Treatment of corneal chemical alkali burns with a crosslinked thiolated hyaluronic acid film

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ABSTRACT

Purpose: The study objective was to test the utilization of a crosslinked, thiolated hyaluronic acid (CMHA-S) film for treating corneal chemical burns.

Methods: Burns 5.5 mm in diameter were created on 10 anesthetized, male New Zealand white rabbits by placing a 1N NaOH soaked circular filter paper onto the cornea for 30s. Wounds were immediately rinsed with balanced salt solution (BSS). CMHA-S films were placed in the left inferior fornix of five injured and five uninjured animals. Five animals received no treatment. At 0h, 48h, 96h, and on day 14 post chemical burn creation, eyes were evaluated by white light imaging, fluorescein staining, and optical coherence tomography (OCT). Corneal histology was performed using H&E and Masson’s Trichrome stains.

Results: Image analysis indicated biocompatible CMHA-S treatment resulted in significant decreases in the areas of corneal opacity at 48h, 96h, and on day 14 postoperatively. A significant increase in re-epithelialization was seen 14 days post injury. CMHA-S treated corneas showed significantly less edema than untreated burns. No pathological differences were observed in corneal histological samples as a result of CMHA-S treatment.

Conclusions: CMHA-S films facilitate re-epithelialization and decrease the area of corneal opacity in our corneal alkali burn rabbit model.

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Abbreviations: ANOVA, analysis of variance; ARVO, The Association for Research in Vision and Ophthalmology; CMHA-S, crosslinked carboxymethylated thiolated hyaluronic acid; CRADA, cooperative research and development agreement; BSS, balanced salt solution; HA, hyaluronic acid; H&E, hematoxylin and eosin; HOA, high order aberration; LASEK, laser-assisted subepithelial keratomileusis; MRMC, medical research and materiel command; NZW, New Zealand White; OCT, optical coherence tomography; ORISE, Oak Ridge Institute for Science and Education; PAD, program area directorate; PBS, phosphate buffered saline; PEGDA, polyethylene (glycol) diacrylate; SEM, standard error of the mean.

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1. Introduction

Regardless of occupational safety measures, clinicians still treat a number of ocular surface injuries caused by heat, acids, or alkali agents. Clinicians also treat patients with severe facial and eyelid burns that result in devastating ocular injuries leading to vision loss and/or blindness [1]. Not only are these injuries still prevalent with alkali or acidic agents representing 11.5-22.1% of ocular traumas [2], chemical and thermal injuries are some of the most clinically difficult to treat [3]. In the case of chemical burns, alkali burns are more severe and caustic as alkali agents penetrate the ocular tissues more rapidly due to lipophilic properties. This results in tissue necrosis and ischemia which often gives the eye a misleading, but quiescent white appearance [4]. From a molecular standpoint, exposure to alkali compounds causes the saponification of fatty acids in cell membranes as they penetrate the corneal stroma and destroy proteoglycans and collagens [4]. As corneal alkali burns heal, the injured tissues secrete proteolytic enzymes resulting in additional and continuous tissue damage [4]. For these reasons, the clinical rehabilitation of corneal alkali burn patients is often very challenging given long term complications due to reoccurring corneal epithelial erosions and chronic inflammation which leads to vision loss [5]. Patients with devastating facial burns frequently have damage to the periorbital tissues (i.e. conjunctiva, periorbital fat, glands and eyelids) that can indirectly lead to similar outcomes as a direct insult. Regardless if the patient is suffering from chemical or thermal burn injury, the vision loss that can occur due to these injuries is the same when the ocular surface is not healed in a timely manner. As a result, clinicians are in need of more advanced therapeutics to treat these patients.

While amniotic membrane, buccal skin grafts, tarsorrhaphies (partial suturing of the eyelids), and bandage contact lenses are available as therapies, these treatments fail in treating the most severe ocular burn patients. In severe injuries, a topical treatment such as ascorbate drops, citrate drops, corticosteroids, or bandage contact lenses may be utilized in conjunction with amniotic membrane transplantation, limbal stem cell transplantation, or a corneal transplant to resolve any corneal scarring or opacity [4]. When a stable ocular surface cannot be restored via limbal stem cell transplantation, a keratoprosthesis placement may be required. Despite interventions, however, suboptimal outcomes still frequently occur. Furthermore, the reliance on topical eye drop administration is not sufficient. Topical ophthalmic drops dissipate almost completely after drop administration [6,7] and must also be applied frequently. This is a problem for burn patients or elderly patients who might not be able to place a drop without assistance. A treatment that eliminates drop administration would not only be an improved treatment option, but would also decrease the burden of care. As a result, there is a need for more sophisticated ophthalmic treatment modalities that eliminate ophthalmic drops as well as promote wound healing. To this end, Jade Therapeutics, Inc. (wholly owned subsidiary of EyeGate Pharmaceuticals, Salt Lake City, UT, USA) has developed a continuous drug delivery system that would eliminate the need for drop administration while promoting wound healing. This drug delivery system is a proprietary thiolated, crosslinked hyaluronic acid (CMHA-S) polymer for ocular use that has been demonstrated to accelerate wound closure following photorefractive keratectomy (manuscript under review). Furthermore, when applied as a liquid gel, this polymer has a residence time on the ocular surface of longer than two hours (manuscript under review) due to unique engineering. This polymer, however, can also be manufactured as a thin, flexible film which can be inserted into the inferior fornix to provide sustained release of therapeutic agents. Crosslinked CMHA-S is a versatile, biocompatible polymer that combines the documented wound healing properties of HA [8] with drug-delivery capabilities to provide an innovative ophthalmic treatment when installed quickly (without the use of suturing or tissue glue) directly to the ocular surface [9]. This type of treatment not only has the potential to provide prolonged therapeutic delivery and promote ocular tissue repair, but also would not rely on self-administration making it extremely beneficial for ocular burn management. Due to the intrinsic healing properties of this polymer, we investigated this novel CMHA-S treatment modality to treat one of the most challenging types of ocular chemical burns, alkali chemical injuries. We hypothesize that crosslinked CMHA-S films are not only safe, but can also provide for the advanced treatment of corneal alkali burns as demonstrated in our ocular alkali chemical model of wound healing.

2. Materials and methods

2.1. Animals

Male New Zealand White (NZW) rabbits (2.5-4.0kg) were purchased from Charles River Laboratories (Wilmington, MA) and randomly grouped (N=5 per group). Sample-size requirement estimates are based on clinical examination scores and testing at the study endpoint. The sample size was estimated using a power analysis (SigmaPlot v12.5). Three treatment groups consisted of animals that received CMHA-S films on uninjured eyes, animals that received CMHA-S films with alkali burns, and animals that received no treatment with CMHA-S films, but had corneal alkali burns. All animal procedures were performed on anesthetized animals. Protocols were reviewed and approved by the United States Army Institute of Surgical Research (USAISR) Institutional Animal Care and Use Committee (IACUC). This study was conducted in compliance with Animal Welfare Regulations, other Federal statutes relating to animals and experiments involving animals, and the principles set forth in the Guide for the Care and Use of Laboratory Animals, National Research Council.

2.2. Nictitating membrane removal

The nictitating membrane was removed three weeks prior to corneal alkali burn creation or film placement. Prior to nictitating membrane removal, ketamine hydrochloride (VetOne™, Boise, ID USA; 35-45mg/kg), buprenorhine SR-LAB (0.5mg/kg), and xylazine (5mg/kg) were administered via intramuscular (IM) injection and animals were placed on isoflurane (1-3.5%). Left eyes of the rabbits were anesthetized.
with 0.5% tetracaine hydrochloride (Alcon, Ft. Worth, TX, USA). A 5% povidone iodine solution (10% povidone-iodine; Purdue Pharma L.P., Stamford, CT, USA) in phosphate buffered saline (PBS; Gibco, Grand Island, NY, USA) was utilized to sterilize the surgical site. A topical antibiotic drop, 0.1% moxifloxacin (Alcon) was administered before placing the eyelid speculum. After the eye was properly draped, a 0.5% tetracaine hydrochloride drop (Alcon) was administered for a second time. Forceps (#12) were employed to grip the nictitating membrane and a small (30G) needle was used to inject 0.1-0.2ml 2% Lidocaine HCl with Epinephrine 1:100,000 USP (Hospira, Inc. Lake Forest, IL, USA) into the posterior and anterior base of the nictitating membrane. Westcott scissors were used to remove the nictitating membrane. A week cell was utilized to remove blood, and a high temperature cautery was used to cauterize the surgical site. Bacitracin Zinc and Polymixin B Sulfate Ophthalmic Ointment USP (Bausch & Lomb, Rochester, NY, USA) was applied at the surgical site. Animals were placed in an oxygen chamber (39-40% oxygen) for recovery. Animals were checked twice daily for three days, treated for pain, and allowed to heal for three weeks.

2.3. Corneal alkali burn creation

Corneal alkali burns 5.5mm in diameter were created on anesthetized rabbits by soaking grade 52, 175 pm thick, 0.015% ash, circular filter paper (Whatman, GE Healthcare Bio-Sciences, and Pittsburg, PA, USA) in 1N NaOH for 60s before placing them on the central cornea for 30s. Corneas were measured with calipers to ensure central burn creation. Burns were immediately rinsed with 10ml balanced salt solution (BSS, Baxter, Deerfield, IL, USA) and debrided.

2.4. CMHA-S film placement

CMHA-S films were fabricated by combining a solution of thiolated carboxymethyl HA in PBS (final concentration, 16mg/ml) with a solution of poly(ethylene glycol) diacrylate (PEGDA; MW 3550) in PBS (final concentration, 15mg/ml). The resulting solution was aliquoted into silicone molds (3mm wide × 9.9mm long × 1mm thick), crosslinked for two hours, and dried at room temperature. Upon re-hydration, the CMHA-S films exhibit swelling to yield an oblong, clear, and flexible film 4mm wide by 15mm long. Dry CMHA-S films were partially rehydrated in sterile BSS for two minutes before being placed in the left inferior fornix. Some films were initially re-hydrated in 0.1% rose bengal dye in sterile BSS before placement for visualization. Animals were monitored daily for film retention and any signs of irritation.

2.5. Corneal imaging

Images were obtained at 0h, 48h, 96h, and on day 14 post injury utilizing a camera (7D, Canon USA., Melville, NY, USA) with a 100mm macro lens. Corneal wounds were stained using sterile fluorescein sodium ophthalmic films USP (Fluoret®, Chaunvin Laboratory, Aubenas, France) dampened with 100µl sterile BSS. The fluorescein solution was allowed to remain on the eye for 10s before being rinsed with BSS. Images of uptake were captured under cobalt blue light post staining. Percentage areas of wound closure and areas of corneal opacity were quantified by a blinded observer as previously described utilizing ImageJ software (NIH) [10]. Each time point was compared to 0h post wounding. Following fluorescein imaging, optical coherence tomography (OCT, Bioptigen Spectral Domain Optical Coherence Tomography (SD-OCT)) images were acquired. OCT images were analyzed with ImageJ software by measuring the widest section of the central cornea [11]. In vivo confocal microscopy images (Heidelberg Retina Tomograph (HRT3) Franklin, MA, USA) were obtained on uninjured, healthy control animals, but were not obtained on injured corneas due corneal opacity.

2.6. Histology

Eyes from euthanized rabbits were enucleated for histology on day 14 and fixed in modified Davidson’s solution (Poly Scientific R&D Corporation, Bay Shore, NY) for 24h before being transferred to 10% neutral buffered formalin (NBF; Thermo Fisher Scientific, Middletow, VA). Globes were bisected through the burn (when present) and placed, cut side down, in a megatissue cassette (Fisher Scientific, Fair Lawn, NJ). Tissues were embedded in paraffin, sectioned (3-4µm), and stained with hematoxylin and eosin (H&E) (Fisher Scientific, Fair Lawn, NJ) or Masson’s Trichrome (Poly Scientific R&D Corporation, Bay Shore, NY) according to the manufacturer’s protocol.

2.7. Statistical analysis

Data sets were analyzed by our in-house statisticians using a repeated measures 2-way analysis of variance (ANOVA) with JMP® 10 software (SAS Institute Inc., Cary, North Carolina, USA). Independent values are presented as mean ± standard error of the mean (SEM). A P-value of <0.05 was regarded as significant for all statistical analyses.

3. Results

3.1. CMHA-S films are retained in the inferior fornix

Although previous studies have demonstrated the ability of crosslinked CMHA-S in a drop formulation to facilitate corneal repair, it was unknown if CMHA-S in a hydrogel film would remain in the inferior fornix long enough to be an effective treatment. Therefore, retention of the film was an important part of our study. Films were rehydrated in sterile BSS prior to placement into the inferior fornix of healthy rabbit eyes. Films were observed to be safely retained and biocompatible in the inferior fornix for 96h after placement and remained structurally intact (Fig. 1). Daily gross observations of the films revealed that, while the films did not completely degrade or dissolve, some films exhibited mild wear and tear likely due to mechanical forces of eye movement. No irritation, redness, or swelling of the eye or ocular structures was observed in any animal due to film placement. Animals were monitored for 14 days post film placement and observed for any signs of pathology.
3.2. CMHA-S films are biocompatible

CMHA-S films were placed in the inferior fornix of animals with uninjured, healthy eyes and compared to healthy untreated (no CMHA-S films) eyes as a control (Fig. 2). White light imaging, fluorescein staining, in vivo confocal microscopy, and corneal surface histology were carried out 14 days post CMHA-S film placement (Fig. 2). White light imaging revealed no signs of corneal opacity. A lack of fluorescein uptake indicated that no corneal epithelial defects were present in either group (Fig. 2). In vivo confocal images as well as H&E and Masson’s Trichrome staining also did not reveal any corneal pathology in either group (Fig. 2).

3.3. CMHA-S films treatment results in a decrease in the area of corneal opacity

After the burn creation CMHA-S film placement, white light images (Fig. 3A) were taken at 0h, 48h, 96h, and on day 14 to observe corneal opacity. At 48h, our results revealed a 17% significant decrease of ("P < 0.05) in the area of corneal opacity in CMHA-S treated eyes while non-treated animals showed only a 4% decrease in corneal opacity (Fig. 3B). This difference continued to increase over time. On day 14 post injury, a 50% significant (""P < 0.001) decrease in opacity was observed in CMHA-S treated eyes while a 16% overall decrease was seen in non-treated injuries (Fig. 3B). Our results revealed significantly smaller areas of corneal opacity in CMHA-S treated injuries versus non-treated injuries and that differences between the two groups increased over time.

3.4. CMHA-S films improve corneal re-epithelialization

Given that previous animal and clinical studies have shown that formulations of crosslinked CMHA-S in a liquid drop facilitates corneal epithelial wound closure [12], a key focus of our study was to determine the effect of CMHA-S film treatment on corneal re-epithelialization. Fluorescein images (Fig. 4A) were taken at 0h, 48h, 96h, and on day 14 after the creation of corneal alkali burns and the placement of CMHA-S films to detect corneal epithelial defects via fluorescein uptake. Corneas treated with CMHA-S films exhibited less fluorescein uptake at 48 and 96h post injury and on day 14.

Fig. 1 – Retention of CMHA-S films in the inferior fornix. CMHA-S films were placed in the inferior fornix of healthy NZW rabbits and monitored daily for retention. CMHA-S films are shown in the inferior fornix at 0 and 48h and on day 14 post placement as indicated by the arrows (→). The data are representative of five total animals.

Fig. 2 – CMHA-S films are biocompatible. Two weeks after the placement of CMHA-S films, white light, fluorescein, and in vivo confocal (epithelium, stroma, endothelium) images were taken of non-treated and CMHA-S treated healthy eyes. Tissues were collected 14 days post film placement and corneal sections stained using H&E and Masson’s Trichrome staining. The data are representative of five animals per group.

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Fig. 3 – CMHA-S films decrease areas of corneal opacity. At 0, 48, and 96h and on day 14 after the creation of a corneal alkali burn and the placement of CMHA-S films, white light images were taken. Images show the area of corneal epithelial wound closure of non-treated wounds (A) and those treated with CMHA-S films (B). The histogram (C) represents the percentage area of corneal opacity at 0, 48, and 96h and on day 14 compared to 0h post injury. The values are expressed as mean ± SEM and are representative of four animals per group. The comparisons of the percentage areas of corneal opacity in CMHA-S treated versus non-treated wounds were performed by utilizing a 2-way repeated measures ANOVA. **P < 0.01.

(Fig. 4B). Fluorescein uptake at 48h indicated that CMHA-S treated wounds exhibited 99% re-epithelialization at 48h compared to approximately 86% re-epithelialization of non-treated control wounds resulting in a 13% percent improvement in re-epithelialization in CMHA-S treated wounds over non-treated wounds.

These injuries did initially re-epithelialize quickly but, also eroded regardless of treatment. By 96h, areas of fluorescein uptake began to erode and became larger (Fig. 4A). Fluorescein uptake revealed that CMHA-S treated wounds were 90% re-epithelialized while non-treated controls were 80% re-epithelialized. By day 14, there was a significant (**P < 0.01) difference in the fluorescein uptake between groups. CMHA-S treated wounds were 83% re-epithelialized and the non-treated controls were 63% re-epithelialized showing a 20% difference in fluorescein uptake (Fig. 4B).

3.5. CMHA-S treated alkali burns exhibited reduced corneal swelling

To determine if treatment with CMHA-S films had an effect on total corneal thickness, OCT images (Fig. 5A) were collected at 0h and on day 14 post injury. OCT images revealed that there were no differences in corneal thickness at 0h showing the

Fig. 4 – CMHA-S films improve epithelial wound closure. At 0, 48, and 96h and on day 14 after the creation of a corneal alkali burn and the placement of CMHA-S films, fluorescein images were taken. Fluorescein imaging results show the area of corneal epithelial wound closure of non-treated wounds (A) and those treated with CMHA-S films (B). The histogram (C) represents the percentage of wound closure at 48h, 96h, and on day 14 compared to day 0. The values are expressed as mean ± SEM and are representative of five animals per group. The comparisons of the percentage areas of corneal opacity in CMHA-S treated versus non-treated wounds were performed by utilizing a 2-way repeated measures ANOVA. **P < 0.01.

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average corneal thickness to be 400μm for both groups (Fig. 5B). At day 14, there was a significant difference (*P<0.05) of approximately 200μm in corneal thickness between burns CMHA-S treated burns (779μm±70 SEM) and non-treated burns (975μm±67 SEM) (Fig. 5B).

3.6 Corneal surface histology appears normal in both treatment groups

H&E staining performed on the central burn revealed no marked differences in corneal pathology between CMHA-S treated and non-treated burns 14 days post injury (Fig. 6). No neovascularization was observed and inflammation was minimal. H&E sections revealed that a thin layer of the epithelium was present at the time of enucleation on day 14, but had not yet returned to full thickness in either group. These results indicate that CMHA-S treatment has no negative consequences on the treatment of these corneal alkali burns.

4. Discussion

The purpose of this study was to assess the use, safety, and effectiveness of CMHA-S films as a more advanced treatment in our corneal alkali burn model. We assessed corneal re-epithelialization, corneal opacity, corneal thickness, and
conical pathology. Our studies revealed not only CMHA-S film biocompatibility, but also that CMHA-S treatment significantly increases corneal re-epithelialization while significantly decreasing areas of corneal opacity and swelling. The re-epithelialization of these burns is of particular interest given the effects wound closure has on the clinical etiology of ocular burns. In our studies, non-treated burns did not completely re-epithelialize at any point during the study unlike CMHA-S treated burns which re-epithelialized by 48h. By 96h post injury, however, corneal epithelial defects had reoccurred in both non-treated and treated groups. It is common to see these reoccurring corneal epithelial erosions in corneal alkali burn progression and was found to be unrelated to CMHA-S film treatment. Studies have noted that corneal erosions occur as early as 72h after a corneal alkali burn and can persist for up to 3 months [13]. The clinical progression of alkali chemical injuries can be divided into four phases: immediate, acute, early reparative, and late reparative [14,15]. After the immediate phase when the injury occurs, the acute phase (days 1–7) begins and a superficial layer of the epithelium forms to protect the cornea from enzymes in tears as the stroma attempts to heal [15]. During the early reparative phase (days 8–20), corneal ulceration is most likely to occur and chronic inflammation begins. By the late reparative phase (after day 20), the injury begins to resolve with either a good or poor prognosis [14,15]. It should be noted that this study focused on wound healing through the early reparative phase and these eyes did not receive additional anti-inflammatory drops during the course of the study. We observed that our model mimicked the human clinical progression, thus providing insight on how CMHA-S treatment affected burn pathology. CMHA-S films appear to specifically improve this process resulting in the complete re-epithelialization by 48h post injury. Even though epithelial defects reoccurred in both treatment groups by 96h, CMHA-S treated wounds were smaller at 96h compared to non-treated controls and were significantly smaller on day 14. Thus, it appears that the CMHA-S films continue to improve re-epithelialization through day 14 resulting in a more favorable outcome. We hypothesize that increased re-epithelialization in CMHA-S treated burns protects the eye from inflammatory mediators and enzymes in the tear film that lead to corneal perforation and thinning [15] and also decreases inflammatory cytokines released by the injured surface epithelium and underlying stroma. The reduction in inflammatory burden may lead to less corneal swelling, edema, and corneal opacity. This is consistent with our results that show significantly less corneal opacity (48h, 96h, and on day 14) and swelling (day 14) in CMHA-S treated burns versus non-treated burns.

Studies have shown that HA-based hydrogels facilitate re-epithelialization in extremity burn injuries [9,12,16]. This is consistent with our findings showing the impact of CMHA-S hydrogel film treatment on corneal burn re-epithelialization. Interestingly, other forms of HA such as non-crosslinked 0.2% HA drops have no long term impact on the healing of corneal alkali burns [17], while a 1% thiolated crosslinked derivative of HA (CMHA-SX) in a drop formulation has been shown to re-epithelialize corneal alkali burns by 48h [12]. Taken together, these results indicate that the specific formulation HA, concentration, and delivery method may impact treatment.

5. Conclusions

Our study is the first to our knowledge in which CMHA-S in the form of a hydrogel has been shown to facilitate the re-epithelialization of corneal alkali burns. Our results demonstrate that CMHA-S films may be a viable treatment to promote re-epithelialization while decreasing corneal opacity and swelling. Future studies aim to characterize the release of HA from the films as well as the exact mechanisms by which CMHA-S is promoting re-epithelialization including the investigation of how cytokines and inflammatory mediators may be modulated.

Declaration of competing/conflicts of interest


Hee-Kyoung Lee: EyeGate Pharmaceuticals, Inc.: Financial Support, Personal Financial Interest, Employment.

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