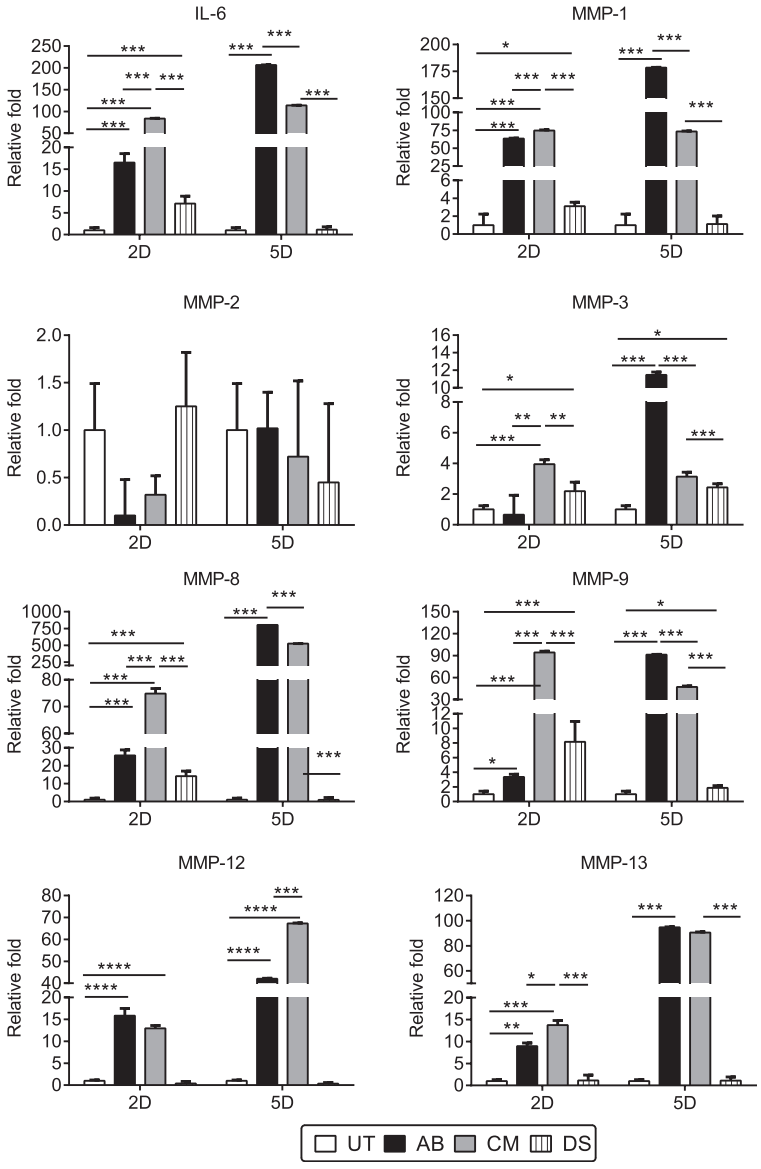

Figure 2. Early increase cytokine and MMP storm in combined model corneas. Relative fold of expression of IL-6 and MMPs-1, -2, -3, -8, -9, -12, and -13, in whole corneas subjected to AB or CM. *Bar graphs* show means ± SEM of one representative experiment with five samples per group/time point (experiment was repeated thrice with similar results). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

was considered statistical significant. These tests were performed using GraphPad Prism 6.0 software.

Results

Concomitant Alkali Burn and Desiccating Stress Leads to Ocular Perforation

Because environmental dry eye is an under recognized variable in the management of severe burned patients, including the ones treated in intensive care units, we have developed a CM of AB and dry eye by subjecting mice to desiccating stress immediately after anesthetic recovery following creation of AB (Fig. 1A). We observed that eyes subjected to CM perforated as early as 2 days post initial injury (20%) and the rate of perforation in some experiments was up to 40% by day 5 (Figs. 1B, 1C), while eyes subjected to AB alone did not perforate. Naïve and DS corneas did not perforate at all (data not shown). Corneas that did not perforate in the initial 5 days post injury did not perforate at later time point (even up to 21 days), showing that the initial inflammatory has the greatest effect on corneal integrity (Fig. 1C). Histologically, perforated corneas subjected to the CM had collapsed anterior chamber, iris, and lens tamponade, total loss of corneal epithelium and massive infiltration of corneal stroma by inflammatory cells that dissected into the cornea from the limbus (Fig. 1D). Microbial cultures of perforated corneas and conjunctivas ($n = 6$)

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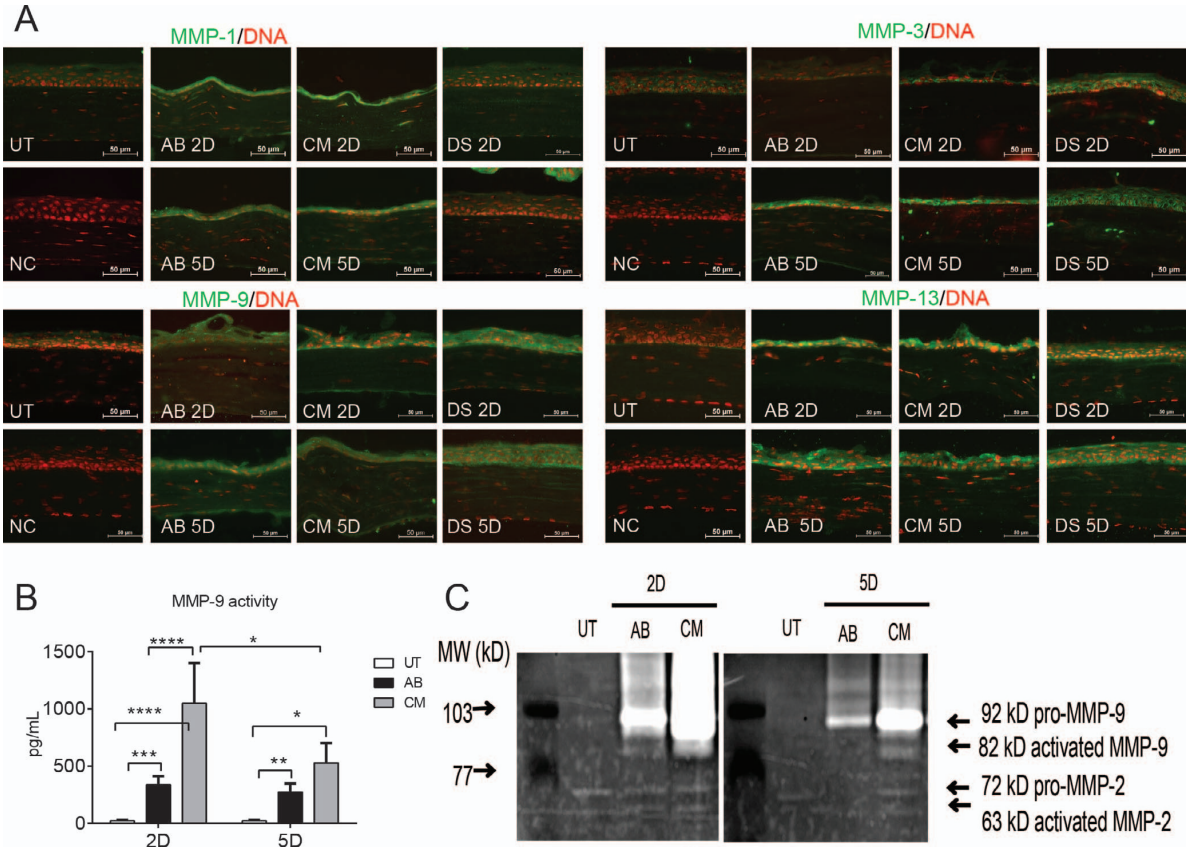

Figure 3. Increased MMP protein expression in combined model of AB and dry eye. (**A**) Representative merged digital images of corneas cryosections immunostained for MMP-1, -3, -13, and -9 (all in *green*) with propidium iodide nuclei counterstaining (*red*) in corneas subjected to AB, DS, or CM. *Scale bars*: 50 μm. (**B**) Matrix metalloproteinase–9 activity in whole-cornea lysates in AB, CM, and untreated group. (**C**) Representative zymogram showing MMP-9 bands whole-cornea lysates in the treatment groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

showed no microorganism growth, confirming the sterile nature of the perforation. Eyes subjected to AB had a central corneal epithelial defect and moderate inflammatory infiltration in the corneal stroma, but no perforation.

We evaluated wound closure by staining corneas with 0.1% fluorescein daily and generated a survival curve of wound closure based on presence/absence of epithelial wound closure, independent of the area left to be closed (Figs. 1E, 1F). The CM corneas had delayed re-epithelization compared to AB alone (40% vs. 100% by day 5, respectively, Fig. 1E). We also graded daily corneal opacification in digital images captured daily by masked examiners using a scale from 0 (transparent cornea) to 5 (complete opaque cornea, Fig. 1G). We observed higher residual cornea opacification in the CM group compared with corneas subjected to AB alone (Fig. 1H).

Taken together, these results show that combining dry eye with AB induces sterile corneal perforation in in up to 40% of the cases, delays wound healing and increases corneal opacity.

Cytokine and MMPs Storm in the Combined Model

It has been recognized for decades that ocular surface chemical/thermal injury stimulates production of tissue degrading enzymes as part of the wound healing cascade.[16,22,24–26] Matrix degrading enzymes, including MMPs have been identified as important factors in the inflammatory and wound healing response of the ocular surface, particularly in dry eye and ocular burns. Their induction during wound healing is thought to play a role in extracellular matrix remodeling, cytokine activation, and regulation of angiogenesis.

Because most of cornea perforations and greatest differences in cornea wound healing were seen early (at 2 and 5 days post injury), we evaluated the levels of RNA transcripts encoding IL-1α, IL-6, and MMPs-1, -2, -3, -8, -9, -12, and -13, by real-time PCR using whole corneas harvested from all groups at these same time points. Our results are presented in Figure 2.

Two days post injury, both AB and CM groups had significantly elevated expression of IL-6 and MMPs compared with untreated corneas; however, CM significantly increased IL-6 (83- vs. 17-fold), MMP-1 (75- vs. 63-fold), MMP-3 (4- vs. 0.7-fold), MMP-8 (75- vs. 26-fold), MMP-9 (95- vs. 4-fold), MMP-13 (14- vs. 9-fold) mRNA transcripts compared with AB alone. Desiccating stress corneas had upregulation of IL-6, MMPs-1, -3, -8, and -9 compared with naïve corneas, however, this increase was significantly lower than the one observed in the AB and CM corneas. Five days post injury, AB corneas had significantly elevated IL-6 and MMPs-1, -3, -8, and -9, mRNA transcripts compared with the CM group (Fig. 2). Interleukin-1α was equally elevated in both CM and AB groups after 2 and 5 days post injury (2.7- vs. 1.7-fold and 2.5- vs. 3-fold, respectively)

compared with control corneas (data not shown). Desiccating stress corneas had significantly elevated expression of MMP-3 and -9 after 5 days of desiccating stress, as we previously reported.[17,19,27]

Matrix metalloproteinase–12, also known as macrophage elastase, and produced by macrophages, fibroblasts, and epithelial cells[28–31] was equally elevated in both AB and CM corneas 2 days after injury (15.85 ± 1.66– vs. 12.97 ± 0.65–fold) but at 5 days its expression significantly increased in the CM group (42.03 ± 0.23– vs. 67.30 ± 0.18–fold, $P < 0.001$).

The immunoreactivity of corneas to collagenases (MMP-1 and -13[32]), MMP-3 (a physiological activator of MMP-9[33]), and -9 were evaluated by immunostaining (Fig. 3A). Minimal staining of MMPs-1, -3, -9 and -13 was noted in the control corneas, while increased immunoreactivity was observed in the corneal epithelium of CM, AB, and DS corneas (DS < AB < CM).

Among the MMPs, MMP-9 plays a prominent role being produced by stressed cornea and conjunctival epithelial cells and has both matrix degrading and proinflammatory activities.[34–36] Matrix metalloproteinase–9 have been reported to delay corneal wound healing.[37] We evaluated MMP-9 activity in whole-cornea lysates using an MMP-9 activity assay and observed that the CM group had significantly higher levels of active MMP-9 than AB both at day 2 and day 5 post injury (Fig. 3B). Gelatin zymography showed increased amounts of both pro and active MMP-9 bands in the CM and AB groups compared with control corneas (Fig. 3C); however, as suspected from the MMP-9 activity assay, greater amounts of pro and activated MMP-9 bands were present in the CM group. Negligible amounts of MMP-2 were present in AB and CM groups and they did not differ from untreated corneas.

These results indicated that the early peak of inflammatory cytokines and MMPs in the CM group coincides with the time period where most of the corneal perforations occur, indicating that the early additive effect of dry eye with AB jeopardizes corneal integrity.

Increased Infiltration of Neutrophils in the CM Group

Polymorphonuclear leukocytes (neutrophils) are the first inflammatory cells to migrate into sites of tissue injury. The pathogenic role of neutrophils in AB can be appreciated as neutrophil-depleted C57BL/6 corneas subjected to AB healed faster than nondepleted controls.[38]

Interleukin-1 is a cytokine produced by epithelium, fibroblasts, and neutrophils[39,40] and participates in collagen degradation by stimulating neutrophils.[41] The chemokine chemokine (C-X-C motif) ligand 1 (CXCL1) is crucial for the recruitment of neutrophils to inflammatory sites.[42] We evaluated expression of IL-1β and CXCL1 in corneas subjected to AB and CM and compared with controls. The CM significantly increased IL-1β (72- vs. 11-fold) and CXCL1 (27- vs. 5-fold) 2 days after injury compared with AB alone. At 5 days, IL-1β levels maintained elevated in CM (393- vs. 267-fold) and similar levels of CXCL1 were seen 5 days post injury in both models (~20-fold; Fig. 4A). Minimal expression of IL-1β was seen in untreated corneas; however, AB and CM increased immunoreactivity in corneal epithelium to IL-1β, which peaked at day 5 post injury.

We evaluated presence of neutrophils in injured corneas by immunohistochemistry using the Gr-1 marker, flow cytometry and also by myeloperoxidase activity assay in whole-cornea lysates. In a naïve cornea, a few resident neutrophils can be found at the limbal area (data not shown). A significant influx of Gr-1$^+$ cells was observed in AB and CM groups 2 and 5 days post injury; the infiltration was not restricted to the limbal area (Fig. 4B, inset), but extended to the central cornea (Fig. 4B). The CM corneas had the highest Gr-1$^+$ cell counts (Fig. 4C) compared with AB and UT corneas 2 days after injury. These results were confirmed by flow cytometry as we observed a significant increase in CD45$^+$ cells in corneas subjected to AB and CM (Fig. 4D); the majority of CD45$^+$ infiltrating cells were Gr-1$^+$ cells. There was a significant increase in the frequency of CD45$^+$Gr-1$^+$ (neutrophils) cells in CM group compared with AB alone 2 days post injury (Fig. 4E), while there was a lower frequency of CD45$^+$F4/80$^+$ cells (macrophages) compared with naïve corneas. The pattern of Gr-1$^+$ infiltration was similar to expression pattern of CXCL1 in cornea. These results are in agreement with the literature that showed that neutrophils can be found in central cornea as soon as 8 hours post injury.[43]

Because neutrophil infiltration peaked 2 days post injury, we measured MPO activity at the same time point, because MPO is the most abundant protein found in neutrophils. Our results, presented in Figure 4G, demonstrate that significantly higher MPO activity than control was seen in CM and AB groups, and MPO activity in CM was higher than AB. Myeloperoxidase activity in naïve control corneas was minimal, confirming the immunohistochemistry and flow cytometry results.

These results indicated that combining dry eye with AB worsens the severity of AB by increasing neutrophil infiltration and activity.

Discussion

Eye injuries can be very costly. In a 10-year review of eye injuries in the United States Army, Navy, and Air Force, Buckingham and colleagues[44,45] showed that the average military eye injury misfortune results in 4.7 to 6.1 days lost from work and has a cost ranging from $4222 to $9724. They also estimated that there was an underreporting of eye trauma data by at least 250%. Another underestimated factor is the cost for ambulatory visits, which is estimated to vary from $8.9 million to $14 million among the Army, Navy, and Air Force.[44] Therapeutic strategies for ocular surface chemical injuries have attempted to favorably modify one or more aspects of this process, during the acute phase. The visual outcomes from these injuries still remain poor in large part due to inadequate control of inflammatory and proteolytic components of the wound healing response.[46–48]

Dry eye can contribute to the severity of chemical and thermal injuries to the cornea, either during the initial healing phase following the burn or during the late phases. Dry eye can worsen the injury through a variety of events: reduced tear clearance and wash out, decrease provision of growth factors, and decrease corneal sensation and corneal exposure. Having all this in mind, we created a combined model where experimental dry eye is induced immediately after creation of AB and anesthetic recovery. We found that inhibition of tear production and exposure to desiccating environment markedly worsened corneal inflammation and matrix degradation, leading to perforation in nearly 40% of eyes. This was accompanied by increased production of IL-1β, IL-6, and MMPs mRNA transcripts as well as increased MMP-9 activity and MMP-1, -3, -13, and -9 immunoreactivity in corneal epithelium and increased frequency and function of neutrophils.

We observed that perforated corneas in the CM group showed detached epithelium, disrupted Bowman layer, and stromal ulceration. Although the basement membrane is not directly subjected to alkaline agents placed on the surface of the eye, its degradation by MMPs (collagenases and gelatinases) secreted by damaged or infiltrating cells contributes to the pathogenic ulceration and perforation of the stroma.[49] Stromal ulceration did not develop following alkali injury, in a

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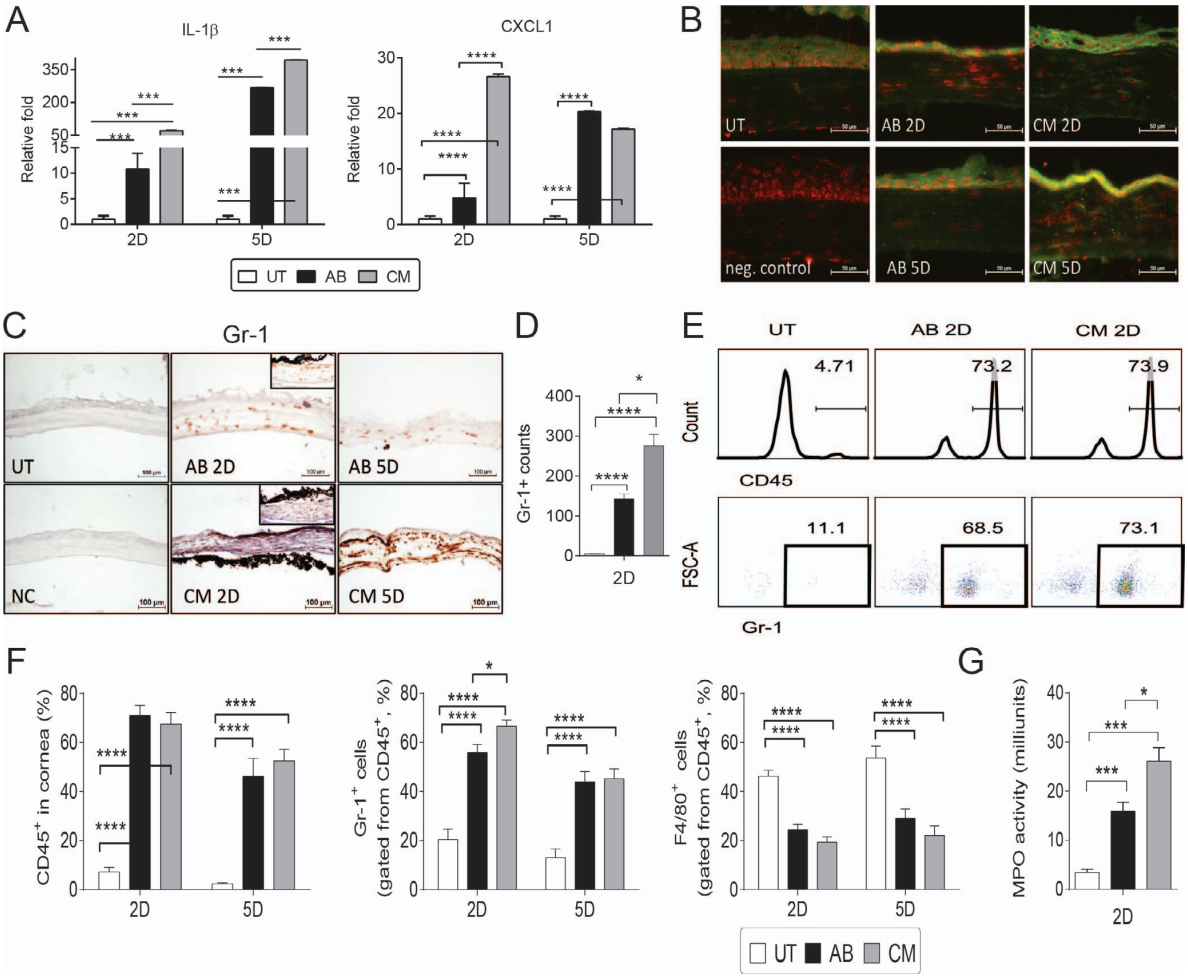

Figure 4. Increased neutrophil infiltration in CM groups. (**A**) Relative fold of expression of IL-1β and CXCL1 in whole corneas subjected to AB, or CM (AB + dry eye). *Bar graphs* show means ± SEM of one representative experiment with five samples per group/time point (experiment was repeated twice with similar results). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (**B**) Representative merged digital images of corneas cryosections immunostained for IL-1β (in *green*) with propidium iodide nuclei counterstaining (*red*) in corneas subjected to AB or CM. *Scale bars*: 50 μm. (**C**) Representative pictures of Gr-1^{+} cells (*red*) of central cornea cryosections among the AB, CM, and control groups used to generate the *bar graph* showing counts in (**D**). *Insets* are representative pictures of limbal area. (**D**) *Bar graphs* (mean ± SEM) of Gr-1^{+} cell counts in whole cornea/groups. (**E**) Representative histogram (*top panel*) and dot plot (*bottom panel*) of CD45^{+} and Gr-1^{+} cells evaluated by flow cytometry. Numbers in the *squares* indicate percentage of positive cells. (**F**) *Bar graphs* (mean ± SEM) of frequency of CD45^{+} and CD45^{+}Gr-1^{+} and CD45^{+}F4/80^{+} cells quantified by flow cytometry in whole-cornea, single-cell suspension. (**G**) Myeloperoxidase activity in whole-corneas lysates 2 days after injury in untreated, AB and CM groups (mean ± SEM). $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. FSC-A, forward side scatter area.

standardized AB rabbit model using sodium hydroxide concentrations equal to or below 1N.[50] Healing after AB is characterized by extended inflammatory cell infiltration of the stroma, persistent epithelial defect, and degradation of the basement membrane and corneal neovascularization in late phases. In tissue sections from humans, different cell types can produce MMPs: neutrophils, stromal fibroblasts, and epithelial cells. It is important to note, however, that the cellular sources of collagenase appear to be somewhat species-dependent.[51] Studies in animal models of chemical or thermal injury revealed that there are very few fibroblasts in newly ulcerating corneas, due to cell death from the initiating agent. In the AB cornea, the epithelium surrounding the wound has long been thought to participate in stromal destruction.

We observed a significant increase in MMPs-1, -8, and -9 mRNA levels in the CM group compared with AB and DS. The activation of p38 MAPK alone has been shown to induce expression of MMP-1 and -3 in an activator protein 1 independent manner by stabilizing the corresponding mRNAs in human skin fibroblasts.[52] We have previously reported that DS upregulates MMPs, in particular MMP-3, and MMP-9 transcripts in the corneal epithelium.[19] The precise regulatory mechanisms inducing MMP production in wound healing have not yet been fully elucidated despite extensive investigations, but are thought to be stimulated by cytokines and growth factors, and cell–matrix or cell–cell interactions. During the repair period of cornea remodeling, the rate of collagen turnover is much higher than it is in the normal cornea,[51] suggesting the involvement of MMPs in the remodeling

process. In a rabbit model of superficial or penetrating injury to the stroma, increased expression of gelatinase (MMP-2), collagenases (MMP-1 and -13), and stromelysins MMP-3 was found.[24]

We found a significant increase in MMP-9 (both RNA and protein level, including activity) in corneas subjected to the combined model, peaking at 2 days post injury. Matrix metalloproteinase–9 has been implicated in both favoring and decreasing re-epithelization: MMP-9KO has been shown to accelerate healing of corneal wounds[37] while delaying re-epithelization in large cutaneous wound.[53,54] In human cornea wounds, MMP-9 is expressed in basal epithelial cells at the leading edge of the migrating epithelial front closing the corneal wound.[36] We have previously reported that human dry eye and experimental desiccating stress stimulates production of MMP-9, as well as other MMPs by the ocular surface epithelia.[19,55–57] MMP-9 was found to degrade the tight-junction protein occludin and to decrease apical epithelial barrier function in the cornea.[55] Matrix metalloproteinase–9 in human tears has also been found to increase in a variety of ocular surface diseases, including sterile corneal ulceration.[26,58–61] In a group of dry-eye patients, we observed a strong positive correlation between tear MMP-9 activity and severity of corneal epithelial disease. Tear MMP-9 activity levels also correlated positively with reduced contrast visual acuity.[21] The significant increase in MMP-9 in the combined model (~100-fold) compared with AB alone may suggest that MMP-9 could be delaying wound healing and facilitating cornea melting. Increased expression of MMP-9 and gelatinase activity has been reported in melted corneas from severe primary Sjögren Syndrome patients.[12] Interestingly, other MMPs were also present in these melted specimens, (intense MMP-1, and -7, and moderate MMP-3, and -8 immunoreactivity was noted).[12] The concentrations of IL-1β, IL-6, and MMP-8 and -9 were significantly upregulated in the tear fluid of the ulcer patients, corroborating our findings.[62]

Neutrophils are the first inflammatory cell responders to migrate toward the site of inflammation. Our results showed a significant influx of polymorphonuclears (PMNs) in the CM group, which coincides with a significant increase in CXCL1 in cornea. During the acute phase in corneal AB, the PMNs began to infiltrate the stroma, exerting their phagocytic functions to clear cellular debris, but at the same time, neutrophils release their proteolytic contents, including MMPs-1, -8, and -9[39] that may cause collateral damage. They enter the cornea from the limbus and move centrally in the superficial stroma somewhat behind the regenerating epithelium.[63] Collagenase breakdown products have been shown to be chemotactic for PMN.[64] Strategies developed to decrease neutrophil infiltration have been shown to improve wound healing following corneal alkali injury.[65,66] Neutrophil depleted C57BL/6 subjected to AB healed faster than nondepleted controls, demonstrating bystander damage to host tissue by PMN.[38]

Taken together our results showed that the stroma ulceration following AB is not simply the passive breakdown of alkali denatured collagen and proteoglycans, is a complex process involving interactions between different cell types, regulation of collagenases, cytokines, and growth factors. Moreover, they show that alkali injury in the context of a dry ocular surface dramatically exacerbates the inflammatory response and increases the rate of corneal perforation. This combined model is therefore more severe than regular AB and can be used to study severe devastating ocular injuries and facial burns that reach the eye, ocular injuries secondary to warfare agents and the effects of prolonged stay in environmental controlled low humidity intensive care unit on disease severity.

Acknowledgments

The authors thank Joel M. Sederstrom for his expert assistance with flow cytometry experiments and Mahira Zaheer and Kevin Tesareski for technical assistance.

Presented in part as abstracts at the annual meeting of the Association for Research in Vision and Ophthalmology, 2010, 2013, and 2014.

Supported by grants from W81XWH-12-1-0616 (CSDP; Department of Defense, Fort Detrick, MD, USA), National Institutes of Health (NIH) Training Grant T32-AI053831 (FB; Bethesda, MD, USA), National Eye Institute/NIH Core Grant EY-002520, Research to Prevent Blindness (New York, NY, USA), NIH funding to Cytometry and Cell Sorting Core at Baylor College of Medicine (NIAID P30AI036211, NCI P30CA125123, and NCRR S10RR024574), The Oshman Foundation (Houston, TX, USA), William Stamps Farish Fund (Houston, TX, USA), and The Hamill Foundation (Houston, TX, USA).

Disclosure: **F. Bian**, None; **F.S.A. Pelegrino**, None; **S.C. Pflugfelder**, None; **E.A. Volpe**, None; **D.-Q. Li**, None; **C.S. de Paiva**, None

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ED: Please verify the accuracy of any edits made to the article summary below.

Combining alkali burn and desiccating stress delays wound healing, induces proinflammatory cytkokines, and matrix metalloproteinases while increasing neutrophil infiltration.

Investigative Ophthalmology & Visual Science

Manuscript under review at “The Ocular Surface” Journal 07-04-15

Differential effects of Dexamethasone and Doxycycline on Inflammation and MMP Production in Alkali-Burned Corneas Associated with Dry Eye

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Keywords: alkali injury, dry eye, neutrophils, MMPs, ocular perforation, doxycycline, dexamethasone; corticoid; lipocalin-2
Figures: 4

Table: 1

Presented in part as abstract at the annual meeting of the Association for Research in Vision and Ophthalmology, 2010, 2013 and 2014.

Abbreviations:

AB=alkali burn

BID=bis in die, i.e., twice a day

BSS=balanced salt solution

CM=combined model (AB+DS)

D=days

Dex=Dexamethasone

Doxy=Doxycycline

DS=desiccating stress

QID=quater in die, i.e., 4 times a day

UT=untreated

M-MuLV=Moloney murine leukemia virus

MMP=matrix metalloproteinase

MPO=myeloperoxidase

NGAL=neutrophil gelatinase associated lipocalin

PMN=polymorphonuclear

Abstract

Alkali burns to the cornea are among the most devastating injuries to the eye. We investigated the effects of topical Dexamethasone (Dex) or Doxycycline (Doxy) on protease activity and sight threatening corneal complications in a combined model (CM) of alkali burn and dry eye. Mice were observed daily for appearance of corneal perforation. Whole corneas were harvested and lysed for RNA extraction. Quantitative real time PCR was performed to measure expression of inflammation cytokines, matrix metalloproteinases (MMP). No perforations were observed in the Dex treated corneas. Doxy treated eyes had 100% of wound closure 2D post-injury, and significant lower corneal opacity scores at days 4 and 5 compared to BSS. Dex-treated corneas showed the lowest corneal opacity score. Dex treatment significantly decreased mRNA levels of IL-1β, IL-6, MMPs -1,-9, -13, and TIMP-1 after 2 days with increased levels of MMP-8, while Doxy treatment significantly decreased IL-1β, IL-6, MMP-8, and -9, compared to BSS-treated corneas. Decreased MMPs -1,-9 and -13 immunoreactivity and gelatinolytic activity were seen in corneas treated with Doxy and Dex compared to BSS vehicle. Increased neutrophil infiltration and MPO activity was noted in the BSS group compared to Dex 2D post-injury. These findings demonstrated that early treatment with anti-inflammatory therapy is very efficacious in preserving corneal clarity and facilitating wound healing, while controlling MMP production and migration of neutrophils.

Introduction

Chemical/thermal injuries to the eye are potentially blinding conditions. Therapeutic strategies for ocular surface alkali injuries have been directed towards promoting epithelial healing and suppressing inflammation and tissue destruction, during the acute phase [1]. Treatment of the chronic phases, including the anatomical sequelae, sometimes require a multi-disciplinary approach and very often, surgical procedures, such as amniotic transplantation or stem cell transplantation [2]. Although these approaches have slightly improved visual outcomes, the visual outcomes from these injuries still remain poor in large part due to inadequate control of inflammatory and proteolytic components of the wound healing response. Among the treatments proposed, several inhibitors of MMPs and inflammation have been used in the past with some success [3-5].

Previous studies have demonstrated a marked anti-collagenolytic effect for the family of tetracycline antibiotics in rat, rabbit, and human corneas, both in vivo and in vitro [6-9]. Doxycycline (Doxy) was discovered in the early 1960s as a semisynthetic long-acting tetracycline derivative useful as a bacterial ribosome inhibitor in a wide variety of microbes. It is also proven to be effective primary treatment for common ocular surface diseases such as acne rosacea, recurrent corneal erosions, and sterile corneal ulceration [10]. Sub-antimicrobial doses have been reported to be effective for treating chronic meibomian gland dysfunction [11], as well as an effective adjunctive treatment for adult periodontitis [12, 13]. Doxy, administered by subconjunctival injections or drops, successfully prevented neutrophil infiltration and promoted healing in corneas subjected to alkali burn from half mustard in an animal model [14, 15].

It has been suggested that early systemic and topical administration of corticosteroids after alkali injury may be of benefit by decreasing inflammation, scarring, and neovascularization in the cornea after alkali burn injuries, improving success rates for patients for whom corneal transplantation is their only alternative [16-18]. However, early reports showing decreased efficacy in deep alkali burns in rabbits has limited its use [16, 19].

Dry eye is comorbidity that markedly worsens the outcome of ocular surface chemical burns by increasing the risk of sterile corneal ulceration. We have previously reported that combining alkali burn with dry eye leads to delayed wound healing, greater corneal opacity, greater production and activity of MMPs, and increased neutrophil infiltration [20].

Herein we describe the use of anti-inflammatory therapy preventing corneal opacification, improving wound healing and decreasing production of MMPs and gelatinolytic

activity in the combined model (CM) of alkali burn and dry eye. Separate groups of mice, subjected to CM for 2 and 5 days were treated bilaterally with either Doxy, Dexamethasone (Dex) or vehicle QID. Topical anti-inflammatory therapy was greatly superior to vehicle treatment without causing corneal perforations.

Materials and Methods

Animals

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it conformed to the standards in the ARVO Statement for the use of animals in Ophthalmic and Vision Research.

Unilateral alkali burn

After systemic anesthesia with isoflurane using a vaporizer (SomnoSuite, Kent Scientific, Torrington, Connecticut), a unilateral alkali burn (AB) was created on the right eye of 6-8 week old C57BL/6 mice. This was achieved by placing one 2.0 mm diameter filter paper disc that had been pre-soaked with 1N NaOH on the central cornea for 10 seconds, followed by extensive rinsing with balanced salt solution (Alcon, Fort Worth, TX, USA), as previously described [21]. Precautions were taken to avoid damage to the peripheral cornea, conjunctiva, and lids. AB was created at day 0 and animals were sacrificed after 2 or 5 days. Naïve mice served as untreated (UT) controls. After anesthetic recovery, mice that received unilateral AB were subjected to desiccating stress (DS) for 2 or 5 days (AB+DS, referred to as the combined model = CM).

DS was induced in female C57BL/6 mice aged 6-8 weeks by sterile subcutaneous injection of 0.5 mg/1 mL scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) QID into alternating flanks and exposure to a drafty low humidity (<30% relative humidity) environment for 2 or 5 days (DS, DS2, and DS5 respectively) as previously described [22]. Mice received no antibiotic eyedrops.

Mice subjected to the combined model for 2 and 5 days were topically treated either with 2μL sodium phosphate Dexamethasone (Dex, 0.1%, Spectrum Laboratory, Gardena, CA), 2μL of freshly prepared Doxycycline USP (Doxy, 0.025%,Sigma-Aldrich, St. Louis, MO) or vehicle (balanced salt solution, BSS, Alcon, Fort Worth, TX) QID and compared to naïve control corneas.

At least 32 animals without corneal perforations were used per group (BSS, Doxy, Dex) and per time point: six for histology, fifteen for real-time PCR, four for myeloperoxidase

assay, four for MMP-9 assay, three for gelatinase zymography. Corneal opacity and wound closure rate were evaluated in twelve live mice that were used for either histology or PCR. Thirty-two naïve control animals were used.

Extended dosing after short-term CM

To evaluate if prolonged anti-inflammatory therapy promoted corneal melting, a distinct group of animals (n=6/group) were subjected to the combined model and dosed with either BSS, Doxy or Dex QID for 5 days. On the 6th day, mice were placed in a normal vivarium room with normal environment (Relative humidity >40-60%) and topical treatment continued for another 16 days, for a total of 21 days of topical regimen.

Clinical Findings: ocular perforation and opacity score

Eyes of all treatment groups were examined daily under a microscope (SMZ 1500, Nikon, Melville, New York) for presence of corneal perforation. Once corneal perforation was observed, mice were euthanized. The number of corneal perforations occurring each day was recorded and survival curves were calculated using Graph Pad Prism 6.0 software (GraphPad Software Incorporation, San Diego, CA). Perforated corneas were not used subsequently in any experiment.

Corneal edema and opacity were graded by in biomicroscopic examination by two masked observers in images taken by a color digital camera DS-Fi1 (Melville, New York) by the method described by Yoeruek [23]. Corneal opacity was scored using a scale of 0-4 where grade 0=completely clear; grade 1=slightly hazy, iris and pupils easily visible; grade 2= slightly opaque, iris and pupils still detectable; grade 3=opaque, pupils hardly detectable, and grade 4= completely opaque with no view of the pupils.

Measurement of corneal epithelial defect

Corneal epithelial healing was assessed daily in the experimental. Briefly, 1 µL of 0.1% liquid sodium fluorescein was instilled onto the ocular surface. Corneas were rinsed with phosphate-buffered saline and photographed with a stereoscopic zoom microscope (SMZ 1500; Nikon, Melville, NY) under fluorescence excitation at 470 nm (DS-Qi1Mc, Nikon, Melville, NY). Corneal epithelial defects were graded in digital images by two masked observers in a categorical manner (present/absent) to generate a survival curve. Biological replicate scores were transferred to excel database where the results were analyzed.

Histology and immunostaining

Mice (n=6/group/time point) were euthanized after 2 or 5 days of the initial injury. Eyes and adnexae each group/time point were surgically excised, embedded in optimal cutting temperature compound (VWR, Suwanee, GA), and flash frozen in liquid nitrogen. Cryosections were prepared as previously described[22] and stained with rat anti-Gr-1 antibody (Ly6G; 1:250, clone 1A8, BD Pharmingen). Cryosections were stained with the primary antibody and appropriate biotinylated secondary antibody (1:100 biotin goat α-rat, BD Pharmingen, San Diego, CA) using a Vectastain Elite ABC kit and Nova Red reagents (Vector, Burlingame, CA). Six sections from each animal /group/time point were examined and photographed with microscope equipped with a digital camera (Eclipse E400 with a DS-Qi1Mc; Nikon, Melville, NY, USA). The numbers of Gr-1 positive ($^{+}$) cells were counted in cornea sections from each animal at 20X magnification and results averaged and expressed as the number of positive cells per cornea.

Immunofluorescent staining was performed in frozen tissue sections with rabbit polyclonal antibody anti-MMP-1 (1:50, NBP1-77209; Novus Biologicals), anti-MMP-9 (1:100; Santa Cruz Biotechnology, Dallas, TX), anti-IL-1β (1:100, Upstate-Millipore Corp., Bedford, MA), anti-neutrophil-gelatinase associated lipocalin (1:50, ab63929, Abcam, Cambridge, MA), goat anti-MMP-13 (1:100 dilution, sc-123630, Santa Cruz Biotechnology, Dallas, TX). Secondary goat-anti rabbit or donkey anti-goat Alexa-Fluor 488 conjugated IgG antibodies were used, as previously described [24]. The images were captured and photographed by a Nikon fluorescence microscope (Eclipse E400 equipped with a DS–F1 digital camera; Nikon, Melville, NY).

RNA isolation and quantitative PCR

Five whole corneas/group/time point/per experiment (total of 3 independent experiments) were excised, minced and total RNA was extracted using a Qiagen MicroPlus RNeasy isolation® Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, quantified by a NanoDrop® ND-2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at -80°C. First-strand cDNA was synthesized with random hexamers by M-MuLV reverse transcription (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Inc., Arlington Heights, NJ), as previously described [22].

Real-time polymerase chain reaction (PCR) was performed with specific Taqman MGB probes (Applied Biosystems, Inc., Foster City, CA) and PCR master mix (Taqman Gene

Expression Master Mix), in a commercial thermocycling system (StepOnePlus™ Real-Time PCR System, Applied Biosystems), according to the manufacturer's recommendations. Quantitative real time PCR was performed using gene expression assay primers and MGB probes specific for murine targets described in Table 1. The hypoxanthine guanine phosphoribosyl transferase (HPRT-1) gene was used as an endogenous reference for each reaction to correct for differences in the amount of total RNA added. The results of quantitative PCR were analyzed by the comparative C_T method where target change = $2^{-\Delta\Delta C_T}$. The results were normalized by the C_T value of HPRT-1 and the relative mRNA level in the untreated group was used as the calibrator.

Myeloperoxidase assay

Myeloperoxidase (Samtani *et al.*) activity was measured using a myeloperoxidase colorimetric activity assay kit as described by the manufacturer (Sigma-Aldrich, CA). Briefly, whole cornea lysates from experimental groups (n=4/group) were homogenized in MPO assay buffer and the homogenate was centrifuged at 14000 ×g for 20 min at 4°C. Total protein concentration was measured by the BCA protein assay as previously described [25]. A 50 µg/sample was mixed with MPO assay buffer and MPO substrate, incubated at room temperature for 2 hours, and then mixed with tetramethylbenzidine probe. Fluorescence was measured at 412 nm using a Tecan Infinite M200, Durham, NC) plate reader equipped with Magellan V6.55 software. Biologic replicate samples were averaged. Results are presented as mean ± SEM milliunits.

MMP-9 activity assay

Whole corneas were excised, rinsed and homogenized in RIPA buffer and the homogenate was centrifuged at 14000 ×g for 20 min at 4°C.Total protein concentration of the whole cornea cell lysate (n=4/group/time point) was measured by BCA protein assay as previously described [22]. Total MMP-9 enzyme activity was measured with an MMP activity assay kit (Biotrak; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol, as previously published [26]. Absorbance was read at 405 nm using a Tecan Infinite M200 plate reader equipped with Magellan V6.55 software. The activity of MMP-9 in a sample was determined by interpolation from a standard curve. Biological replicates were averaged and results presented as mean ± SEM (pg/ml).

Gelatin zymography

The relative amount of MMP-9 in whole cornea lysates was measured by gelatin zymography, using a previously reported method [9, 25]. Whole cornea lysates prepared for MMP-9 activity (20 μg/sample, n=3/group/time point) were fractionated in an 8% polyacrylamide gel containing gelatin (0.5 mg/mL). Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

Statistical Analysis

Two-way analysis of variance (ANOVA) with Bonferroni post hoc testing was used for statistical comparisons of gene expression. $P \leq 0.05$ was considered statistical significant. These tests were performed using GraphPad Prism 6.0 software (GraphPad Incorporation, San Diego, CA).

Results

Clinical effects of anti-inflammatory therapy on a combined model of alkali burn and dry eye

It is well recognized that alkali burns to the cornea induce a significant amount of inflammation and MMPs [16, 27]. Corneal scarring and opacification is are sight-threatening sequelae of severe chemical burns. In these cases, corneal transplantation may be required to corneal clarity [18], however, these eyes have high risk of graft failure. We have previously reported the deleterious effect of combining alkali burn with dry eye [20, 28]. Herein we subjected mice to unilateral alkali burn and concomitant desiccating stress and topically treated them with either Dex or Doxy and compared the effects to BSS treated corneas.

We evaluated corneal opacification in digital images by masked observers using a scale from zero (transparent cornea) to 5 (complete opaque cornea). We also evaluated wound closure daily for the duration of the experiment. Representative images for corneal opacity and wound closure are shown on Fig. 1A-B. Dex-treated corneas had the lowest corneal opacity score among all treatment groups (Fig. 1C).

Doxy-treated corneas healed significantly faster than BSS-treated corneas; with 100% wound closure 2 days post-injury (Fig 1D, $p<0.001$). Dex-treated corneas showed delayed cornea wound healing, with 60% of corneas showing areas of epithelial defect, while 75% of BSS treated corneas had epithelial defects 5 days post-injury.

BSS- and Doxy-treated corneas subjected to the combined model showed early corneal perforations as early as day 1 after the initial insult; however, more BSS-treated corneas perforated than Doxy-treated (27 vs. 14%, $p=0.03$, Fig. 1E). Dex-treated corneas did not perforate for the duration of the experiment.

Our results confirm that alkali injury in the context of a dry ocular surface dramatically exacerbates the inflammatory response and increases the rate of corneal perforation, while treatment with Doxycycline or Dexamethasone is efficacious in preventing this sight-threatening complication.

Extended anti-inflammatory therapy for up to 21 days does not lead to corneal perforations

Conflicting results of prolonged use of glucorticoids promoting corneal perforation after alkali burn in animal models have been reported [16, 27, 29, 30]. To investigate if prolonged anti-inflammatory treatment in mice after this combined model would also lead to corneal

perforation, we subjected a separate group of mice to alkali burn and dry eye for 5 days; and subsequently placed them in a normal environment (RH 40-70%) for 16 days. Mice were continuously treated with either BSS, Doxy or Dex for a total of 21 days and corneal opacity, wound closure and rate of corneal perforation were evaluated.

We observed that if BSS- or Doxy-treated corneas did not perforate in the first 5 days, they did not perforate at all (Fig. 1E), indicating that this is the critical period for corneal ulceration leading to perforation. Dex-treated corneas did not perforate even after continuous treatment for up to 21 days, indicating that prolonged therapy for up to 21 days does not cause corneal melting.

Doxy-treated corneas showed the fastest wound healing, but no difference in corneal opacity scores compared to vehicle treated eyes (Fig.1C-D). While prolonged treatment with Dex showed incomplete wound closure similar to BSS controls even after 21 days, it yielded significantly lower corneal opacity scores (Fig. 1C). These results indicate that in mice subjected to alkali burn and dry eye develop more corneal disease than alkali burn alone and that topical anti-inflammatory therapy does not promote corneal perforation.

Controlling host response with anti-inflammatory therapy in a combined model of dry eye and alkali burn

Our previous results showed that combining alkali burn and dry eye led to significantly increased levels of IL- and MMPs-1, -3, -8, -9, and -13 transcripts in the cornea compared to alkali burn alone (manuscript in press). Next we evaluated the efficacy of anti-inflammatory therapy with Doxy or Dex at 2 and 5 days post initial injury controlling expression of inflammatory cytokines and MMPs. Corneas subjected to alkali burn and dry eye and topically treated with BSS showed a significant increase in IL-6, MMPs -1, -3, -8, -9, -13 and TIMP-1 compared to naïve corneas. MMPs -1, -3, -8, -9,-13 and TIMP-1 transcripts peaked at 2 days and decreased by 5 days, while IL-6 continued to increase up to day 5 (Fig. 2). No changes in MMP-2, TIMP-2, TIMP-3 or TIMP-4 were observed (data not shown).

Doxy treatment corneas significantly decreased MMPs -8, -9, -13 two days post-injury, while Dex treatment significantly decreased IL-6, MMPs -1, -3, -9, -13 and TIMP-1 but significantly increased MMP-8 RNA transcripts (up to a 1000 fold). Doxy treatment showed greater suppression of IL-6, and MMPs -8, -9, and MMP-13 five days after the initial injury than BSS (Fig. 2).

The immunoreactivity of corneas to collagenases (MMPs -1, -13) and MMP-9 was evaluated by immunostaining (Fig. 3). Minimal levels of MMP-1, -9 and -13 were present in the control corneas. Increased reactivity against these MMPs in the corneal epithelium was

seen BSS treated corneas. Both Doxy and Dex-treated corneas showed decreased immunoreactivity of corneal epithelium to MMP-1, MMP-9 and MMP-13 compared to BSS treatment (Fig. 3).

MMP-9KO mice have been noted to heal epithelial defects faster than wild-type mice, indicating that MMP-9 can delay corneal wound healing [31]. MMP-9 activity was evaluated by gelatin zymography, which showed increased amounts of both pro and active MMP-9 bands in the BSS group compared to control corneas (day 2 > day 5). Dex and Doxy groups showed no activated MMP-9 bands and had reduced pro-MMP-9 bands, in both day 2 and 5 post injury samples. Negligible amounts of MMP-2 were present in all groups (Fig. 3B).

These results confirm the efficacy of broad anti-inflammatory therapy in controlling MMP production and level of gelatinases in the cornea after alkali burn and dry eye. The corticosteroid Dexamethasone showed greater anti-inflammatory effect than Doxycycline.

Dexamethasone decreases lipocalin-2-MMP-9 complex formation

Neutrophil-gelatinase associated lipocalin (NGAL), also known as Lipocalin 2, belongs to the lipocalin family. It is involved in cell growth, migration, differentiation and has anti-microbial properties [32-34]. It is released by the kidney tubular cells under stressful conditions [35] and expressed in the colonic epithelia during cancer and colitis [36]. NGAL exists as a monomer (25kDa), a homodimer (46kDa) or as a heterodimer (125kDa)[37]. The heterodimer form is composed of NGAL and MMP-9 and it is known to protect MMP-9 from degradation in a concentration dependent manner [33].

We investigated the expression of NGAL in the combined model by immunostaining frozen sections. While barely detected in naïve corneas, increased immunoreactivity to NGAL was observed in the epithelium of BSS treated corneas. Treatment with Dex or Doxy decreased NGAL immunoreactivity (Fig. 3A). We also observed that a 125-kDa band in our zymography gels, corresponding to the NGAL-MMP-9 complex, was markedly decreased in Dex-treated corneas compared to BSS 2 days after injury. Doxy had a moderate effect decreasing it (Fig. 3B).

Anti-inflammatory therapy decreases neutrophil infiltration in CM

Neutrophils are the first cells to respond to a site of injury. Several enzymes and proteases, including collagenases, are store within the cytoplasmic granules of neutrophils. Elegant experiments showed that depletion of neutrophils dramatically improved the fate of

alkali burn injuries [19, 38]. Doxycycline and Dexamethasone have been shown to decrease migration of polymorphonuclear leukocytes (PMN) [6, 19, 39, 40]. We have previously reported that combining alkali burn with dry eye significantly increases neutrophil infiltration in wounded corneas [20, 28]. We observed increased immunoreactivity of MMP-8 in the corneal epithelium, stroma, and endothelium after 2 and 5 days of the combined model (Fig. 4A). In addition, we investigated the expression of the cytokine IL-1 and the chemokine CXCL1 since they have been implicated in the migration of neutrophils [41, 42]. Dex treatment in the combined model significantly decreased the early peak of IL-1β and CXCL1 2 days after initial injury as well as at day 5. Doxycycline was more efficacious than vehicle decreasing both mediators by day 5 (Fig. 4B).

To investigate if topical treatment would decrease neutrophil infiltration, we performed immunohistochemistry in cornea cryosections using the Gr-1 marker, and investigated myeloperoxidase activity assay in whole corneal lysates. In naïve corneas, a few resident neutrophils were found at the limbal area (data not shown). A significant influx of Gr-1$^+$ cells was observed in BSS-treated corneas 2 and 5 days post-injury; the infiltration was not restricted to the limbal area, but extended to the central cornea (Fig. 4D). Dex-treated corneas had a significant decrease in Gr-1$^+$ cell counts compared to BSS corneas both days 2 and 5 after injury (Fig. 4D, 4E).

Myeloperoxidase activity has been used as a parameter of neutrophil infiltration. [43]. We measured MPO activity in corneal lysates in treated groups 2 days after the injury. Significantly higher MPO activity was seen in BSS treated corneas, and significantly lower MPO activity was present in Dex groups, confirming the IHC results (Fig. 4F). These results confirm that Dex treatment increases production of MMP-8 while at the same time decreases influx of neutrophils and production of myeloperoxidase that is associated with decreased CXCL1 expression.

Discussion

Alkali burns of the cornea are potentially blinding conditions. Alkali can penetrate rapidly into the eye and cause intense inflammation. There is a consensus that the prognosis of an alkali burn depends on the area of the insult, the penetration of the agent and early treatment controlling host response and inflammation. The visual outcomes from these injuries still remain poor in large part due to inadequate control of inflammatory and proteolytic

of components of the wound healing response, leading to corneal perforation, corneal opacification and corneal neovascularization.

We have previously found that inhibition of tear production and exposure to a desiccating environment markedly worsened corneal inflammation and matrix degradation, leading to perforation in nearly 30% of corneas acutely subjected to alkali burn [20]. Herein we describe how topical therapy with broad anti-inflammatory agents (Doxycycline and Dexamethasone) are efficacious in decreasing corneal perforations, production and activation of MMPs and neutrophil infiltration.

Therapeutic strategies for ocular surface chemical injuries have attempted to favorably modify the immune host response by decreasing inflammation during the acute phase. Treatments have shown some efficacy include corticosteroids, citrate, and oral doxycycline [4, 16, 19, 27, 29, 30, 44-46]. The management of alkali burns is often lengthy. Corneal transplants may be needed to preserve the globe or improve vision in eyes with corneal perforation or scarring and depending on the residual scarring and opacity [17, 47].

Our study showed that corneas treated with Doxycycline healed faster than controls, had a small number of corneal perforations and decreased mRNA levels of MMP-9, MMP-13, TIMP-1, IL-1β and CXCL1 (at day 5) while decreasing immunoreactivity of MMPs-1, -9 and 13 and levels of gelatinases in the cornea. Doxycycline has been extensively used in vitro and in vivo to decrease MMPs after inflammatory stimuli [7, 10, 25, 48, 49]. Oral Doxycycline is a FDA-approved drug to treat periodontitis [50], another disease where MMPs are involved. We have previously reported that doxycycline decreases levels of MMP-9 mRNA transcripts, and prevents DS-induced increase in inflammatory cytokines IL-1 and TNF-α [25]. The inhibitory effect of doxycycline on MMP-9 was also confirmed on osmotically stressed human corneal epithelia cells [51]. Doxycycline functions as a noncompetitive inhibitor of MMPs by interacting with the zinc or calcium atoms within the structural center of these enzymes that is required for their stability [52]. Chelation of these metal ions by Doxycycline could be responsible for the decreased activity of collagenolytic enzymes [6].

Dexamethasone significantly increased MMP-8 transcript levels while this was not observed in the Doxycycline and BSS treated groups. MMP-8 is rarely detected in corneal epithelium [7], but reports have shown increased immunoreactivity in context of inflammation [53, 54]. MMP-8, MMP-9 and lipocalin-2 are some of the many proteins stored in peroxidase negative granules in neutrophils [55]. MMP-8 facilitates neutrophil infiltration in many models, including LPS stimulation of the cornea [56] [57, 58]. However, MMP-8 deficient mice showed delayed healing of cutaneous skin wounds that was accompanied by increased inflammation and expression of MMP-1, MMP-13 and MMP-9 [59]. Based on these findings, we are

planning future experiments to investigate the role of MMP-8 during Dexamethasone treatment in the combined model of alkali burn and dry eye.

We observed an increase in NGAL immunoreactivity in corneal epithelium and a 125-kDa band in the zymography that was markedly inhibited by Dexamethasone and moderately by Doxycycline compared to BSS vehicle. This 125kDa corresponds to the NGAL-MMP-9 complex [32, 34]. NGAL binds covalently to MMP-9 and this complex has been reported to protect MMP-9 from degradation and preserve MMP-9 activity in a concentration-dependent manner [32, 37]. Levels of serum NGAL-MMP-9 complex were increased in patients with active ulcerative colitis, correlated with neutrophil infiltration and it has been proposed as a surrogate marker of mucosal healing [60]. NGAL immunoreactivity increased in the colonic epithelium in inflammatory and neoplastic, colorectal diseases [36]. Increased levels of NGAL-MMP-9 have been reported in tears upon awakening [61] and observed in tears of Sjögren Syndrome patients [62]. Therefore, the presence of NGAL-MMP-9 complex in BSS treated corneas may indicate a mechanism to preserve MMP-9 activity. Dexamethasone has another mechanism of action in addition to decreasing the MMP-9 itself.

Dexamethasone treatment paradoxically decreased TIMP-1 while Doxycycline treatment did not. We did not found any increase in the other TIMPs. TIMP-1 binds MMP-9, while TIMP-2 binds MMP-2 and MMP-14. Once recognized as negative regulators of MMPs, TIMPs are now recognized as bi-functional regulators [63]. TIMP-2 exogenous application resulted stimulated wound closure [64], while overexpression of TIMP-1 in a transgenic mouse of cutaneous wound decreased re-reepithelization.

Our results showed that topical steroid therapy greatly decreases inflammatory cytokines (IL-1β and IL-6), chemokine (CXCL1) and expression and activity of MMPs. It is interesting to note that Dexamethasone was more potent than Doxycycline. Dexamethasone greatly preserved corneal clarity, decreased neutrophil infiltration and expression of the neutrophil chemoattractant, CXCL1. Our results are in agreement with Saud and colleagues who showed an improvement in corneal opacity with no corneal perforations in rabbits subjected to alkali burn and subconjunctival injections of the corticosteroid triamcinolone [29].

Davis and colleagues showed that treatment of human alkali burns with topical steroids for longer than 10 days after the initial injury was safe and did not induce corneal melting [44]. Among the anti-inflammatory treatments after alkali burn, the use of a topical steroid is by far the most controversial, specifically its use after the 6th day post-injury. While several animal models (rabbits and more recently, mice) have been used to study alkali burns, there is no consensus regarding the strength of alkali used to induce the burn (0.5N, 1N, 2N of NaOH), mode of application (pre-wet discs vs. topical instillation of alkali as

eyedrops) or the duration of the contact (in case of pre-wetted discs, 30 seconds to 2 minutes) and volume of irrigation agents after the injury (from “brief irrigation” to “copious”, to “2 liters”). The diversity in models makes generalization more difficult regarding steroid-induced corneal perforations. While Donshik and colleagues described that treatment with Dexamethasone sodium phosphate was just partially efficacious in preventing deep corneal ulcers and descemetoceles in rabbits, they used a strong (2N) sodium hydroxide-embedded filter paper and very brief rinsing [16]. Chung and colleagues showed that in a milder model in rabbits (0.1N NaOH filter discs), 0.1% Dexamethasone was able to control inflammation without inducing corneal perforation, although it retarded corneal reepithelization [65].

The type of corneal injury is also important to take into consideration. Thermal burns treated early with either Medroxyprogesterone or Prednisolone showed a significant reduction on deep ulcers in albino rabbits compared to vehicle-treated corneas, but not if treatment was started 6 days post-injury [66]. Another consideration is the animal itself. It seems that alkali burns in rabbits will evolve to perforation more frequently than in mice. We have not observed corneal perforations after alkali burns in mice unless these mice are subjected to a desiccating stress and cholinergic blockade [20]. In our experiment with prolonged dosing of Dexamethasone (up to 21 days), we did not observe any corneal perforations. Another interesting consideration when evaluating corneal wounds is the size of wound itself. In our preliminary results, we observed that large corneal burns (>3mm) induced greater production of MMPs than smaller burns (2mm; data not shown). So it is possible that efficacy of anti-inflammatory agents will also vary according to the size of initial lesion.

Conclusions

Taken together our results showed that the stroma ulceration following alkali burn is not simply the passive breakdown of alkali denatured collagen and proteoglycans, is a complex process involving interactions between different cell types, regulation of collagenases, cytokines and a chemokine. Topical anti-inflammatory therapy appears to be a valuable addition to inhibit sight threatening corneal ulceration and perforation.

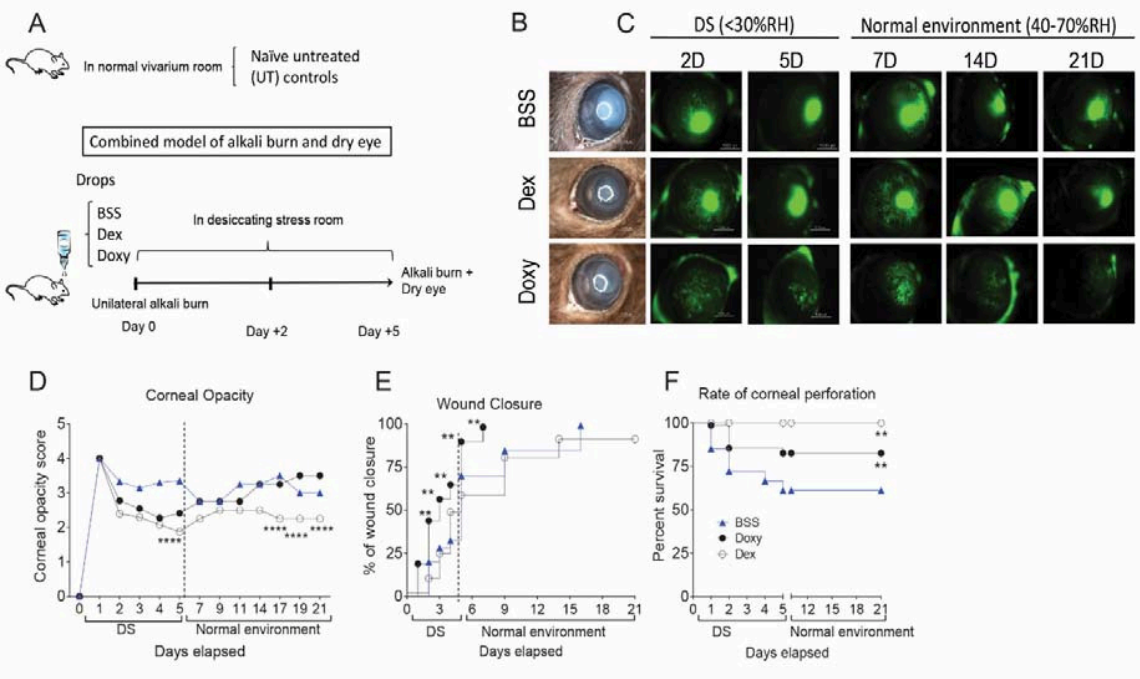
Acknowledgements: We want to thank Mahira Zaheer and Kevin Tesareski for expert technical assistance.

Support: Supported by W81XWH-12-1-0616 (CSDP), NIH Training Grant T32-AI053831 (FB), NEI/NIH Core Grant EY-002520, RPB Research to Prevent Blindness, The Oshman Foundation, William Stamps Farish Fund and The Hamill Foundation.

Role of the Sponsor: The funding agency had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Figure legends

Figure 1. Anti-inflammatory therapy improves clinical parameters in a combined model (CM) of alkali burn and dry eye.



A. Schematic of experimental design of the combined model of alkali burn and drye. A unilateral alkali burn (AB) was created on the right cornea as described in materials and methods. Mice were then subjected to desiccating stress and topically treated with either balanced salt solution (BSS), Dexamethasone (Dex) or Doxycycline (Doxy). Control mice were kept in a normal vivarium room (untreated animals, UT).

B. Digital images of bright field pictures alkali burn and dry eye combined model treated with either balanced salt solution (BSS), Dexamethasone (Dex) or Doxycycline (Doxy) 5 days (5D) post-injury. Scale bar=1000μm

D. Representative digital images of corneas 5 days stained with 0.1% sodium fluorescein after creation of alkali burn lesion and induction of dry eye topically treated with BSS, Dex or Doxy for 5 days and then topically treated for 16 days in normal environment. Scale bar=1000μm

D. Corneal opacity score in CM corneas topically treated with BSS, Dex or Doxy.

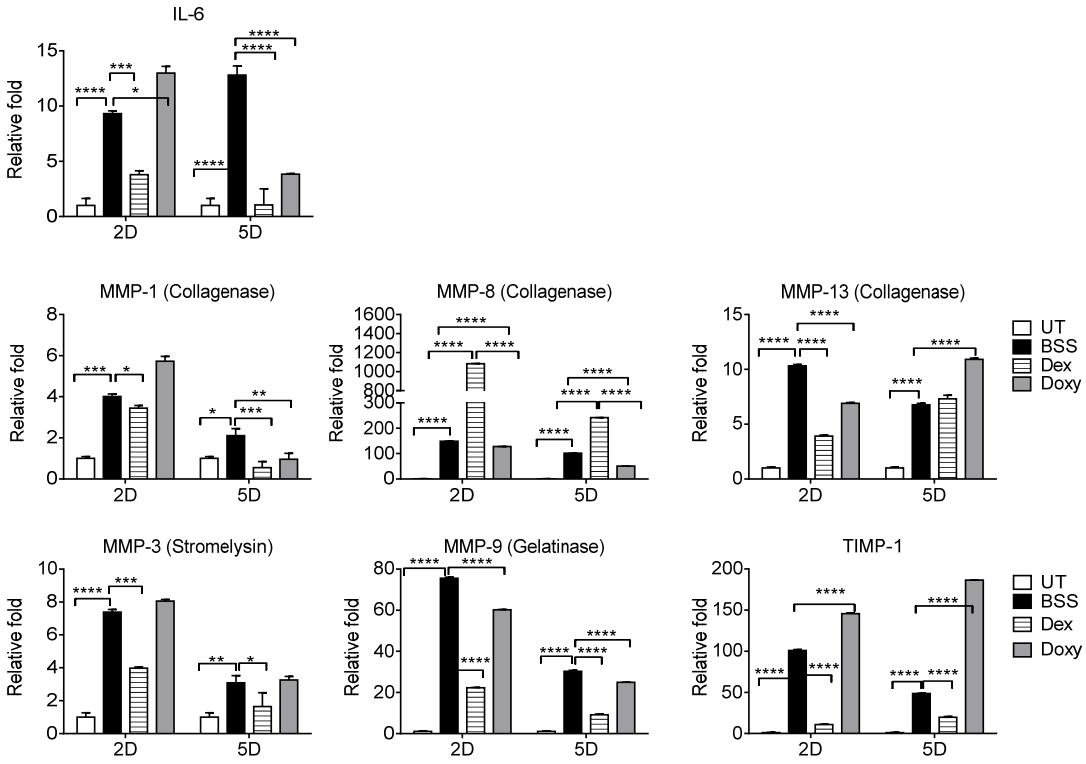
E. Survival rate of wound closure in CM corneas topically treated with BSS, Dex or Doxy.

F. Rate of ocular perforation in eyes subjected to CM topically treated with BSS, Dex, Doxy.

*p<0.05, **p<0.01; ***p<0.001, ****p<0.0001

BSS= corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex= corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy= corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, Dex+Doxy= corneas subjected to alkali burn and dry eye and alternately treated topically with Dexamethasone and Doxycycline, D=days.

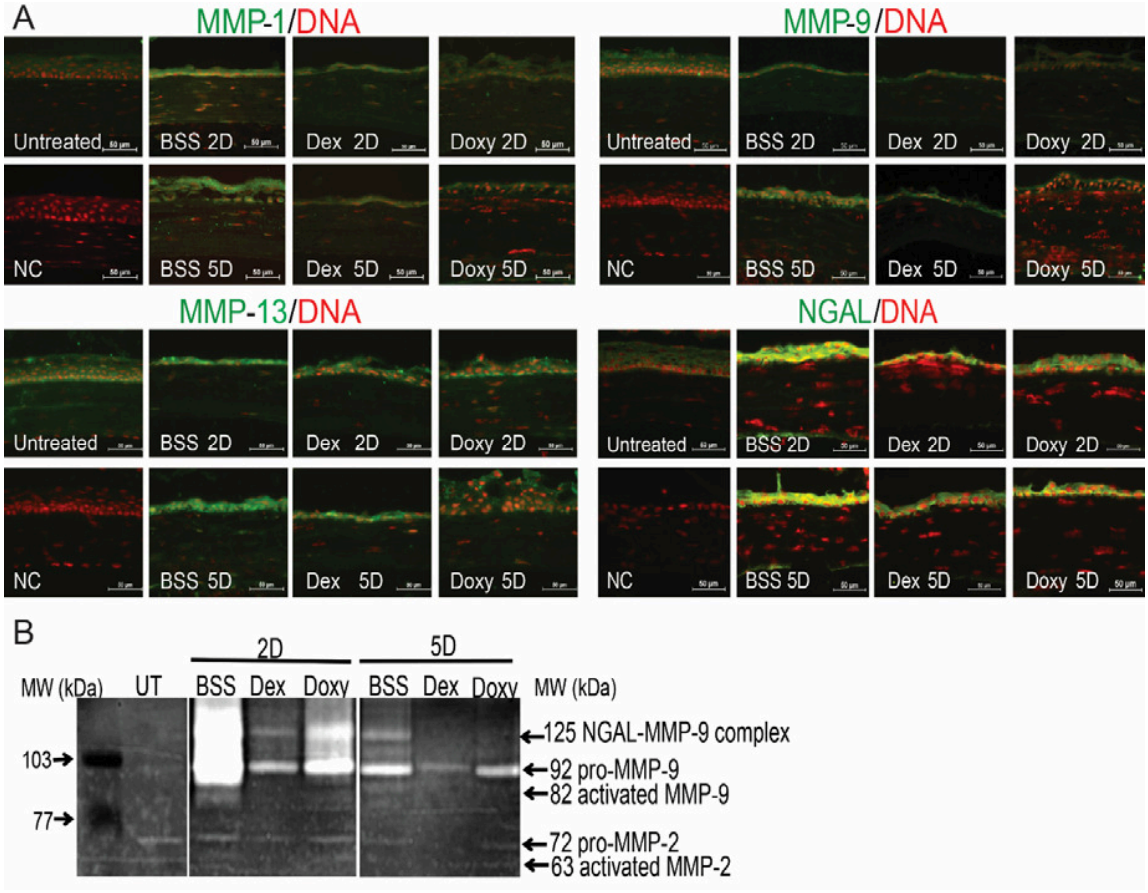
Figure 2. **Inflammatory cytokines and MMPs decrease after anti-inflammatory therapy in a combined model (CM) of alkali burn and dry eye.**



Relative fold of expression of IL-6, collagenases (MMPs -1, -8 and 13), the stromelysin MMP-3, the gelatinase MMP-9 and TIMP-1 in whole corneas subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy. Bar graphs show means ± SEM of one representative experiment with four samples per group/time point (experiment was repeated three times with similar results). UT = untreated cornea, BSS = corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex = corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy = corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, D=days.

*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001

Figure 3. Anti-inflammatory therapy decreases MMP protein expression and gelatinolytic activity.

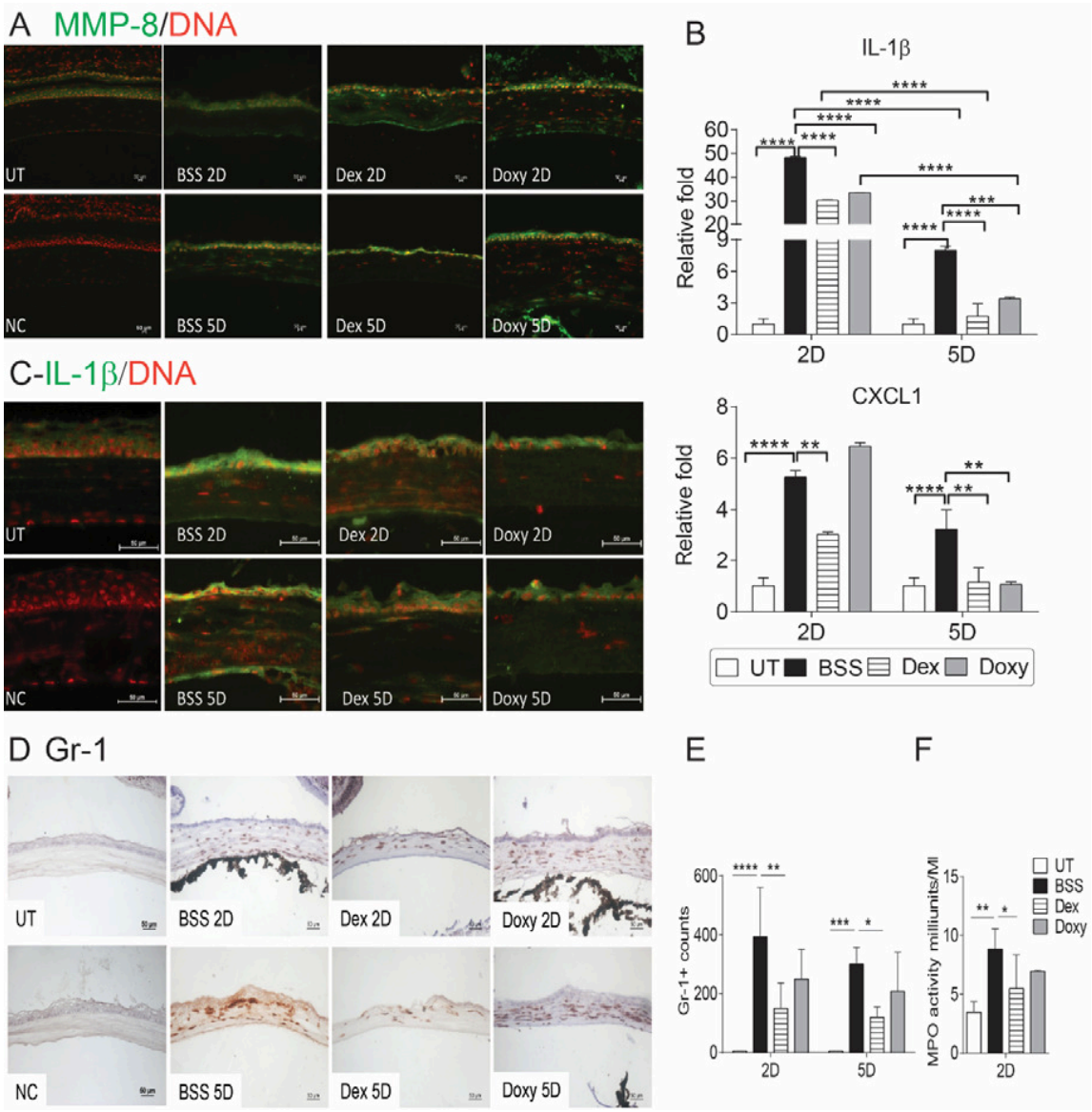


A Representative merged digital images of cornea cryosections immunostained for MMP-1, MMP-9, MMP-13 and NGAL (all in green) with propidium iodide nuclei counterstaining (Reim *et al.*) subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy for 2 or 5 days (2D or 5D, respectively). Scale bar=50μm.

B Representative zymogram showing MMP-9 and NGAL-MMP-9 bands of whole corneal lysates in the treatment groups.

UT=untreated cornea, BSS= corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex= corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy= corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, D= days, NGAL= Neutrophil-gelatinase associated lipocalin, NC=negative control.

Figure 4. Anti-inflammatory therapy decreases neutrophil infiltration



A. Representative merged digital images of corneal cryosections immunostained for MMP-8 (in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in corneas subjected to the combined model topically treated with BSS, Dex or Doxy. NC= negative control. Scale bar=100μm.

B. Relative fold of expression of IL-1β and CXCL1 in whole corneas subjected to alkali burn and dry eye topically treated with BSS, Dex or Doxy. Bar graphs show means ± SEM of one representative experiment with five samples per group/time point (experiment was repeated three times with similar results). UT=untreated cornea

*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001

C. Representative merged digital images of central cornea cryosections immunostained for IL-1β (in green) with propidium iodide nuclei counterstaining (Reim *et al.*) in corneas subjected to combined model topically treated with BSS, Dex or Doxy. NC=negative control. Scale bar=50μm.

D. Representative images of Gr-1$^+$ cells (Reim *et al.*) of central cornea cryosections from animals subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy used to generate the bar graph showing counts in D.

E. Bar graphs (mean±SEM) of Gr-1$^+$ cell counts in whole cornea/groups.

F. Myeloperoxidase (Samtani *et al.*) activity in whole corneas lysates from animals subjected to a combined model of alkali burn and dry eye topically treated with BSS, Dex or Doxy 2 days after injury (mean±SEM).

*p<0.05; ***p<0.001; ****p<0.0001.

UT = untreated cornea, BSS = corneas subjected to alkali burn and dry eye and treated topically with balanced salt solution, Dex = corneas subjected to alkali burn and dry eye and treated topically with Dexamethasone, Doxy = corneas subjected to alkali burn and dry eye and treated topically with Doxycycline, D = days. NC=negative control

Table 1. Oligonucleotide primers used for real-time PCR.

Gene Name	Symbol	Assay ID*
Interleukin 1 beta	IL1-β	Mm00434228
Interleukin 6	IL-6	Mm99999064
Chemokine (C-X-C motif) ligand 1	CXCL1	Mm04207460
Matrix metalloproteinase 1	MMP-1	Mm00473493
Matrix metalloproteinase 2	MMP-2	Mm00439506
Matrix metalloproteinase 3	MMP-3	Mm00440295
Matrix metalloproteinase 8	MMP-8	Mm00439509
Matrix metalloproteinase 9	MMP-9	Mm00442991
Matrix metalloproteinase 13	MMP-13	Mm00439491
Tissue inhibitor of metalloproteinase-1	TIMP1	Mm00441818
Tissue inhibitor of metalloproteinase-2	TIMP2	Mm00441825
Tissue inhibitor of metalloproteinase-3	TIMP3	Mm00441826
Tissue inhibitor of metalloproteinase-4	TIMP4	Mm00446568
Hypoxanthine guanine phosphoribosyl transferase 1	HPRT-1	Mm00446968

* Identification number from Life Technologies (www.lifetechnologies.com).

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