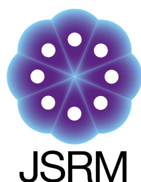
Regenerative Therapy 20 (2022) 51–60



Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

# Hyaluronic acid hydrogels crosslinked via blue light-induced thiol-ene reaction for the treatment of rat corneal alkali burn

Sun Kyoung Park [a], Minji Ha [b], Eun Jeong Kim [a], Youngyoon Amy Seo [c], Hyun Jong Lee [d], David Myung [c], Hyun-Seung Kim [a], Kyung-Sun Na [a, *]

[a] Department of Ophthalmology, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea
[b] HanGil Eye Hospital, Incheon, Republic of Korea
[c] Ophthalmology, Byers Eye Institute at Stanford University School of Medicine, Palo Alto, CA, USA
[d] Chemical and Biological Engineering, Gachon University, Seongnam-si, Gyeonggi-do, Republic of Korea

ARTICLE INFO

*Article history:*
Received 16 November 2021
Received in revised form
23 February 2022
Accepted 8 March 2022

*Keywords:*
Corneal burn
Corneal inflammation
Hyaluronic acid
Thiol-ene reaction
Photo-click chemistry
*In situ*-Forming hydrogel

ABSTRACT

To assess corneal inflammation from alkali chemical burns, we examined the therapeutic effects of *in situ*-forming hyaluronic acid (HA) hydrogels crosslinked via blue light-induced thiol-ene reaction on a rat corneal alkali burn model. Animals were divided into three groups ($n = 7$ rats per group): untreated, treated with 0.1% HA eye drops, and treated with crosslinked HA hydrogels. Crosslinking of HA hydrogel followed by the administration of HA eye drops and crosslinked HA hydrogels were carried out once a day from days 0–4. Corneal re-epithelialization, opacity, neovascularization, thickness, and histology were evaluated to compare the therapeutic effects of the three groups. Further investigation was conducted on the transparency of HA hydrogels to acquire the practical capabilities of hydrogel as a reservoir for drug delivery. Compared to untreated animals, animals treated with crosslinked HA hydrogels exhibited greater corneal re-epithelialization on days 1, 2, 4, and 7 post-injury ($p = 0.004$, $p = 0.007$, $p = 0.008$, and $p = 0.034$, respectively) and the least corneal neovascularization ($p = 0.008$). Histological analysis revealed lower infiltration of stromal inflammatory cells and compact collagen structure in crosslinked HA hydrogel-treated animals than in untreated animals. These findings corresponded with immunohistochemical analyses indicating that the expression of inflammatory markers such as α-SMA, MMP9, and IL1-β was lower in animals treated with crosslinked HA hydrogels than untreated animals and animals treated only with 0.1% HA eye drops. With beneficial pharmacological effects such as re-epithelization and anti-inflammation, *in situ*-forming hyaluronic acid (HA) hydrogels may be a promising approach to effective drug delivery in cases of corneal burn injuries.

© 2022, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

## 1. Introduction

Effective treatment of corneal chemical burns is pivotal in ophthalmic emergency because these injuries, spanning in severity from irritation to entire destruction of the ocular surface epithelium, often entail corneal opacification, limbal ischemia, and loss of vision [1]. Alkalis increase the pH of tissue and result in saponification of fatty acids in cell membranes, eventually leading to cellular destruction [2]. Following destruction of the corneal epithelium, alkali may penetrate the stroma and anterior chamber, eventuating in further tissue damage and inflammation [2]. Therefore, timely management of corneal inflammation plays a vital role in determining the severity of injury and preventing tragic consequences.

The treatment of corneal burns is directed at decreasing inflammation, limiting matrix degradation, and promoting re-epithelialization of the cornea [1,3–5]. Changes in conventional medical treatments which are limited to topical anti-inflammatory drugs including corticosteroids, cyclosporine, and supplementary biological fluids, such as autologous serum or amniotic membrane suspensions, have substantially plateaued in the recent decades

* Corresponding author. Department of Ophthalmology, Yeouido St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 10, 63-ro, Yeongdeungpo-gu, Seoul, 07345, Republic of Korea. Fax: 82-2-590-1693.
*E-mail address:* drna@catholic.ac.kr (K.-S. Na).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2022.03.005

2352-3204/© 2022, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

[2,5]. A major obstacle in treating ocular diseases includes providing and maintaining the optimal concentration of a drug on the ocular surface over a long period of time [6]. Although topical instillation is the most preferred route of administration, the bioavailability of the applied substance is less than 5% due to lacrimal turnover, blinking reflex, nasolacrimal drainage, and/or ocular barriers [7].

Hyaluronic acid (HA), a high-molecular-weight glycosaminoglycan and extracellular matrix component, enhances water retention on the ocular surface and promotes cell proliferation, which leads to accelerated epithelial wound healing [8–10]. The relatively high viscosity of HA allows for improved tear-film stability and reduction of washout from the ocular surface [11]. Hence, HA has become a salient treatment modality to be predominantly used as a component of tear supplements and protection of the corneal endothelium during ophthalmic surgery [11]. However, the drawback to this modality is that 90% of instilled HA washes out within an hour [12]. To overcome the low bioavailability and inconvenience of topical ophthalmic preparations, there has been growing interest in the development of novel biomaterials using tissue engineering to modify the molecular weight, viscosity, and hydrophobicity of HA [13]. Among the various approaches to the discovery of novel biomaterials, the crosslinking procedure has lured much attention with its ability to increase the residence time of macromolecules [14,15].

*In situ*-forming HA hydrogels crosslinked via blue light-induced thiol-ene reaction in the presence of riboflavin phosphate (RFP) have recently been introduced [15]. The thiol-ene “click” reaction is a highly efficient reaction between a thiol and an alkene to form an alkyl sulfide which is mediated by free radicals, and it is used to crosslink conjugated HA and other biomolecules [15,16]. Lee et al. has demonstrated that blue light exposure successfully initiate HA hydrogel crosslinking via thiol-ene reactions in a similar manner to that of UV light [15]. An *in vitro* study of cytocompatibility of hydrogels following a light-induced fabrication procedure has exemplified that gel materials did not affect cell viability [15]. In this study, we aimed to evaluate the therapeutic effects of *in situ*-forming HA hydrogels crosslinked via visible light-induced thiol-ene reaction on corneal inflammation using a rat corneal alkali burn model (Fig. 1).

## 2. Methods

### 2.1. Reagents and chemicals

Unless otherwise noted, all chemicals and solvents were of analytical grade and were used as per manufacturer's instructions. Dimethyl sulfoxide (DMSO), fluorescamine, sodium hydroxide solution (1.0 N), bovine serum albumin (BSA), fibronectin, cholera subunit A, insulin, Triton-X, and Cell Counting kit-8 were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1 × phosphate-buffered saline (PBS) pH 7.4, 0.1% HA, HA hydrogel, Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F-12) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), insulin-transferrin-selenium (ITS), Dulbecco's phosphate-buffered saline (DPBS), antimycotic antibiotic, and paraformaldehyde (PFA) were purchased from Thermo Fisher Scientific (Massachusetts, USA).

### 2.2. Measurement of the mechanical properties

The rheological properties of 0.1% HA eye drops, HA hydrogel precursor, and crosslinked HA hydrogels were measured by a rheometer (MCR 301, Anton Paar, Germany) equipped with a 25 mm parallel plate and a solvent trap for preventing water evaporation. Each sample with 100 μm volume was mounted onto the rheometer. First, the storage and loss moduli were measured using amplitude sweep from 0.1 to 100% with a fixed 1 Hz frequency to determine the linear viscoelastic region, and we

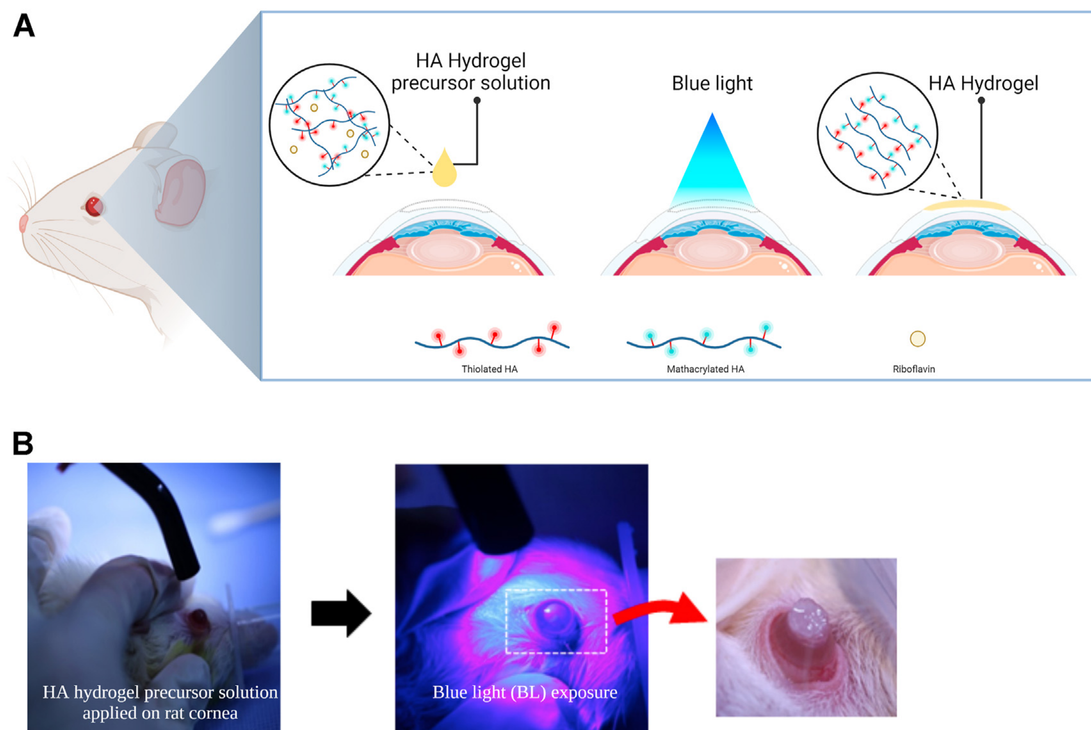



**Fig. 1. (A)** Schematic images of hyaluronic acid (HA) hydrogel formation crosslinked via blue light-induced thiol-ene reaction of thiolated HA (SH-HA) and methacrylated HA (MA-HA) with riboflavin phosphate (RFP). **(B)** Fabrication process of *in-situ* HA hydrogel on rat cornea through blue light induced thiol-ene reaction.

identified that 1% strain was appropriate. Then, the storage and loss moduli were measured using frequency sweeps from 0.1 to 10 Hz with a fixed 1% strain. The temperature was maintained at 37 °C during the experiments.

### 2.3. Measurement of HA hydrogel transparency

HA hydrogel transmission spectra were measured in the range of 300–700 nm using SpectraMax M Series Multi-Mode Microplate Reader (Sunnyvale, CA, USA). HA hydrogels were fabricated in 96-well plates using 100 μL of precursor solution with blue light exposure. After recording absorbance measurements, 150 μL of PBS was added to each well, followed by an incubation of the solution. The absorbance spectra were measured after 0, 2, 3, and 24 h by removing incubation solution.

### 2.4. Animals and corneal alkali burn model

A total of 21 male Sprague Dawley (SD) rats (7 weeks old, 200–300 g) were purchased from Saeronbio Inc. Anesthesia was achieved by intraperitoneal injection of ketamine hydrochloride (30 mg/kg). Alkali burn injury was performed on the right eye and was induced by pressing a qualitative filter paper disc (3.0 mm in diameter, Hyundal micro) containing 1 N NaOH onto the central cornea for 30 s. After removal of the filter paper disc, the corneal surface was carefully rinsed with 20 mL of physiological saline solution for 5 min.

Study protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (IRB No: YEO-2020-004FA), and this study was conducted in compliance with Animal Welfare Regulations.

The animals were randomly divided into three groups ($n = 7$ per group). Sample size requirement estimates are based on clinical examination scores and testing at the study endpoint. In the first group of animals, no further treatment was applied to the injured eyes. In the second group, 0.1% HA eye drops (Tearin free®, DHP Korea Co., Ltd., Korea) were applied onto the injured corneal surface. In the third group, HA hydrogels were crosslinked *in situ*. Methacrylated HA (MA-HA) and thiolated HA (SH-HA) were dissolved in PBS at 10 mg/mL, and the solutions were mixed at a 1:1 ratio. RFP was dissolved in PBS and added to the mixed HA solution. The mixture was applied to the injured cornea, and crosslinking was induced *in situ* using blue light. Exposure times of 40 s for blue light were implemented using a commercial 1.5 W dental LED curing blue light (Fig. 1B). Instillation of HA eye drops and crosslinking of HA hydrogel were conducted once daily on days 0–4. Photographs of the corneas were taken on day 0, 1, 2, 3, 4, and 7 post-injury. Animals were sacrificed on day 7 post-injury. The experimental eyes were enucleated and embedded within a mold. In alkali injury experiments, healthy corneas served as controls.

### 2.5. Histological examinations

After completion of study (on day 7), tissues were isolated. The cornea was carefully separated from the eye while avoiding damage and washed in 1 × PBS. Tissues were fixed using 4% PFA solution overnight at 4 °C and then washed in 1 × PBS. Tissue segments were dehydrated in graded ethanol, embedded in paraffin, and cut into 3- to 4-μm-thick sections using a microtome.

To study postoperative inflammatory cell infiltration, tissue sections were stained with hematoxylin-eosin (H-E). For H-E staining, tissue sections (3–4 μm) were rehydrated by immersing in xylene (3 × 5 min) followed by 100% (twice), 95%, 85%, and 70% ethanol for 1 min each, deionized water for 1 min, and hematoxylin (BBC Biochemical) and eosin (BBC Biochemical) solutions for 10–20 s before clearing with pure xylene.

For immunohistochemistry, tissue sections (3–4 μm) were rehydrated by immersing in xylene (3 × 5 min), 100% (twice), 95%, 85% and 70% ethanol for 5 min each, and deionized water for 5 min. Antigen retrieval buffer (citrate buffer, pH 6.0) was preheated to 92–95 °C. Slides were immersed in the pre heated solution for 5–10 min. Tissues were encircled with a hydrophobic barrier using a barrier pen. Tissues were further blocked with 2.5% normal horse serum (Vector) for 30 min–1 h. The slides were washed and incubated with primary antibodies: mouse anti-CK3 monoclonal antibody (ab68260, Abcam, Cambridge, UK; 1:50 dilution), mouse anti-alpha smooth muscle actin monoclonal antibody (ab7817, Abcam, Cambridge, UK; 1:100 dilution), rabbit IL-1 beta polyclonal antibody (ab9722, Abcam, Cambridge, UK; 1:1000 dilution), and mouse anti-MMP9 monoclonal antibody (ab58803, Abcam, Cambridge, UK; 1:1000 dilution) at 4 °C overnight. The next day, sections were washed three times in 1 × PBS followed by incubation with anti-mouse/rabbit IgG secondary antibodies (MP-7500, Vector, Burlingame, CA, USA) at room temperature (20–22 °C) for 1 h in the dark. For negative controls, nonimmune serum at 1:100 dilution was used instead of the specific primary antibody. After three washes in 1 × PBS, visualization was performed using a freshly prepared DAB (K5007, DAKO, USA) substrate-chromogen solution for 30–40 s, followed by three washes in 1 × PBS. Contrast staining was performed using hematoxylin (BBC Biochemical). Sections were observed and imaged with a Trinocular Microscope (Olympus BH2 BHS-312, Shinjuku, Tokyo, Japan).

### 2.6. Evaluation of corneal re-epithelialization

The extent of the corneal chemical injury was calculated using the following equation and expressed as a percentage (%):

$$E\,(\%) = W_s / W_t$$

Where $W_s$ is the extent of the cornea stained with fluorescence dye, $W_t$ is the extent of the whole cornea, and E represents the ratio indicating the extent of the corneal injury relative to the whole cornea. Denuded epithelium was stained with fluorescein dye, and it was visible under blue LED light. Blue LED light photos of rat corneas following alkali burn were taken on days 1, 2, 3, 4, and 7 post-injury. Measurements were processed using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). The extent of corneal chemical injury at baseline was assumed to be identical in all three experimental groups and was presented as a percentage (%).

### 2.7. Evaluation of corneal opacity and neovascularization

The degree of corneal opacity and neovascularization was measured after administration of 0.1% HA eye drops, crosslinked HA hydrogels, or no treatment to evaluate the efficacy of corresponding treatments. Two researchers (K.S.N and S.K.P.) assessed the high resolution photographs of the cornea and scored the degree of relevant parameters on day 7 post-injury using grading measures previously described by Larkin et al. [17]. The mean value of two measurements was obtained as a representative value. All data analyses were performed in a manner blinded to treatment groups. Corneal opacity was graded as follows: 0, completely transparent; 1, minimal loss of transparency; 2, moderate loss of transparency but iris vessels visible on retroillumination; 3, iris vessels not visible but pupil outline visible; 4, pupil outline not visible. Corneal neovascularization was graded as follows: 0, no vascularization of

graft; 1, vessel growth to 25% of graft radius in any quadrant; 2, vessel growth to 50% of graft radius; 3, vessel growth to 75% of graft radius; 4, vessel growth to center of graft.

### 2.8. Statistical analysis

One-way ANOVA followed by post hoc analysis was used to analyze the differences in corneal re-epithelialization and opacity between untreated injured corneas, injured corneas treated with 0.1% HA eye drops, and injured corneas treated with crosslinked HA hydrogels. Similarly, one-way ANOVA followed by post hoc analysis was used to compare the mean values of corneal neovascularization among groups, and Pearson's Chi-square test was used to compare the fraction of moderate or severe neovascularization among groups. P-values $< 0.05$ were considered statistically significant. Data were analyzed using SPSS statistical software, version 22, (SPSS Inc., USA).

## 3. Results

### 3.1. Rheological characterization of HA hydrogel

For the 0.1% HA eye drop and HA hydrogel precursor solution, the loss moduli (G″) were higher than the storage moduli (G′) from 0.1 to 10 Hz of frequency overall (Fig. 2A and B). Moreover, the values of G′ and G″ for solutions increased with increasing frequency. The results suggest that the ratio of the viscous component to stress is higher than elastic stress to strain, which is normal for materials in the liquid state. On the other hand, G′ was higher than G″ when the HA hydrogel precursor solution was irradiated by blue light. G′ of crosslinked HA hydrogel maintained about 200 Pa regardless of frequency (Fig. 2C). The rheological properties of blue light-irradiated HA hydrogel precursor solution indicate hydrogel formation by crosslinking through the combination of riboflavin phosphate and blue light irradiation.

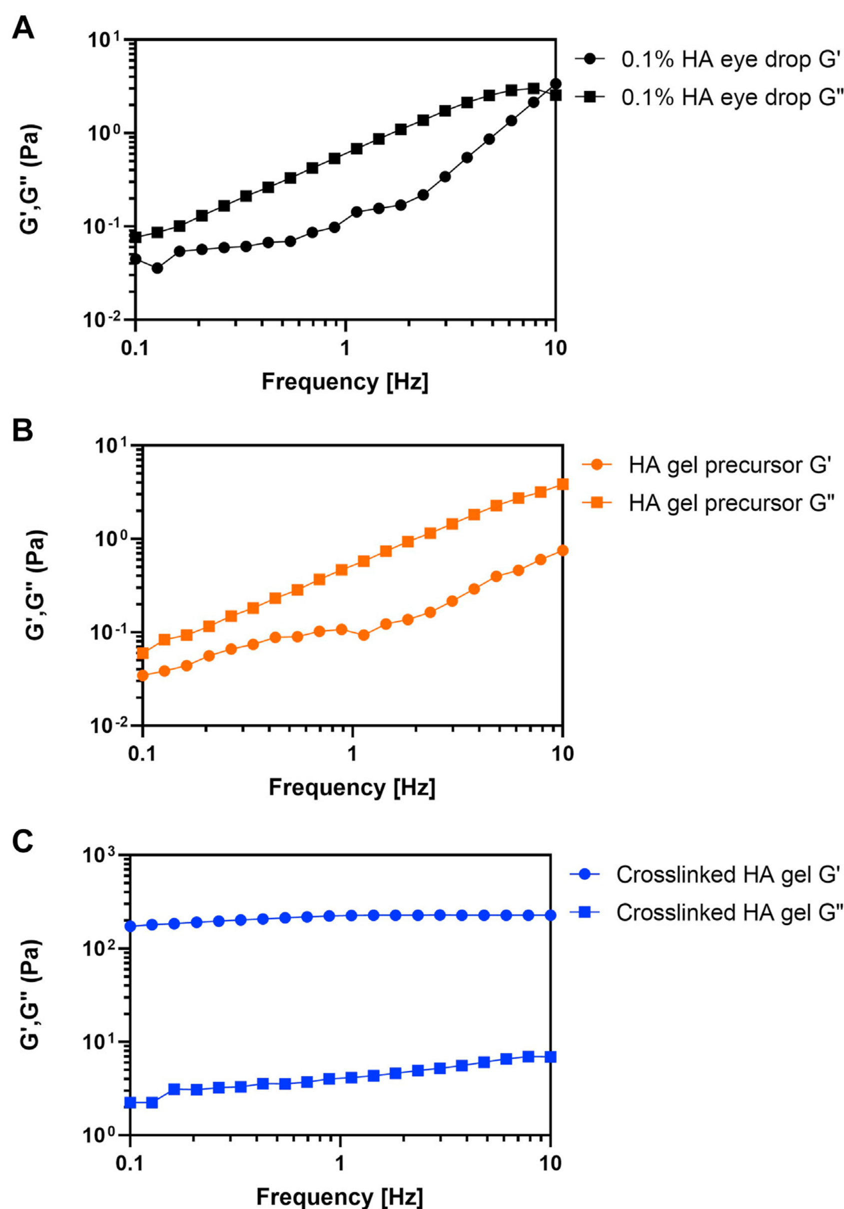



**Fig. 2.** Dynamic moduli (G′: storage modulus and G": loss modulus) plotted as a function of the frequency of **(A)** 1.0% HA eye drop, **(B)** HA hydrogel precursor solution, and **(c)** Crosslinked HA hydrogel from 0.1 to 10 Hz of frequency.

### 3.2. Transparency of HA hydrogel

Given that HA hydrogels are placed directly on the cornea, material transparency is a critical factor for practical application. As the HA hydrogels used in our study contained RFP which imparts yellow color, we measured the transmittance of HA hydrogels and obtained macroscopic photographs of these gels to evaluate the maintenance of corneal transparency.

Macroscopic photographs of the mixed HA solution, HA hydrogel precursor, and crosslinked HA hydrogels were placed over text to determine the respective transparencies (Fig. 3A). The text was clearly visible through crosslinked HA hydrogels, mixed HA solution, and HA hydrogel precursor. Although the texts were clearly shown under the three types of HA, the final color of the gels differed depending on the formulation method. For instance, crosslinking led to a loss of the original yellow color of the HA hydrogel precursor. That is, RFP undergoes a change in chemical structure upon blue light irradiation, resulting in a gel with a comparatively lighter yellow color. Given the remnants of the yellow color of RFP after crosslinking, we incubated the hydrogels in PBS to assess the time taken for the yellow color of the hydrogels to completely fade.

We measured HA hydrogel transmittance in 96-well plates at 0, 2, 4, and 24 h after crosslinking induced by 40 s of blue light irradiation. At 0 h after crosslinking, hydrogel transmittance was nearly 100% at visible spectrum wavelengths of 500–700 nm. Sharp decreases in transmittance at 370 and 445 nm were observed, indicating that the hydrogel contained residual RFP in the hydrogel matrix. During incubation of hydrogels in PBS solution, the RFP in the matrix diffused into the incubation solution. After 2 h of incubation, the transmittance at 370 and 445 nm increased, pinpointing to a lower amount of RFP in the hydrogel matrix. At 4 h after crosslinking, the yellow color derived from RFP had mostly disappeared almost to the point of complete transparency (Fig. 3A). The transmittance of RFP at 4 h and 24 h after crosslinking were almost identical, and the transmission spectra of HA hydrogels over time are presented in Fig. 3B. Based on the transmission spectra and gross examination of transparency, the yellow color of RFP did not appear to affect the overall transparency of HA hydrogels.

### 3.3. Corneal re-epithelialization

Facilitating re-epithelialization is of paramount importance in corneal wound healing. Here, we examined the extent of corneal erosion after chemical injury for 7 days. While the extent of corneal erosion decreased gradually over time in all experimental groups, corneal re-epithelialization was markedly observed in the group treated with crosslinked HA hydrogels. Significant differences in the extent of corneal erosion were observed between the untreated group and the group treated with crosslinked HA hydrogels on days 1, 2, and 4 post-injury ($p = 0.013$, $p = 0.014$, and $p = 0.032$, respectively) (Fig. 4T). Representative images of untreated corneas along with corneas treated with 0.1% HA eye drops and crosslinked HA hydrogels at baseline and on days 1, 2, 3, 4, and 7 post-injury are presented in Fig. 4A–S.

### 3.4. Corneal opacity and neovascularization

Corneal inflammation upon injury results in vision loss due to corneal opacification and neovascularization. Therefore, an investigation of corneal opacity and neovascularization is pertinent to study the overall efficacy of HA gels in various forms. First, the mean values of corneal opacity grading on day 7 post-injury in the untreated group, group treated with 0.1% HA eye drops, and group treated with crosslinked HA hydrogels were $2.7 \pm 0.6$, $2.9 \pm 0.3$, and $2.8 \pm 0.1$, respectively ($p = 0.647$, $p = 0.952$, and $p = 0.822$, respectively, for pairwise comparisons) (Fig. 4U). No significance differences were observed between groups. Subsequently, the mean values of corneal neovascularization grading in the untreated group, group treated with 0.1% HA eye drops, and group treated with crosslinked HA hydrogels were $2.2 \pm 0.6$, $2.2 \pm 0.6$, and $1,4 \pm 1.0$, respectively ($p = 1.000$, $p = 0.175$, and $p = 0.175$, for pairwise comparisons) (Fig. 4V). Grade 2 or higher, which indicated vessel growth over 25% of the graft radius, accounted for 71%, 86%, and 14% of the untreated group, group treated with 0.1% HA eye drops, and group treated with crosslinked HA hydrogels, respectively ($p = 0.515$, $p = 0.031$, and $p = 0.008$, respectively, for pairwise comparisons) (Fig. 4W). Although no significant differences in mean values of grading among groups were shown, the fraction of moderate or severe neovascularization was lower in the group treated with crosslinked HA hydrogels compared to the untreated group and group treated with 0.1% HA eye drops.

### 3.5. Histology

To explore the histological effects of the different treatments on injured corneas, we conducted a histological analysis. As illustrated in Fig. 5A, normal corneas are comprised of three to five layers of epithelial cells and compact collagen fibrils in their stroma. H-E staining of untreated injured corneas and corneas treated with 0.1%

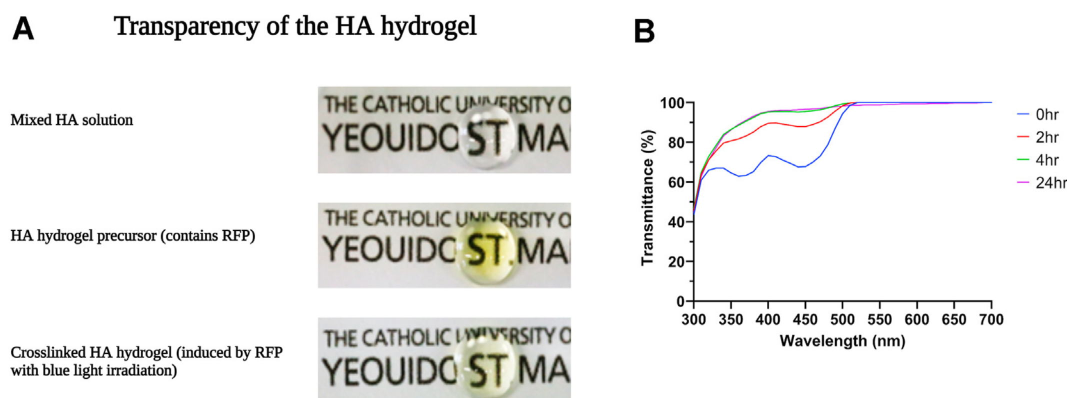



**Fig. 3.** **(A)** Macroscopic photographs of three materials over the text; mixed HA solution, HA hydrogel precursor, and crosslinked HA hydrogel. **(B)** Transmission spectra of RFP at 0, 4, 12, and 24 h after crosslinking.

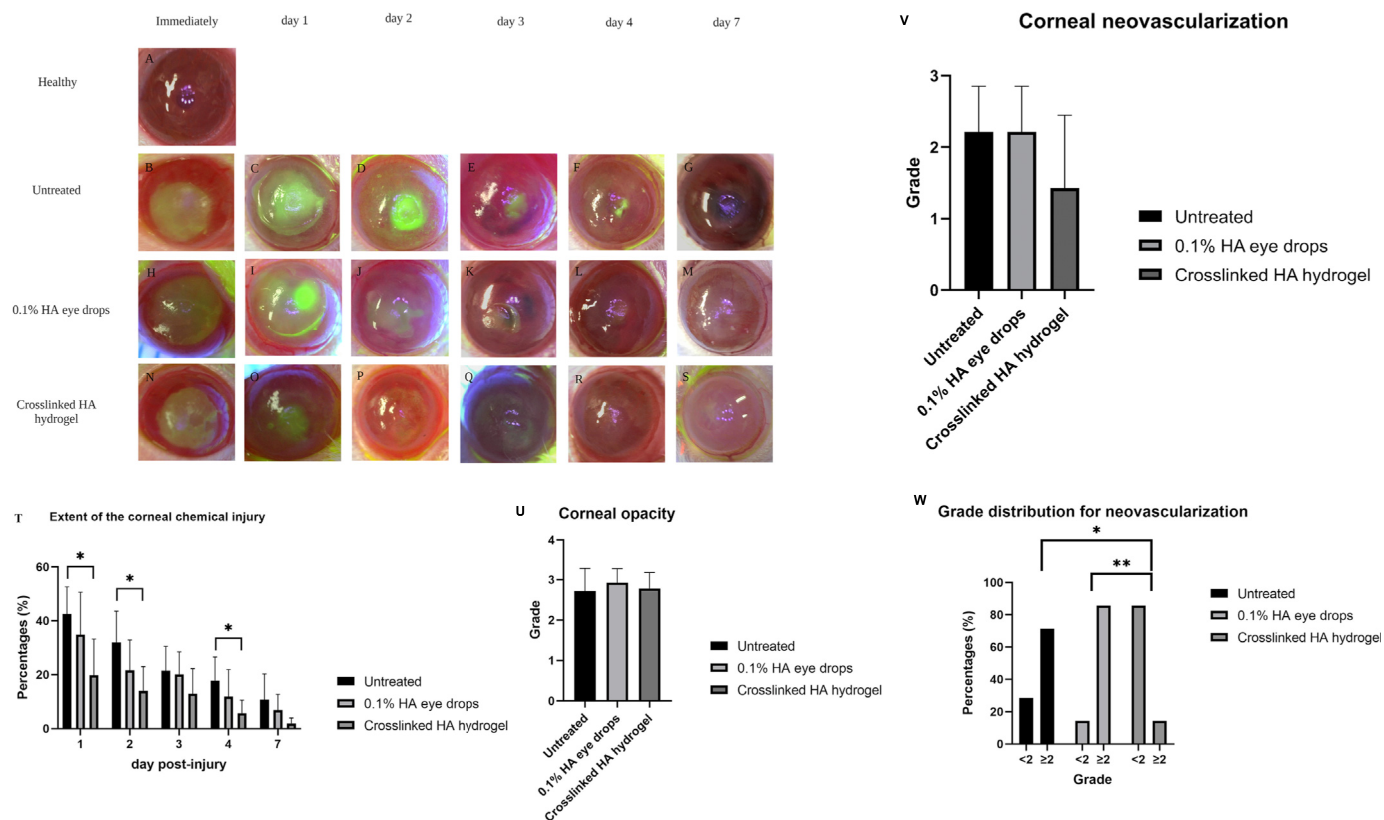



**Fig. 4.** Macroscopic images and quantified clinical parameters of injured and treated corneas. (A) Representative photographs of healthy corneas. (B, H, N) Corneas immediately after the injury with 1 N NaOH. (C–G) Untreated corneas on days 1, 2, 3, 4, and 7 post-injury. (I–M) Corneas treated with 0.1% HA eye drops on days 1, 2, 3, 4, and 7 post-injury. (O–S) Corneas treated with crosslinked HA hydrogel on days 1, 2, 3, 4, and 7 post-injury. (T) Quantification of corneal chemical injury in individual experimental groups of animals over time. Significant differences (* $P < 0.05$) between group of untreated and crosslinked HA hydrogel were found. (U) Quantification of corneal opacification and (V) corneal neovascularization. (W) Grade distribution for corneal neovascularization in each group. Significant differences (* $P < 0.05$, and ** $P < 0.01$) between group of crosslinked HA hydrogel and untreated/or 0.1% HA eye drops were found.

HA eye drops revealed greater infiltration of stromal inflammatory cells and looser stromal collagen structure compared to that in the crosslinked HA hydrogel-treated group; these effects were more pronounced in the untreated group (Fig. 5(B–D)).

Immunohistochemistry was performed on day 7 after corneal alkali injury to qualitatively assess the wound healing process and tissue inflammatory reactions of injured corneas (Fig. 5F–H, J–L, N–P, R–T). As opposed to high α-SMA expression in the untreated group, less prominent expression of α-SMA was identified in healthy corneas when detected in the anterior stroma injured corneas. In the process of wound healing and tissue repair, mature myofibroblasts secrete collagens and extracellular matrix materials that form fibrotic scars [17]. Thus, α-SMA is used as a marker of activated myofibroblasts, and α-SMA expression implies activated fibrosis in the corneal stroma. MMP9 production is stimulated by the pro-inflammatory cytokine IL1-β, and these factors play a role in corneal matrix degradation [18]. Hence, expression of MMP9 and IL1-β in the anterior stroma is associates to inflammation. Strong expressions of MMP9 and IL1-β were observed in inflammatory cells and keratocytes of untreated injured corneas. In other words, the expression of MMP9 and IL1-β was lower in injured corneas treated with 0.1% HA eye drops and crosslinked HA hydrogels than in untreated injured corneas. MMP9 and IL1-β expression was hardly detected in normal corneas. Expression of CK3, a cytokeratin specific for corneal epithelium, was identified in the epithelium of injured corneas and healthy corneas. As re-epithelialization had already occurred by day 7 post-injury, no differences in the expression of CK3 among groups were seen.

## 4. Discussion

This study aimed to investigate the therapeutic effects of *in situ*-forming hyaluronic acid (HA) hydrogels crosslinked via blue light-induced thiol-ene reaction on corneal inflammation using a rat corneal alkali burn model. We observed superior re-epithelialization in corneas treated with 0.1% HA eye drops or crosslinked HA hydrogels compared to that in untreated corneas. This result supports previous reports indicating that HA plays an important role in each phase of corneal epithelial wound healing, including proliferation and migration of corneal epithelial cells and fibroblasts, anti-inflammation, and wound repair [18–21]. HA exhibited dose-dependent stimulatory effects on epithelial migration in organ cultures of rabbit corneas [10]. Similar results were observed in vivo studies using rabbit corneas [22,23]. Concentration dependence for wound healing is considered to reflect the pharmacological effects of HA [22]. Higher concentrations of HA may have more enhanced therapeutic effects than the 0.1% concentration. However, we did not intend to determine the dose-dependent therapeutic effects of HA but the difference in therapeutic effects of the various formulations. Therefore, we selected 0.1% HA eye drops as a representative comparative medication for the cross-linked HA hydrogels.

Histological analyses conveyed superior corneal stromal organization in terms of thickness and collagen structure and less infiltration of inflammatory cells in corneas treated with cross-linked HA hydrogels compared to that in untreated corneas and corneas treated with 0.1% HA eye drops. Among all groups, the

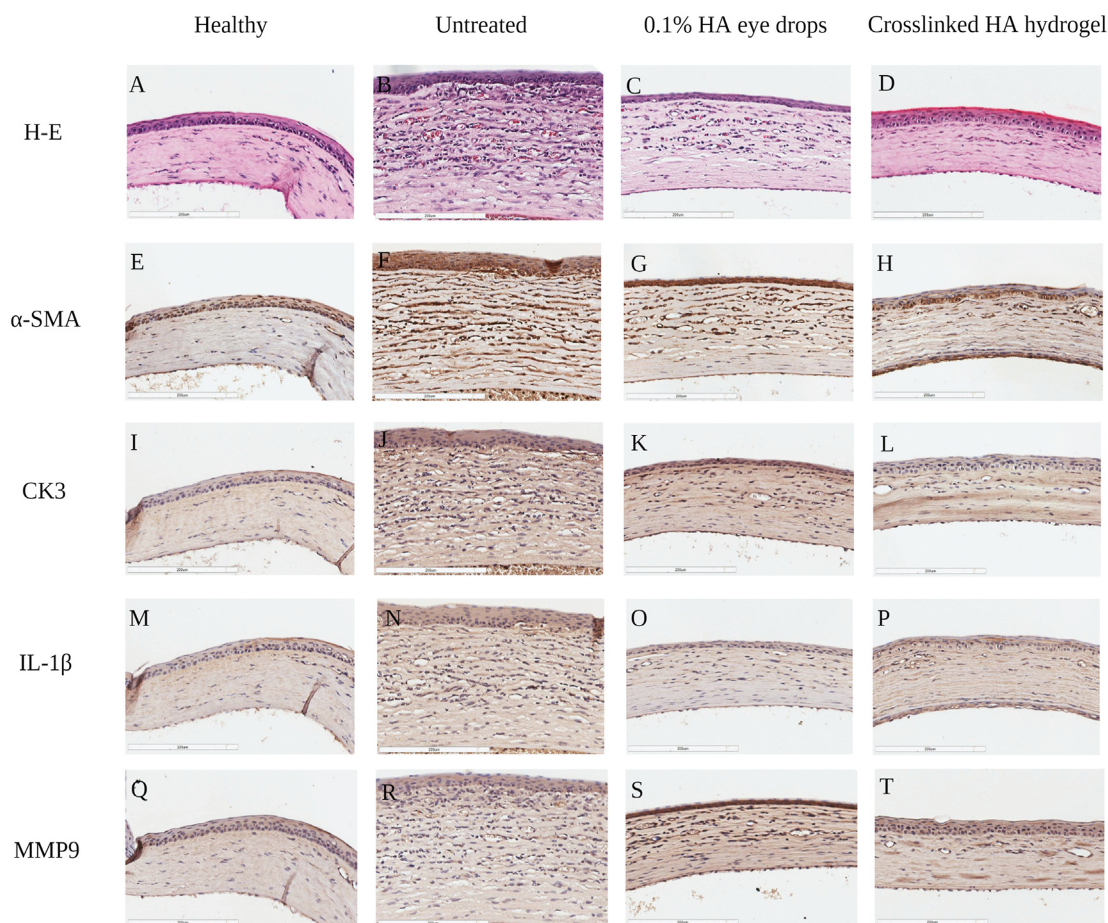



**Fig. 5.** Representative images of H-E and immunohistochemistry staining. **(A)** H-E stained healthy cornea showed infiltration of inflammatory cells. **(B)** H-E staining of untreated injured corneas and **(C)** corneas treated with 0.1% HA eye drops revealed increased infiltration of stromal inflammatory cells and loosened stromal collagen structure as well as increased corneal thickness compared to **(D)** crosslinked HA hydrogel group, and it was more pronounced in untreated group. The expression of α-SMA, MMP9, IL-1β, and CK3 was detected in **(E, I, M, Q)** heathy corneas, **(F, J, N, R)** untreated corneas, **(G, K, O, S)** corneas treated with 0.1% HA eye drops or **(H, L, P, T)** with crosslinked HA hydrogel. Scale bar: 200 μm.

crosslinked HA hydrogel-treated group exhibited the least corneal neovascularization on day 7 post-injury in terms of mean grading value and severity, which lures attention to the anti-inflammatory effects of HA. Although the functions of HA are determined by its molecular weight [24], HA in its native form as a large polymer has anti-inflammatory and immunosuppressive properties [25]. CD44, the main receptor for hyaluronan, plays a crucial role in fibroblast migration and inflammatory responses in a wound environment. However, the interaction between CD44 and HA is necessary to activate the wound healing process as neither CD44 nor HA alone can induce cell migration and promote wound healing [26]. Further work is merited to investigate the mechanisms of CD44 activation in the early phase of corneal chemical injury.

No significant differences were observed in corneal opacification on day 7 post-injury between all groups, and at least two factors may underpin this observation. First, alkali substances penetrate the eye rapidly, in as little as 5–15 min [2]. Due to the quick speed of this process, tissue denaturation is inevitable if pH neutralization with copious irrigation is achieved immediately after the injury, though the inflammatory response can be controlled with medical treatment in the acute or subacute phase. Secondly, various factors influence the rate of corneal wound healing following chemical injury including the inflammatory response and structural damage to the epithelial basement membrane [1], which may lead to corneal wound healing taking months or years depending on these factors. We suppose that 7 days may have been insufficient to observe improvements in corneal opacification/scarring and neovascularization. Studies with longer durations are therefore warranted.

Numerous results in corneal wound healing efficacy have been observed according to types of corneal wound. One study reported that eye drops with HA and high potassium concentration combined were reported to promote corneal epithelial wound healing in a mechanical scraping model but not in an alkali burn model [27]. We assert that this could be due to differences in the severity of corneal wounds between simple corneal abrasion and alkali burn injuries. Compared to mechanically denuded wounds, alkali burn injuries are more severe and penetrative due to saponification from alkali. Damaged tissues secrete proteolytic enzymes as part of an inflammatory response, leading to collateral damage termed liquefactive necrosis [2]. Since the absorption of drugs instilled in the eye may be impeded by eye barriers and continuous turnover of tears, eye drops must be instilled frequently and/or at high concentration to exert sufficient therapeutic effects [28]. The rapid degradation and short residence time of HA have limited its clinical application [29]. To extend contact time with the damaged corneal tissue and improve bioavailability, chemical modifications of HA, such as hydrogels, polymer micelles, biodegradable nanocapsules, HA-coated nanospheres, and niosomes have been developed [28].

Among these, hydrogels contain a large amount of water in their 3D network of hydrophilic polymer chains [30]. Since water is the greatest component of the human body, a hydrogel, which can

absorb large quantities of water, has advantages of increased biocompatibility compared to other types of biomaterials [31–33]. In the liquid state, the diffusivity has a finite value, whereas the diffusivity of the molecules in the gel is completely zero. The diffusivity depends on the rate of crosslinking, and it would affect the therapeutic potential regarding releasing molecules. In the study by Kim et al. [34], the matrix degradation of HA hydrogels, which was fabricated in the same manner as the one used in our study, was evaluated. HA hydrogel with a 1:1 ratio of SH-HA to MA-HA maintained its original structure without degradation on day 4 after crosslinking, and the degradation process lasted for two weeks. Although, HA polymer solution also contains a large amount of water as HA hydrogels could, since the degradation rate of HA polymer solution is faster than HA hydrogel, the therapeutic efficacy of HA polymer solution in wound circumstances is much less than HA hydrogel.

In our animal study, we identified the difference in therapeutic efficacy between HA hydrogels and liquid-stated 0.1% HA eye drops. Moreover, hydrogel is considered to have great potential when applied for biomedical purposes due to its similarities to the body tissues [31–33]. Meanwhile, we conducted crosslinking, once daily, on days 0–4 despite the slow degradation rate of HA hydrogels. Since eyelid tension or blinking rate can act as variables on the residence time of HA hydrogels, it might unexpectedly fall off early. Moreover, the rat eyes were too small to determine whether HA hydrogels were in place or not. In this respect, it is necessary to customize HA hydrogel to have desired mechanical properties for each individual, considering the eyelid tension or blinking rate. Additionally, one might also expect that the changes in material properties vary according to stress relaxation and this will lead to different results. However, we focused on the in vivo therapeutic effect of crosslinked HA hydrogel rather than the detailed characteristics of the hydrogel in this study. Further studies of the stress relaxation for the treatment are warranted.

There are adhesive biomaterials that have been commercialized recently. For example, ReSure (Ocular Therapeutix Inc. MA, USA), a polyethylene glycol based synthetic adhesives, is FDA-approved sealant in the United States, which is designed to seal full-thickness corneal incisions in ocular surgery. According to the FDA summary of safety and effectiveness data for ReSure, it was fully degraded within approximately 81 h (3.4 days) *in vitro*, and was no longer present by day 2 in vivo. Since the degradation rate of ReSure is faster than that of HA hydrogel, ReSure may be a suitable material as a sealant for clear corneal incision in which reepithelialization is completed within 3 days. However, it is not suitable for severer wounds in which the material is required to be in place much longer to exert sufficient therapeutic effect [35]. Meanwhile, Lee et al. identified that there is no link between cells and HA hydrogel, unlike in ReSure [15].

In recent decades, several trials have been initiated to develop drug-loaded HA formulations due to the viscosifying and mucoadhesive properties of HA as well as its protective role in the cornea and conjunctival epithelium [36]. Most formulations involve the encapsulation of drugs in nanocapsules that can be instilled as eye drops, but the rapid drainage caused by tear turnover still limits ocular bioavailability. HA hydrogels have therefore been studied as drug carriers in the form of films or membranes, injectable gels, and micro/nanoparticles for sustained release of drugs [37]. Yoon et al. [38] developed UV-triggered photo-crosslinked HA nanoparticles (c-HANPs) which demonstrated high stability in a physiological buffer and released the loaded drug, paclitaxel (PTX) (an anticancer drug), in a sustained manner. In addition, a comparative study probed drug release kinetics between drug-loaded liposomes into hydrogels and drug-loaded liposomes alone [37]. Latanoprost-liposome loaded HA hydrogels released the drug in a more sustained manner compared to liposomes alone. These results highlight the potential of nanocomposite hydrogels as efficient drug carriers that permit sustained drug release at the ocular level. Several trials have been conducted using crosslinking approaches to improve the integrity of HA, resulting in enhancement of its bioavailability [37–42]. Photo-crosslinking reactions are often used to obtain hydrogels with HA because crosslinked structures improve stability and allow the polymer chains of HA to break down more gradually, leading to an ideal candidate for biomedical applications, such as wound dressing, artificial corneas, or contact lenses [24,43].

Although HA in isolation is insufficient to promote wound healing following severe chemical injuries, we aimed to investigate the pure pharmacological effects of HA and the possibility of using HA as a drug delivery platform. For treatment of chemical injuries, prolonged administration of multiple medications including topical steroids, antibiotics, ascorbate, citrate, cycloplegics, antihypertensives, vascular endothelial growth factor (VEGF) inhibitors, serum eye drops, and topical lubricants is necessary. Despite the use of these medications, however, chemical burns may produce several sequelae such as potential corneal ulceration/perforation, corneal scarring, limbal stem cell deficiency, corneal neovascularization, conjunctival scarring/symblepharon, dry eye, and lid malposition from cicatricial changes [2,27]. To mitigate these effects, extensive efforts have been devoted in identifying more effective treatments such as anti-angiogenic or stem cell-based therapies [44–51]. Combining HA with these medications may result in synergetic effects because the residence time of medications on the ocular surface can be prolonged, thereby allowing sustained drug release due to the mucoadhesive and viscosifying properties of HA.

An advantage of the *in situ*-forming HA employed in this study included the elimination of the need to consider and tailor the shape and curvature of the cornea. Moreover, the height of HA hydrogel can be easily controlled by the volume of the precursor solution. However, since living cells were simultaneously irradiated when the cornea was induced by blue light, it is in point of fact that safety issues of blue light should be considered. Blue light is a light source commonly used in slit lamp microscopy for ophthalmic examination. Since blue light is used to irradiate the human eye for several minutes during slit lamp microscopic examinations with time taken for crosslinking of only 40 s, phototoxicity associated with blue light is likely negligible. Even more, harmful effects on ocular tissues including the cornea, lens, and retina may be abated compared to UV-triggered crosslinking which is frequently used for conventional crosslinking reactions. Lee et al. [15] signified that blue light posed the same or even greater effectiveness in activating the thiol-ene crosslinking reaction with RFP compared to that of UV.

## 5. Conclusion

All in all, we presented pure pharmacological effects of HA for corneal re-epithelialization and anti-inflammation, as well as the potential of harnessing *in situ*-forming HA hydrogel as an optimized formulation for corneal injury and drug delivery. Although progress has been made in understanding the therapeutic impact of HA on corneal alkali burns, there is still a paucity of research on the utilization of *in situ*-forming HA hydrogels for drug delivery. Future studies should focus on the synergistic effects of epidermal growth factor (EGF)- or keratinocyte growth factor (KGF)- loaded HA in corneal wound healing and evaluate their therapeutic effects in various animal models, such as diabetic rats and alkali burn models. Moreover, determining the optimal intensity and irradiation time of blue light in clinical settings is imperative to minimize damage to surrounding ocular tissues including the cornea, iris, lens, and retina.

## Author contributions

S.K.Park contributed to the text writing, and discussion of the results. M. Ha designed and performed the experiments. E.J.Kim managed the rats, performed sample preparations, performed histological examinations, helped with experiments, and wrote the manuscript. Y.A.Seo helped with experiments. H.J.Lee performed examinations for chemical property of materials, and helped writing the manuscript. D. Myung, and H.S.Kim participated in the discussion of the results. K.S.Na supervised the project, formulated the hypothesis, designed experiments, analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

## Funding

This study was supported by a grant from the National Research Foundation of Korea (NRF) (2019R1F1A1061421 and 2022R1A2C2006109) and by a grant from the Catholic University Yeouido St. Mary's Hospital in the program year of 2021. The funders had no role in the study design, data collection and analysis, decision on publishing, or preparation of the manuscript.

## Declaration of competing interest

The authors declare that they have no conflicts of interest with contents of this article.

## Acknowledgments

We would like to express our gratitude to Jong Sung Kim, veterinarian for providing support with in vivo studies.

## References

[1] Wagoner MD. Chemical injuries of the eye: current concepts in pathophysiology and therapy. Surv Ophthalmol 1997;41(4):275–313.
[2] Fish R, Davidson RS. Management of ocular thermal and chemical injuries, including amniotic membrane therapy. Curr Opin Ophthalmol 2010;21(4): 317–21.
[3] Javorkova E, Trosan P, Zajicova A, Krulova M, Hajkova M, Holan V. Modulation of the early inflammatory microenvironment in the alkali-burned eye by systemically administered interferon-γ-treated mesenchymal stromal cells. Stem Cell Dev 2014;23(20):2490–500.
[4] Soiberman U, Kambhampati SP, Wu T, Mishra MK, Oh Y, Sharma R, et al. Subconjunctival injectable dendrimer-dexamethasone gel for the treatment of corneal inflammation. Biomaterials 2017;125:38–53.
[5] Wu CL, Chou HC, Li JM, Chen YW, Chen JH, Chen YH, et al. Hyaluronic acid-dependent protection against alkali-burned human corneal cells. Electrophoresis 2013;34(3):388–96.
[6] Irimia T, Ghica MV, Popa L, Anuţa V, Arsene AL, Dinu-Pîrvu CE. Strategies for improving ocular drug bioavailability and corneal wound healing with Chitosan-based delivery systems. Polymers (Basel) 2018;10(11).
[7] Patel A, Cholkar K, Agrahari V, Mitra AK. Ocular drug delivery systems: an overview. World J Pharmacol 2013;2(2):47–64.
[8] Lapcík Jr L, Lapcík L, De Smedt S, Demeester J, Chabrecek P. Hyaluronan: preparation, structure, properties, and applications. Chem Rev 1998;98(8): 2663–84.
[9] Nakamura M, Hikida M, Nakano T, Ito S, Hamano T, Kinoshita S. Characterization of water retentive properties of hyaluronan. Cornea 1993;12(5): 433–6.
[10] Nishida T, Nakamura M, Mishima H, Otori T. Hyaluronan stimulates corneal epithelial migration. Exp Eye Res 1991;53(6):753–8.
[11] Posarelli C, Passani A, Del Re M, Fogli S, Toro MD, Ferreras A, et al. Cross-linked hyaluronic acid as tear film Substitute. J Ocul Pharmacol Therapeut 2019;35(7):381–7.
[12] Mochizuki H, Yamada M, Hato S, Nishida T. Fluorophotometric measurement of the precorneal residence time of topically applied hyaluronic acid. Br J Ophthalmol 2008;92(1):108–11.
[13] Valachová K, Volpi N, Stern R, Soltes L. Hyaluronan in medical practice. Curr Med Chem 2016;23(31):3607–17.
[14] Griffith GL, Wirostko B, Lee HK, Cornell LE, McDaniel JS, Zamora DO, et al. Treatment of corneal chemical alkali burns with a crosslinked thiolated hyaluronic acid film. Burns 2018;44(5):1179–86.
[15] Lee HJ, Fernandes-Cunha GM, Myung D. In situ-forming hyaluronic acid hydrogel through visible light-induced thiol-ene reaction. React Funct Polym 2018;131:29–35.
[16] McCall JD, Anseth KS. Thiol-ene photopolymerizations provide a facile method to encapsulate proteins and maintain their bioactivity. Biomacromolecules 2012;13(8):2410–7.
[17] Larkin DF, Calder VL, Lightman SL. Identification and characterization of cells infiltrating the graft and aqueous humour in rat corneal allograft rejection. Clin Exp Immunol 1997;107(2):381–91.
[18] Calienno R, Curcio C, Lanzini M, Nubile M, Mastropasqua L. In vivo and ex vivo evaluation of cell-cell interactions, adhesion and migration in ocular surface of patients undergone excimer laser refractive surgery after topical therapy with different lubricant eyedrops. Int Ophthalmol 2018;38(4):1591–9.
[19] Inoue M, Katakami C. The effect of hyaluronic acid on corneal epithelial cell proliferation. Invest Ophthalmol Vis Sci 1993;34(7):2313–5.
[20] Voigt J, Driver VR. Hyaluronic acid derivatives and their healing effect on burns, epithelial surgical wounds, and chronic wounds: a systematic review and meta-analysis of randomized controlled trials. Wound Repair Regen 2012;20(3):317–31.
[21] Saranraj P, Naidu M. Hyaluronic acid production and its applications A review. Int J Pharm Biol Arch 2013;4(5):853–9.
[22] Camillieri G, Bucolo C, Rossi S, Drago F. Hyaluronan-induced stimulation of corneal wound healing is a pure pharmacological effect. J Ocul Pharmacol Therapeut 2004;20(6):548–53.
[23] Nakamura M, Hikida M, Nakano T. Concentration and molecular weight dependency of rabbit corneal epithelial wound healing on hyaluronan. Curr Eye Res 1992;11(10):981–6.
[24] Tripodo G, Trapani A, Torre ML, Giammona G, Trapani G, Mandracchia D. Hyaluronic acid and its derivatives in drug delivery and imaging: recent advances and challenges. Eur J Pharm Biopharm 2015;97(Pt B):400–16.
[25] Litwiniuk M, Krejner A, Speyrer MS, Gauto AR, Grzela T. Hyaluronic acid in inflammation and tissue regeneration. Wounds 2016;28(3):78–88.
[26] Svee K, White J, Vaillant P, Jessurun J, Roongta U, Krumwiede M, et al. Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. J Clin Invest 1996;98(8):1713–27.
[27] Ho WT, Chiang TH, Chang SW, Chen YH, Hu FR, Wang IJ. Enhanced corneal wound healing with hyaluronic acid and high-potassium artificial tears. Clin Exp Optom 2013;96(6):536–41.
[28] Suárez-Barrio C, Etxebarria J, Hernáez-Moya R, Del Val-Alonso M, Rodriguez-Astigarraga M, Urkaregi A, et al. Hyaluronic acid combined with serum Rich in growth factors in corneal epithelial Defects. Int J Mol Sci 2019;20(7).
[29] Prestwich GD, Kuo JW. Chemically-modified HA for therapy and regenerative medicine. Curr Pharmaceut Biotechnol 2008;9(4):242–5.
[30] Ahmadian E, Dizaj SM, Eftekhari A, Dalir E, Vahedi P, Hasanzadeh A, et al. The potential applications of hyaluronic acid hydrogels in biomedicine. Drug Res (Stuttg) 2020;70(1):6–11.
[31] Daniele MA, Adams AA, Naciri J, North SH, Ligler FS. Interpenetrating networks based on gelatin methacrylamide and PEG formed using concurrent thiol click chemistries for hydrogel tissue engineering scaffolds. Biomaterials 2014;35(6):1845–56.
[32] Chai Q, Jiao Y, Yu X. Hydrogels for biomedical applications: their characteristics and the mechanisms behind them. Gels 2017;3(1).
[33] Billiet T, Vandenhaute M, Schelfhout J, Van Vlierberghe S, Dubruel P. A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. Biomaterials 2012;33(26):6020–41.
[34] Kim H, Koh W-G, Lee HJ. Effects of basic fibroblast growth factor combined with an injectable in situ crosslinked hyaluronic acid hydrogel for a dermal filler. React Funct Polym 2021;164:104933.
[35] Shirzaei Sani E, Kheirkhah A, Rana D, Sun Z, Foulsham W, Sheikhi A, et al. Sutureless repair of corneal injuries using naturally derived bioadhesive hydrogels. Sci Adv 2019;5(3):eaav1281.
[36] Debbasch C, De La Salle SB, Brignole F, Rat P, Warnet JM, Baudouin C. Cytoprotective effects of hyaluronic acid and Carbomer 934P in ocular surface epithelial cells. Invest Ophthalmol Vis Sci 2002;43(11):3409–15.
[37] Widjaja LK, Bora M, Chan PN, Lipik V, Wong TT, Venkatraman SS. Hyaluronic acid-based nanocomposite hydrogels for ocular drug delivery applications. J Biomed Mater Res A 2014;102(9):3056–65.
[38] Yoon HY, Koo H, Choi KY, Chan Kwon I, Choi K, Park JH, et al. Photo-crosslinked hyaluronic acid nanoparticles with improved stability for in vivo tumor-targeted drug delivery. Biomaterials 2013;34(21):5273–80.
[39] Manzi G, Zoratto N, Matano S, Sabia R, Villani C, Coviello T, et al. “Click” hyaluronan based nanohydrogels as multifunctionalizable carriers for hydrophobic drugs. Carbohydr Polym 2017;174:706–15.
[40] Grimaudo MA, Nicoli S, Santi P, Concheiro A, Alvarez-Lorenzo C. Cyclosporine-loaded cross-linked inserts of sodium hyaluronan and hydroxypropyl-β-cyclodextrin for ocular administration. Carbohydr Polym 2018;201:308–16.
[41] Choi SC, Yoo MA, Lee SY, Lee HJ, Son DH, Jung J, et al. Modulation of biomechanical properties of hyaluronic acid hydrogels by crosslinking agents. J Biomed Mater Res A 2015;103(9):3072–80.
[42] Calles JA, Tártara LI, Lopez-García A, Diebold Y, Palma SD, Vallés EM. Novel bioadhesive hyaluronan-itaconic acid crosslinked films for ocular therapy. Int J Pharm 2013;455(1–2):48–56.
[43] Segura T, Anderson BC, Chung PH, Webber RE, Shull KR, Shea LD. Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern. Biomaterials 2005;26(4):359–71.

[44] Zavala J, López Jaime GR, Rodríguez Barrientos CA, Valdez-Garcia J. Corneal endothelium: developmental strategies for regeneration. Eye (Lond) 2013;27(5):579–88.
[45] Zahir-Jouzdani F, Soleimani M, Mahbod M, Mottaghitalab F, Vakhshite F, Arefian E, et al. Corneal chemical burn treatment through a delivery system consisting of TGF-β(1) siRNA: in vitro and in vivo. Drug Deliv Transl Res 2018;8(5):1127–38.
[46] Yao L, Bai H. Review: mesenchymal stem cells and corneal reconstruction. Mol Vis 2013;19:2237–43.
[47] Shin YJ, Hyon JY, Choi WS, Yi K, Chung ES, Chung TY, et al. Chemical injury-induced corneal opacity and neovascularization reduced by rapamycin via TGF-β1/ERK pathways regulation. Invest Ophthalmol Vis Sci 2013;54(7):4452–8.
[48] Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol Ther 2012;20(1):14–20.
[49] Prockop DJ, Oh JY. Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. J Cell Biochem 2012;113(5):1460–9.
[50] Friedman M, Azrad-Lebovitz T, Morzaev D, Zahavi A, Marianayagam NJ, Nicholson JD, et al. Protective effect of TLR4 ablation against corneal neovascularization following chemical burn in a mouse model. Curr Eye Res 2019;44(5):505–13.
[51] Estrella-Mendoza MF, Jiménez-Gómez F, López-Ornelas A, Pérez-Gutiérrez RM, Flores-Estrada J. Cucurbita argyrosperma seed Extracts attenuate angiogenesis in a corneal chemical burn model. Nutrients 2019;11(5).