



FULL-LENGTH ARTICLE

Basic Research

Effect of mesenchymal stromal cells encapsulated within polyethylene glycol-collagen hydrogels formed in situ on alkali-burned corneas in an *ex vivo* organ culture model



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ABSTRACT

Background aims: Corneal inflammation after alkali burns often results in vision loss due to corneal opacification and neovascularization. Mesenchymal stem cells (MSCs) and their secreted factors (secretome) have been studied for their anti-inflammatory and anti-angiogenic properties with encouraging results. However, topical instillation of MSCs or their secretome is often accompanied by issues related to delivery or rapid washout. Polyethylene glycol (PEG) and collagen are well-known biomaterials used extensively in scaffolds for tissue engineering. To effectively suppress alkaline burn-induced corneal injury, the authors proposed encapsulating MSCs within collagen gels cross-linked with multi-functional PEG-succinimidyl esters as a means to deliver the secretome of immobilized MSCs.

Methods: Human MSCs were added to a neutralized collagen solution and mixed with a solution of four-arm PEG-N-hydroxysuccinimide. An *ex vivo* organ culture was conducted using rabbit corneas injured by alkali burn. MSCs were encapsulated within PEG-collagen hydrogels and injected onto the wounded cornea immediately following alkali burn and washing. Photographs of the ocular surface were taken over a period of 7 days after the alkali burn and processed for immunohistochemical evaluation. Samples were split into three groups: injury without treatment, MSCs alone, and MSCs encapsulated within PEG-collagen hydrogels.

Results: All corneas in *ex vivo* organ culture lost their transparency immediately after alkali burn, and only the groups treated with MSCs and MSCs encapsulated within PEG-collagen hydrogels recovered some transparency after 7 days. Immunohistochemical analysis revealed increased expression of vimentin in the anterior corneal stroma of the group without treatment indicative of fibrotic healing, whereas less stromal vimentin was detected in the group containing MSCs encapsulated within the PEG-collagen hydrogels.

Conclusions: PEG-collagen hydrogels enable the encapsulation of viable MSCs capable of releasing secreted factors onto the ocular surface. Encapsulating MSCs within PEG-collagen hydrogels may be a promising method for delivering their therapeutic benefits in cases of ocular inflammatory diseases, such as alkali burn injuries.

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Introduction

The severity of chemical injuries to the external eye can vary from mild irritation to complete destruction of the ocular surface, which leads to corneal opacification, persistent ulceration, angiogenesis and

vision loss [1]. The most severe form of chemical injury arises from alkaline solutions, resulting in the saponification of fatty acids in cell membranes and cellular disruption [2]. With damage to the epithelium, the alkaline agent penetrates the corneal stroma, where it destroys proteoglycans and collagen fibers. Injury management usually focuses on decreasing inflammation, preventing angiogenesis and promoting re-epithelialization of the cornea [2–4]. Intense polymorphonuclear leukocyte infiltration of the corneal stroma can dissolve stromal collagen in the acute phase of alkali burns [5].

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Corticosteroids are excellent inhibitors of polymorphonuclear leukocytes and prevent further damage to stromal melting; however, they are often associated with epithelial wound healing retardation, increased intraocular pressure and development of cataract or secondary infection [6–8]. Although a number of treatment modalities have been tested, such as antibiotics, ascorbic acid, collagenase inhibitors and surgery, structural and functional restoration of the cornea from alkali burn injuries remains a challenge [2,9].

Mesenchymal stem cells (MSCs) are multipotent stromal cells derived from the mesoderm that can differentiate into a variety of cell types [10]. MSCs and their secreted factors (secretome) have been studied for their multi-functional properties, from tissue repair/regeneration to immunomodulatory/anti-inflammatory functions [11–13]. MSCs have been shown to be a promising treatment modality for corneal chemical burns [4,14,15]. However, because of dilution into tears or rapid washout through the nasolacrimal pump, topical instillation of MSCs or their secretome does not always yield favorable treatment results [16,17]. Thus, to achieve clinical application, the development of biomaterial matrices to deliver and retain MSCs on the ocular surface remains crucial. Conventional two-dimensional (2D) systems for stem cell culture have demonstrated a non-physiological environment that could affect cell signaling [18]. Recently, stem cell cellular and acellular niches have been emphasized with regard to cell function [19]. Cross-linked collagen has not only been used to prepare scaffolds for tissue repair and engineering by increasing stability and controlling degradation but has also been shown to act as a three-dimensional MSC niche that mimics the *in vivo* environment [18,20–22].

The authors have been using an organ-cultured rabbit cornea model to investigate a series of modalities that take advantage of the therapeutic benefits of MSCs and their secretome, subsequently applying their findings to mitigating the deleterious sequelae of alkaline burns on the ocular surface [23]. In this study, the authors chose to encapsulate bone marrow-derived human MSCs within collagen gels cross-linked by multi-arm polyethylene glycol (PEG)-N-hydroxysuccinimide (NHS) MSCs (Figure 1) to deliver these cells and their beneficial factors to the corneal surface.

Methods

Materials

Unless otherwise noted, all chemicals and solvents were of analytical grade and used as advised by the manufacturer. Four- and eight-arm PEG-succinimidyl glutarate were purchased from JenKem Technology (Dallas, TX, USA). Collagenase, dimethyl sulfoxide, fluorescamine, sodium hydroxide solution (1.0 N), bovine serum albumin, fibronectin, cholera subunit A, insulin, Triton X and Cell Counting Kit 8 were purchased from Sigma-Aldrich (St Louis, MO, USA). Phosphate-buffered saline (PBS) pH 7.4, 10× PBS, Slide-A-Lyzer dialysis kit (3.5 kDa molecular weight cutoff), collagen I bovine protein

solution (5 mg/mL), collagenase, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, insulin-transferrin-selenium, Dulbecco's PBS (DPBS), antibiotic/antimycotic, Live/Dead viability/cytotoxicity staining kit and paraformaldehyde (PFA) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Collagen cross-linking by four- and eight-arm PEG-NHS

The pH of type I bovine collagen was neutralized using a solution of 1 M sodium hydroxide solution, deionized water and 10× PBS in a 3:57:20 ratio as previously described [24]. Briefly, the 5 mg.mL⁻¹ collagen solution was mixed with the neutralization solution in a 3:2 ratio so that the final concentration of collagen was 3 mg.mL⁻¹. Collagen consists of two alpha-1 chains and one alpha-2 chain, each of which contains approximately 1000 amino acids. Since conjugation of the click chemistry functional groups occurs with the primary amine of lysine by PEG-NHS ester reaction, the number of lysine residues is important for determining the concentration of each chemical. Lysine constitutes 2.5–3.0% of collagen, amounting to a total of 75–100 lysine residues in the triple helix, and 3 mg of collagen corresponds to 7.5×10^{-9} mol and 7.5×10^{-7} mol of primary amine. The four-arm PEG-NHS has four NHS groups per molecule; the molar equivalent amount of NHS groups is 1.875 mg per 3 mg of primary amine group in collagen. Thus, 18.75 µL of 100 mg.mL⁻¹ of four-arm PEG-NHS provided the equivalent amount of NHS groups. Four-arm PEG-NHS was dissolved in PBS at 100 mg.mL⁻¹, and the solution was then mixed with the neutralized collagen solution at the desired concentration (0.2% w/v, 0.4% w/v, 0.8% w/v and 1.6% w/v) for 30 seconds at room temperature. Subsequently, the collagen gels were incubated for 10–15 min at 37°C for completion of gelation.

Human MSC culture and cell encapsulation in PEG-collagen hydrogels

As previously described, MSCs (PT-2501) were purchased from Lonza (Basel, Switzerland), and the cells expressed CD29, CD44, CD105, CD166, CD90 and CD73, but not CD14, CD34, CD45 or CD19 [23]. MSCs were cultured in a culture medium (10% v/v fetal bovine serum and 1% v/v antibiotic/antimycotic solution in DMEM) and incubated at 37°C in 5% carbon dioxide. For MSC encapsulation, MSCs were added to the neutralized collagen solution in the fourth passage, resulting in a final concentration of 1.0×10^5 cells.mL⁻¹ in the PEG-collagen hydrogel, followed by the addition of four-arm PEG-NHS solution.

Cell encapsulation within hydrogels on porcine corneas

To evaluate PEG-collagen hydrogel on the cornea, the authors formed the PEG hydrogel using collagen conjugated to fluorescein isothiocyanate (FITC). MSCs were incubated with CellTracker (Thermo Fisher Scientific) for 30 min. After incubation, the authors

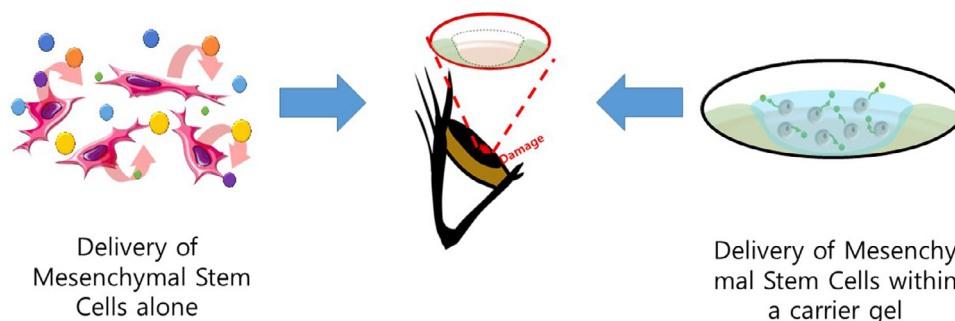


Fig. 1. Schematic diagram showing the two main approaches to delivering the benefits of bone marrow-derived human MSCs. MSCs were either administered onto ex vivo corneal wounds topically or encapsulated within PEG-collagen hydrogels. (Color version of figure is available online).

encapsulated the cells in the PEG-collagen hydrogel following the same procedure described earlier, and the final PEG concentration was 4 mg/mL. As a negative control, the authors applied only collagen conjugated to FITC. The corneas were washed three times with PBS and immersed in PFA 4% for 1 h and sucrose 10% and 20% for 2 h. Next, the corneas were placed into a tissue freezing medium and sectioned for histological evaluations.

To avoid collagen autofluorescence masking the results, corneas were incubated with Alexa Fluor 647 IgG fraction monoclonal mouse anti-fluorescein (Thermo Fisher Scientific) in blocking solution (1:100) and left for 2 h at room temperature. The sections were washed three times with PBS, and a solution of 4',6-diamidino-2-phenylindole (DAPI) in PBS (1:1000) was added for 5 min. After washing with PBS 3 times, the sections were mounted and observed under confocal microscopy. The encapsulated cells were observed by exciting the CellTracker dye (Thermo Fisher Scientific) under confocal microscopy (Zeiss LSM 880; Carl Zeiss Meditec AG, Jena, Germany).

Human MSC biocompatibility and morphology

In evaluating whether PEG-collagen hydrogels provided a suitable substrate for their growth, the authors examined cell viability, cellular morphological changes and phenotypes of MSCs through *in vitro* cell culture experiments. The sustained viability of MSCs using Live/Dead staining (Thermo Fisher Scientific) revealed that the gels with different concentrations were cytocompatible. PEG-collagen hydrogels were formed in a 48-well plate as previously mentioned and incubated with supplemented keratinocyte serum-free medium overnight. Afterwards, 2×10^5 MSC cells were encapsulated and cultured within the hydrogels for 2 days. Live/Dead solution (Thermo Fisher Scientific) was added, and the cells were evaluated under an inverted microscope. At 2 days, 4 days and 8 days, the authors collected the supernatant, and the remaining hydrogel was degraded by the addition of collagenase 2.5 mg/mL for 10 h. After this time, the suspension was centrifuged at $350 \times g$, and the degraded hydrogel was collected for protein analysis (Luminex; Luminex Corporation, Austin, TX, USA).

The conditioned medium from encapsulated MSCs was collected and centrifuged at $500 \times g$ for 10 min. This assay was performed by the Human Immune Monitoring Center at Stanford University. Human 73-plex eBiosciences with modifications were used, closely following the manufacturer's recommendations. Beads were added to a 96-well plate and washed in an ELx405 washer (BioTek Instruments, Inc, Winooski, VT, USA). Samples were added to the plate containing the mixed antibody-linked beads and incubated at room temperature for an hour, followed by overnight incubation at 4°C with smooth and consistent shaking. Here, cold (4°C) and room temperature incubation plates were executed on an orbital shaker at 500–600 rpm. Following overnight incubation, plates were washed in an ELx405 washer (BioTek Instruments, Inc). The biotinylated detection antibody was then added at room temperature with consistent shaking for 75 min. The same washing protocol described earlier was performed before adding streptavidin-phycoerythrin. The same washing protocol was used after incubation at room temperature for 30 min, and the reading buffer was added to the wells. Each sample was measured in duplicate. Plates were read using a Luminex 200 (Luminex Corporation) or a FlexMAP 3D instrument (Luminex Corporation) with a lower bound of 50 beads per sample per cytokine. The Custom Assay Chex control beads that were added were purchased from Radix BioSolutions (Georgetown, TX, USA), and all well supernatants were analyzed by Luminex multiplex assay (Luminex Corporation) at the Human Immune Monitoring Center at Stanford University. Using the cell number for each condition, the raw mean fluorescence intensity was normalized and compared.

To evaluate the morphology of MSCs encapsulated within the PEG-collagen hydrogels, samples were fixed with 4% PFA and

permeabilized with 0.1% Triton X (Sigma-Aldrich) at each time point. The cells were stained with Alexa Fluor 647 phalloidin (A12379; Thermo Fisher Scientific) and incubated for 20 min. After washing three times with DPBS, 5 mg/mL of DAPI solution in DPBS was added, followed by a 5-min incubation. The glass plates were mounted, and the cell morphology was observed using fluorescence confocal microscopy. Lamellipodia were visually quantified as thin cytoplasmic sheets extending at the front of the cells, whereas filopodia were visually quantified as finger-like projections at the edges of the cells.

Rabbit corneal organ culture

Rabbit eyes were obtained from VisionTech (Sunnyvale, TX, USA) within 2 h after death, transported to the laboratory at 4°C and used immediately. Fresh eyeballs were disinfected with 10% povidone-iodine solution twice and washed in PBS. The ocular surface of the eyeballs was air-dried for 1 min. Meanwhile, Whatman filter paper (Whatman Sigma-Aldrich, USA) was punched with a 5-mm diameter biopsy punch (Integra LifeSciences, Princeton, NJ, USA), and the circular-cut filter papers were completely soaked in 1 M sodium hydroxide solution for 10 seconds. The sodium hydroxide-absorbed filter paper was placed on the center of the cornea for 1 min, and the eyes were rinsed with flowing water for 5 min immediately after the removal of the filter paper.

For the air–liquid interface organ culture system, the authors followed the method described in previous studies using a rabbit organ culture model [23,24]. After the injury, corneoscleral preparations of the wounded corneas were made from the globes with a 1-mm scleral rim, grasping only scleral rims to avoid touching the limbus. Excised corneas were immediately transferred onto individual agar plugs to maintain normal culture and nutritional support. Agar plugs were made from a 1:1 mixture of serum-free medium containing double-strength antibiotic/antimycotic and 2% agar in distilled water. The agar plugs were made using polydimethylsiloxane (PDMS) molds. The base and curing agents of Sylgard 184 (Dow Corning, Midland, MI, USA) were mixed at a 10:1 weight ratio, and the 10 mL round-bottom tubes were posted to the PDMS precursor mixture. The tubes were removed after PDMS curing, and the PDMS mold was autoclaved before use. Wounded corneas on the agar plugs were placed in a 12-well plate with 1 mL of complete serum-free culture medium, which was sufficient in bringing the medium to the level of the limbal area. DMEM/F-12 containing 120 µg/mL penicillin G, 200 µg/mL streptomycin sulfate and insulin-transferrin-selenium pre-mix was used as the culture medium. Samples were incubated at 37°C in a 5% carbon dioxide atmosphere, and the cornea apical surface was irrigated to remove debris and maintain moisture. The culture medium was also changed daily.

For the MSC group, MSCs (1.0×10^5 cells) were directly seeded onto the wounded corneal tissue. For the MSCs encapsulated in the PEG-collagen hydrogel group, MSCs (1.0×10^5 cells) were added to the neutralized collagen, and four-arm PEG-NHS solution was added at a final volume of 1 mL to match the MSC numbers in both groups. The four-arm PEG (0.8%) hydrogel was topically applied onto the corneal wounded surface to form an *in situ* hydrogel on the corneal surface. Photographs of fluorescein-stained corneas were taken with a smartphone ophthalmic imaging adapter (Paxos Scope; IrisVision, Pleasanton, USA) equipped with a $\times 15$ magnifying lens and a blue light-emitting diode in the same manner as previously reported [23].

Immunofluorescence staining

Upon conclusion of the *ex vivo* organ culture experiment, the corneas were cut in half, fixed in 4% PFA for 15 min at 4°C and processed with graded sucrose solutions and 15% sucrose for 2 h. Afterwards, they were left in a 30% sucrose solution overnight at 4°C. Subsequently, corneas were embedded in the optimal critical temperature

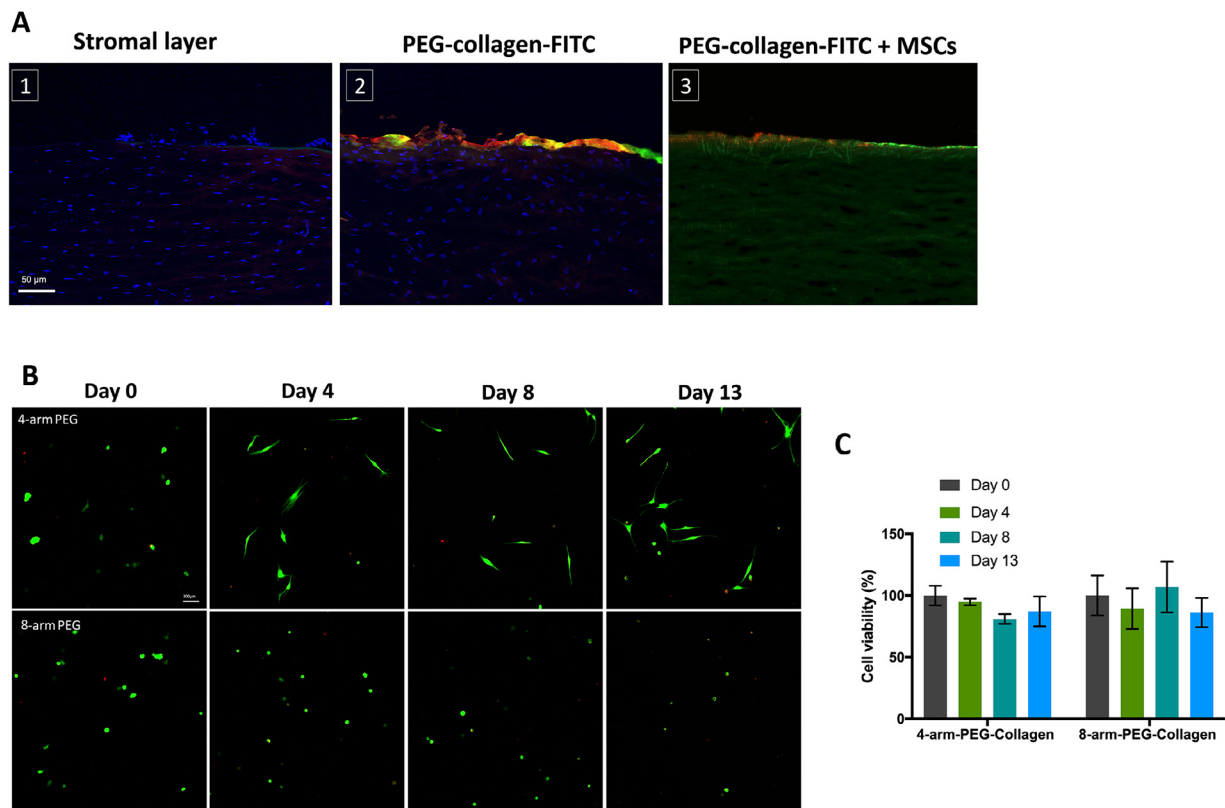


Fig. 2. (A) Presence of PEG-collagen hydrogel on the stromal layer of *ex vivo* porcine cornea. Some degree of corneal autofluorescence = can be observed. PEG-collagen-FITC (green) and anti-FITC antibody (red) confirmed that the gel adheres to the stromal layer of the cornea. MSCs are observed on the stromal layer after application of the gel. (B) MSC viability using the Live/Dead assay after encapsulation in four- and eight-arm PEG-collagen gel at 0.4% w/v at 13 days of culture. Live cells were stained green, whereas dead cells were stained red. (C) Quantification of Live/Dead assay shows that cell viability remained around 100% at 13 days of culture. (Color version of figure is available online).

compound, and 18- μ m sections were cut with a cryostat (Leica CM3050 S research cryostat; Leica Biosystems, Wetzlar, Germany). The eyes were sectioned and permeabilized for 30 min with 5% normal goat serum and 0.5% Triton X (Sigma-Aldrich). Next, anti-vimentin antibody 1:100 (ab137321; Abcam, Cambridge UK) was added to the corneal sections and incubated overnight in the same permeabilization solution. After 3 PBS washes, secondary antibodies (1:1000) and Alexa Fluor 546-coupled goat anti-rabbit IgG (Thermo Fisher Scientific) were added for 2 h. Finally, after washing with PBS, DAPI was added for 5 min in PBS solution (1:1000). The corneas were mounted and observed using confocal microscopy (Zeiss LSM 880; Carl Zeiss Meditec AG). Negative controls were not incubated with primary antibodies. Images were taken with a confocal microscope at $\times 10$ magnification. Additionally, Alexa Fluor 647 (Thermo Fisher Scientific) was conjugated to the PEG-collagen following the manufacturer's protocol to visualize the collagen gels formed on the corneal wounds by fluorescence microscopy. Briefly, Alexa Fluor 647 reactive dye (Thermo Fisher Scientific) was mixed with collagen, incubated for 2 h at 4°C and dialyzed via a Slide-A-Lyzer dialysis kit (Thermo Fisher Scientific) overnight in PBS at 4°C. Finally, the four-arm PEG-NHS dissolved in PBS was added to the neutralized collagen.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation. Each experiment was performed at least three times, unless otherwise indicated. Statistical evaluation was performed using one-way analysis of variance, Kruskal–Wallis test and uncorrected Dunn's test for multiple comparisons. Results with $P < 0.05$ were considered statistically significant. Statistical analysis was performed using the statistical software Prism 7 (GraphPad Software, San Diego, CA, USA).

Results

Adhesion of PEG-collagen hydrogel on corneal stroma and gel biocompatibility

The authors' goal was for the PEG-collagen hydrogel to adhere to the corneal stroma while providing a favorable physiological environment for MSCs. The authors applied NHS ester chemistry to cross-link low concentrations of exogenously applied collagen type I using multi-arm PEG and expected the NHS group to also react with the primary amine groups on the collagen of porcine corneal stroma. First, the authors evaluated whether the PEG-collagen hydrogel could bind to an *ex vivo* corneal stromal layer after removing the epithelial layer. To visualize the gel, collagen was conjugated with FITC before cross-linking with PEG. After washing in PBS 3 times, the authors observed that the gel could form and be retained on the stromal surface (Figure 2A). To confirm the results, the authors incubated the corneas with anti-FITC antibody 647. Figure 2A shows the 488 and 647 emission confirming that the green fluorescence stems from the gel and not from the autofluorescence of corneal collagen. Next, the authors aimed to evaluate the presence of MSCs delivered by the gel onto the cornea. Figure 2A shows how encapsulated MSCs marked with 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate CellTracker (Thermo Fisher Scientific) are observed on the *ex vivo* corneal stroma after application of the PEG-collagen hydrogel.

To analyze the gels' biocompatibility, MSCs were encapsulated in the gels, and a Live/Dead cell assay (Thermo Fisher Scientific) was performed (Figure 2B). Cell viability was compared with cells encapsulated 30 min after gelation. PEG-collagen gels demonstrated excellent biocompatibility after 13 days of encapsulation. Cells encapsulated in the four-arm PEG-collagen gel spread during the evaluated time frame, but cells encapsulated in eight-arm PEG-

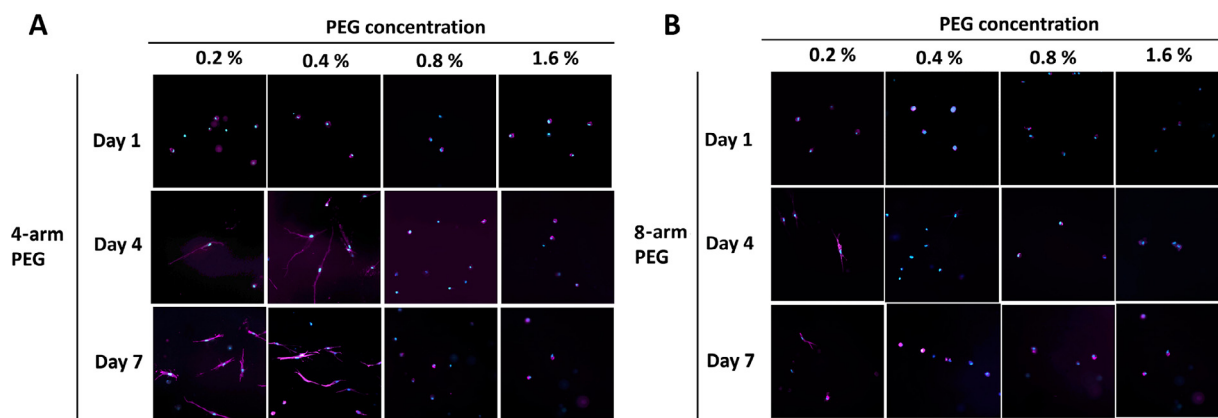


Fig. 3. MSC morphology encapsulated in (A) four-arm and (B) eight-arm PEG-collagen hydrogels on day 1, day 4 and day 7. Immunofluorescence staining was performed with F-actin (magenta) using Alexa Fluor 647 phalloidin and a nuclear counterstain with DAPI (blue). F-actin = filamentous actin. (Color version of figure is available online).

collagen gel did not spread very well. Cell viabilities for four- and eight-arm PEG-collagen hydrogels were both high, at nearly 100% for 13 days (Figure 2C). No significant difference in cell viability was observed between the four- and eight-arm PEG-collagen hydrogels.

MSC morphology encapsulated on PEG-collagen hydrogels at varying PEG concentrations

Next, the authors evaluated MSC behavior as a function of time, PEG arm number and concentration. Figure 3 shows filamentous actin staining of MSCs encapsulated within PEG-collagen hydrogels. At day 1, the cells maintained a round shape in the case of both four- and eight-arm PEG-collagen at all concentrations. At day 4, cells encapsulated in four-arm PEG-collagen at 0.2% w/v and 0.4% w/v and eight-arm PEG-collagen at 0.2% w/v showed spread morphology compared with the other conditions. No changes in cell morphology were observed at day 7 for all evaluated groups. These results suggest that the functionality and concentration of the cross-linker can modulate cell spreading. A lower degree of cell spreading occurred with higher PEG concentration. In addition, the authors found the cells to be less spread in eight-arm PEG-collagen at the same concentration as the four-arm PEG-collagen hydrogel, indicating that the number of PEG arms plays a modulatory role in encapsulated cell behavior.

MSC secretome production comparison between PEG-collagen hydrogel encapsulation and cells on tissue culture plate

The production of growth factors and cytokines for encapsulated MSCs in four-arm PEG-collagen hydrogels as a function of PEG concentration was evaluated after 2 days, 4 days and 8 days. The authors chose to work with four-arm PEG-collagen at two different concentrations: 0.2% w/v and 0.8% w/v and observed that cells encapsulated in four-arm PEG 0.2% spread more readily compared to cells encapsulated in 0.8% PEG that remained round during the evaluated time frame. The authors, questioning whether cell morphology impacted secretome production, compared secretome production of encapsulated cells with cells plated on tissue culture polystyrene (TCP). Figure 4 reveals how the cells plated in the 2D substrate produced relatively lower amounts of protein (growth factors, chemokines and cytokines) at all evaluated times compared with those of encapsulated cells. Cells encapsulated in 0.2% four-arm PEG-collagen hydrogels secreted higher protein concentrations at all times compared with the 2D condition and 0.8% four-arm PEG-collagen hydrogels. Nevertheless, cells encapsulated in 0.8% four-arm PEG-collagen secreted more protein compared with those cultured in 2D conditions. The amount of protein secreted increased with time for all groups. Such results imply how the behavior and environment of

cells hold important modulatory roles for the production of MSC-secreted factors.

The authors then analyzed the levels of protein secretion by cells under the different conditions. Figure 4B and Table 1 show that a distinct secretion profile was observed for MSCs encapsulated in the gels and plated on TCP. The study demonstrated that the secretion of growth factors by cells encapsulated in the gels were significantly greater than those cultured in 2D conditions. Vascular endothelial growth factor (VEGF) production was increased in cells encapsulated in four-arm PEG-collagen hydrogels with 0.2% PEG content compared with cells encapsulated in four-arm PEG-collagen hydrogels with 0.8% PEG content. By contrast, basic fibroblast growth factor was increased in cells encapsulated in four-arm PEG-collagen with 0.8% PEG compared with four-arm PEG-collagen hydrogels with 0.2% PEG. Cells plated on the TCP plates exhibited an increase in pro-inflammatory proteins compared with those encapsulated within the gels.

Corneal alkaline burn organ culture model

Given that MSCs encapsulated in PEG-collagen hydrogels produced an increased concentration of secreted factors compared with cells under 2D conditions, the authors examined how such finding impacts healing after alkali burn in a rabbit corneal organ culture model. Topically applied MSCs and MSCs encapsulated in PEG-collagen hydrogel were applied to the injured corneal surfaces, which were then monitored for epithelial wound healing and stromal scarring. In an organ culture setup, an air–liquid interface is created on the corneal surface, where nutrients make their way to the ocular surface either through diffusion from an underlying agar plug on the posterior side of the cornea or through capillary wicking from a moat of culture medium in which the corneal rim is partially submerged along its periphery, as previously described [23]. When MSCs are applied topically, the cells are likely to slide and settle onto the underlying culture well surfaces in contact with the culture medium. Thus, any secreted factors from the MSCs would actively condition the medium and nourish the cornea through a capillary wicking effect. The MSCs encapsulated in the PEG-collagen hydrogel were applied topically and underwent *in situ* gelation on the corneal surface. Thus, factors secreted from MSCs within the gel should directly affect wound healing. There were three rabbit corneas per treatment group. Photographs of the ocular surface taken over a period of 7 days after the alkali burn demonstrated that corneal damage occurred in a time-dependent manner. To observe the progress of wound healing, daily photographs were taken under white light as well as blue light with fluorescein dye applied to the cornea. Corneas treated with MSCs alone or MSCs encapsulated in PEG-collagen hydrogels had similar rates of wound closure, and by day 6, all

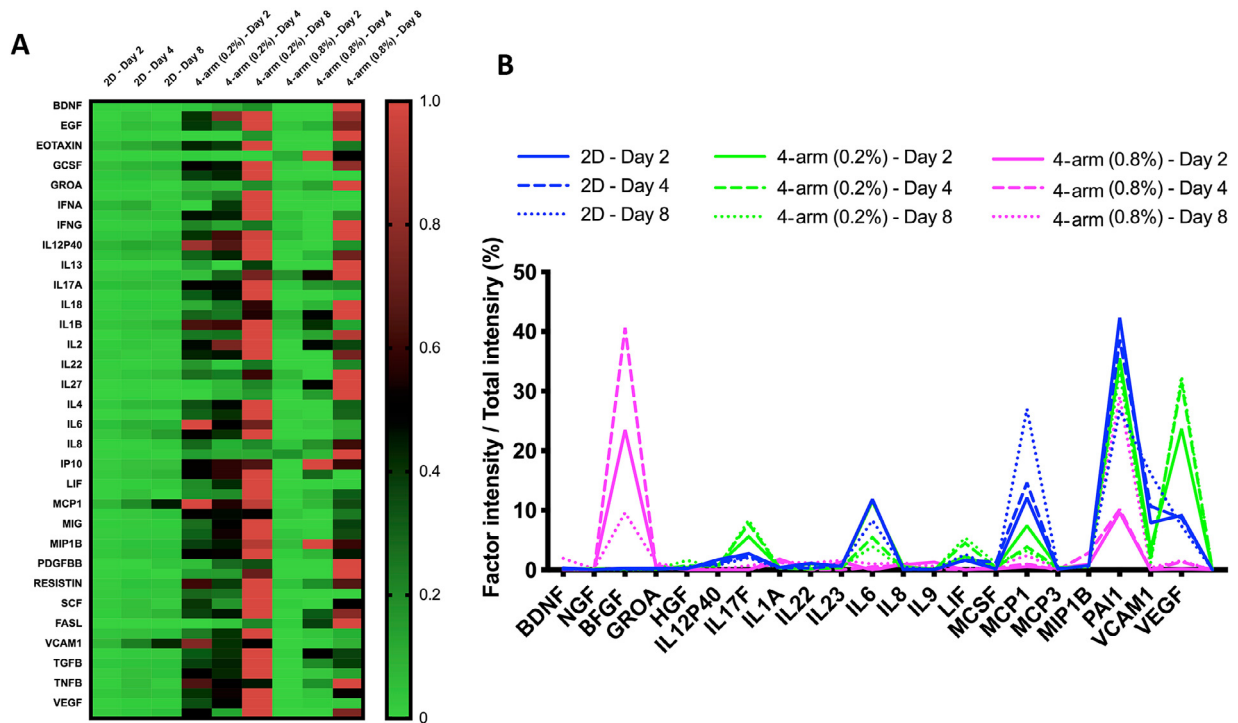


Fig. 4. (A) Heat map showing secretome production by MSCs encapsulated in four-arm PEG-collagen hydrogels at 0.2% w/v and 0.8% w/v. (B) Qualitative secretome profile showing the most abundant proteins secreted by the cells in different conditions. BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IFN- α , interferon alpha; IFN- γ , interferon gamma; IP-10, interferon gamma-induced protein 10; LIF, leukemia inhibitory factor; MCP-1, monocyte chemoattractant protein 1; MCP-3, monocyte chemoattractant protein 3; M-CSF, macrophage colony-stimulating factor; MIP-1 β , macrophage inflammatory protein 1 beta; NGF, nerve growth factor; PAI-1, plasminogen activator inhibitor 1; PDGF-BB, platelet-derived growth factor BB; TGF- β , transforming growth factor beta; TNF- β , tumor necrosis factor beta; VCAM-1, vascular cell adhesion protein 1. (Color version of figure is available online).

corneal wounds had healed in both groups (Figure 5). In the injury-only group, however, corneal wounds were not completely healed until the end of the experiment and still exhibited some diffuse punctate staining (day 7).

The corneas became opaque almost immediately after the alkali burn. At the conclusion of the experiment, the authors quantified corneal transparency by placing printed letters on a sheet of paper placed below the corneal tissue culture dish. Representative images of MSCs and MSCs encapsulated in the PEG-collagen hydrogel highlighted how stromal haze formation was relatively lower in the treated groups compared with the non-treated group. Corneal opacity remained in all three groups, but it was most significant in the injury-only (no treatment) group and least significant in the group treated with MSCs encapsulated within the PEG-collagen hydrogel. MSCs only and MSCs encapsulated within PEG-collagen hydrogel both facilitated similar rates of corneal epithelial wound healing. Corneal haze was graded manually by authors KN, HJL and YAS and was as follows for the injury, MSC and MSC-encapsulated-in-PEG-collagen-hydrogel groups: 1.2 ± 0.2 , 3.0 ± 0.6 and 3.6 ± 0.5 , respectively,

with 0 representing totally opaque and 5 representing totally transparent corneas

Immunofluorescence confocal imaging demonstrated that anterior stromal vimentin (green) was highest in the untreated corneas and lowest in those treated with MSCs encapsulated within four-arm PEG-collagen gels (Figure 6A). The study also demonstrated basal expression of vimentin in keratocytes in unwounded normal corneas. After quantifying the expression of vimentin, a difference between the MSC-only group and the gel-encapsulated group was observed, but the difference was not statistically significant (Figure 6B).

Discussion

In this study, the authors successfully encapsulated MSCs within a PEG-collagen hydrogel as an injectable biomaterial for corneal alkali burn treatment. The authors optimized the concentration of PEG-collagen hydrogel as a vehicle for cell delivery on the ocular surface. Based on the authors' results, PEG-collagen hydrogels formed *in situ* exhibited good adherence to the corneal surface and satisfactory MSC

Table 1

Top five factors secreted by MSCs under different culture conditions.

	2D			Four-arm (0.2%)			Four-arm (0.8%)		
	Day 2	Day 4	Day 8	Day 2	Day 4	Day 8	Day 2	Day 4	Day 8
1	PAI-1	PAI-1	MCP-1	PAI-1	PAI-1	PA-1	bFGF	bFGF	PAI-1
2	MCP-1	MCP-1	PAI-1	VEGF	VEGF	VEGF	PAI-1	PAI-1	bFGF
3	IL-6	IL-6	VCAM-1	IL-6	IL-17F	IL-17F	IL-1A	MIP-1 β	MCP-1
4	VEGF	VCAM-1	IL-6	MCP-1	IL-6	LIF	IL-23	VEGF	BDNF
5	VCAM1	VEGF	VEGF	IL-17F	LIF	IL-6	IL-9	IL-1A	IL-23

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; LIF, leukemia inhibitory factor; MCP-1, monocyte chemoattractant protein 1; MIP-1 β , macrophage inflammatory protein 1 beta; PAI-1, plasminogen activator inhibitor 1; VCAM-1, vascular cell adhesion protein 1.

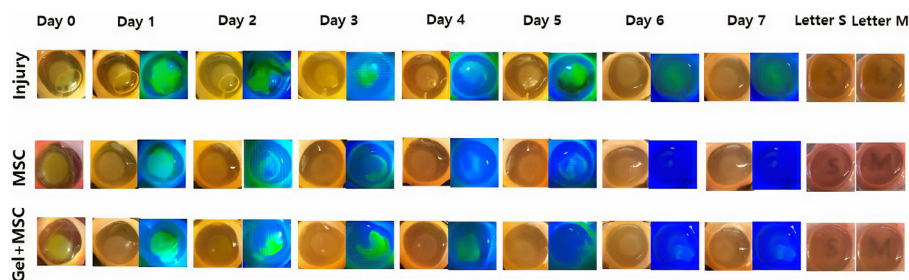


Fig. 5. Representative rabbit cornea organ cultures in the three treatment groups following alkali burn until day 7. Corneas treated with MSCs healed faster and were more transparent than those of the control group after 1 week. Corneas treated with MSCs encapsulated within PEG–collagen hydrogel exhibited the highest degree of transparency. Fluorescein dye was used to track the closure of epithelial wounds. (Color version of figure is available online).

survival after encapsulation. Data from an alkali burn organ culture model demonstrated that PEG–collagen hydrogels may be aybesuitable cell vehicles for the delivery of MSCs to promote epithelial healing and reduce corneal haze.

MSCs have garnered significant interest because of their immunomodulatory capacity, which allows for their use in allogeneic settings [11,25]. A large body of evidence has shown that MSCs secrete a variety of growth factors, chemokines and cytokines [26–29]. Given that their secretome has benefits in the absence of the cells themselves, the cell-free secretome therapy has appealed as a therapeutic option. However, properties of the secretome vary widely depending on the host age and niche [25,26]. It is unclear whether the secretome of MSCs grown *in vitro* provides tissue-repairing and immunomodulatory effects [25]. Previous studies strongly support the safety of MSC use in cell-based therapies by supporting that no tumors have originated from systemic infusions of MSCs [30,31]. Autologous and allogeneic MSCs have been used in a number of clinical trials for a wide range of clinical conditions. A promising method to deliver MSCs onto the corneal surface involves the use of scaffolds to adhere them to target surfaces while also mimicking their three-dimensional physiological environment. Previously, the authors showed that MSCs cultured on electrospun fibers composed of gelatin (to mimic the collagenous matrix of the cornea) and polycaprolactone enhanced healing in corneal fibroblast cells *in vitro* as well as in a rabbit corneal alkali burn organ culture model [23].

In the present study, the authors selected PEG–collagen hydrogels formed *in situ* as delivery vehicles for MSCs. Encapsulation of cells in

hydrogels has some advantages over seeding onto a prefabricated porous scaffold [22]. This strategy allows for cells suspended in a liquid precursor solution to be delivered *in vivo* directly to the site of interest with a simple injection. If hydrogels encapsulated with MSCs adhere to the ocular surface, they can be directly applied to the corneal burn site without the need for sutures or glue. Encapsulating cells in biodegradable hydrogels presents a number of advantages, including ease of handling and a highly hydrated tissue-like environment for cell and tissue growth [32,33]. Consequently, collagen has been commonly used to create scaffolds for many other clinical applications [34,35]. Collagen—a major component of the extracellular matrix—is one of the most well-established natural polymers for biomedical applications [32]. Despite its demonstrated potential, its mechanical properties remain difficult to control, which can pose a challenge [36]. Synthetic polymer-based materials such as PEG have also been developed as scaffolds. One of PEG's most prominent advantages is its tunability—the ability to control its physical and chemical properties, particularly in the setting of cross-linked networks, including mechanical strength and degradation kinetics [33]. PEG is widely used for its ability to be easily modified and create variable structures with different reactive groups [37–39]. In addition, PEG-based materials are bioinert and are used in Food and Drug Administration-approved ocular sealants [40]. Combining both natural and synthetic polymers into hydrogels has gained widespread attention for tissue engineering and drug delivery owing to their high water content, tissue-like elasticity and biocompatibility [41,42].

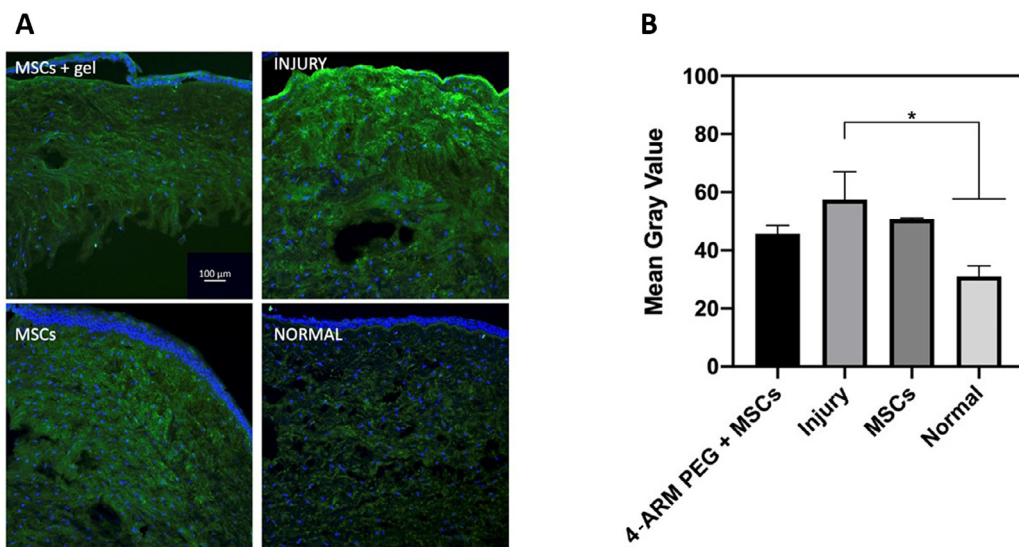


Fig. 6. (A) Fluorescent confocal microscopy images of alkali-burned rabbit corneas in the normal, injury-only, MSC and MSC-encapsulated-in-PEG–collagen–hydrogel groups upon completion of the organ culture experiment. Tissue was stained for vimentin (green) and DAPI (blue). (B) Quantification of vimentin expression in the anterior stroma of normal, injury-only, MSC and MSC-encapsulated-in-PEG–collagen–hydrogel groups. (Color version of figure is available online).

In the current study, the authors observed that PEG-collagen hydrogel cytocompatibility was high, with nearly 100% viability for 2 weeks. The authors measured cell proliferation in the hydrogel after 2 days, 4 days and 8 days (see supplementary Figure 2). The authors observed that cell proliferation decreased over time in the hydrogel, probably as a result of hydrogel degradation. Although the authors understand the importance of these cells maintaining their properties and stemness, here they focused on evaluating secretome release and hope to pursue this line of inquiry in a future study. The authors tried to simplify the study by evaluating encapsulation parameters, including PEG concentration. In the future, the authors aim to assess the impact of gel mechanical properties on cell differentiation and spreading.

The authors found that single encapsulated MSCs showed dendritic morphology by 8 days within the PEG-collagen hydrogel. MSCs undergo spontaneous differentiation in response to certain physical cues in the environment, such as matrix stiffness and mechanical forces [43]. The actin cytoskeleton in MSCs is composed of a large number of thin, parallel microfilament bundles that extend across the entire cytoplasm, and it is still unclear whether cytoskeletal changes of MSCs affect their fate [44]. The authors found that the cells encapsulated within PEG-collagen hydrogels were viable and that their morphology changed in response to their surrounding environment. The authors used four-arm PEG (0.2%) in organ culture because they surmised that MSCs in the lower PEG concentration and with the lower PEG number may spread more freely and favor a stem-like cellular phenotype in the softer gel and consequently affect the secretory properties of the MSCs. The authors found that the cells could migrate from the hydrogel to the plate as a consequence of hydrogel degradation (see supplementary Figure 1). However, the authors' experimental design offered no space for the cells to migrate vertically within the hydrogel covering the entire well, which spanned approximately 300–400 μm .

In a previous study, the authors reported on the rheological characterization of these PEG-collagen hydrogels *in vitro* as well as regeneration of mechanically injured epithelium over the PEG-collagen hydrogels formed *in situ in vivo* [45]. Here, the authors conducted an *ex vivo* rabbit organ culture experiment to analyze the therapeutic effects of MSCs encapsulated in PEG-hydrogels after alkali burn injury to the cornea. Corneal organ culture creates a biomimetic air–liquid interface along with nutrient flow that emulates the tear film (cell culture medium encircling a corneal rim) and aqueous humor (agar plug posterior to the cornea) [46]. Epithelial wound closure was measured with blue light and fluorescein dye, which stains epithelial defects. Scarring and corneal haze were also evaluated through rabbit corneal organ culture experiments. MSCs alone and MSCs encapsulated within PEG-collagen gels promoted faster epithelial wound healing rates and reduced corneal opacification, compared with injured corneas without treatment. Topically instilled MSCs tended to slide off the cornea and grow in the culture medium in which the corneal rim was soaked, and the secretome-enriched medium could reach the cornea via capillary action and diffusion through the underlying agar matrix. PEG-collagen gels, on the other hand, were successfully maintained on the curved surface of the cornea along with the encapsulated cells, without flowing off into the underlying medium. Based on the epithelial wound healing data of the group consisting of PEG-collagen gels with encapsulated MSCs, epithelial wound closure appears slightly delayed compared to topically applied MSCs. However, epithelial healing was achieved by day 5, and corneal transparency was improved compared with the corneas treated with MSCs only and the untreated corneas at day 7. Of note, the topical application of MSCs in organ culture does not have a strong physiologic correlate, since an aqueous suspension of cells are likely to be washed from the ocular surface relatively rapidly and in large part through the lacrimal system, making the

bioavailability of any MSC secreted factors lower *in vivo* than in the *ex vivo* model used here.

In the authors' proteomic analysis of MSC-secreted factors, protein content was normalized to the number of cells and thereafter compared with the 2D conditions for the collected secretome. The normalization allowed the authors to indirectly investigate and characterize the released secretome of encapsulated MSCs based on the PEG concentration and encapsulation time. The authors observed that the secretome released by these cells increased at lower PEG concentrations and over time. The observed increase of VEGF in the MSC secretome in the context of the known anti-angiogenic properties of MSCs requires further explanation. The MSC secretome has a multitude of factors, the makeup of which can be modulated toward pro-angiogenic and pro-inflammatory versus anti-angiogenic and anti-inflammatory depending on culture conditions [47]. Thus, the secretome has factors that may have opposing effects, but with certain factors being at a higher effective concentrations. A previous study has reported that MSCs promote vasculogenesis [48], whereas other groups have shown that they inhibit angiogenesis [49]. Another previous study has reported that an exogenous VEGF isoform (VEGF-B) promotes corneal nerve fiber regeneration as well as restoration of sensation and healing of the epithelium [50]. In the authors' group's recent publication, they showed that the MSC secretome alone, with and without a viscoelastic gel carrier, could prevent corneal neovascularization after alkali burn injury in rodents *in vivo* [51]. In this context, the authors hypothesized that the MSC secretome produced by MSCs encapsulated within PEG-collagen gels may also facilitate corneal epithelial wound healing and similarly prevent neovascularization, although further *in vivo* testing is required to confirm this. In addition to the therapeutic potential of the MSC secretome, exosomes have also been well studied for their ability to accelerate corneal epithelial wound healing [52].

Immunofluorescence imaging of corneal cryosections revealed differences in the expression of vimentin between the 3 groups. The injury-only group exhibited increased vimentin expression in the stroma, as did the group treated with MSCs alone, albeit to a lesser degree. The gel-encapsulated MSC group had the lowest degree of stromal vimentin expression of all groups, which is suggestive of the beneficial effects this delivery modality may have on MSC activity and, in turn, long-term stromal scarring. A previous study by Chaurasia *et al.* [53] reported that vimentin, a major class of intermediate filament proteins, plays an essential role in the early and intermediate stages of myofibroblast formation during corneal wound healing. The researchers showed that there was an increase in stromal cells expressing vimentin at higher levels at 1 week after photorefractive keratectomy in rabbits, followed by α -smooth muscle actin by 2 weeks and desmin by 4 weeks. As the authors of the current study tested eyes 1 week after corneal burns, they selected vimentin as the marker of fibrotic wound healing. This result correlates with the authors' macroscopic observations of corneal haze in organ culture over 8 days. The highest levels of vimentin expression and corneal haze were seen in the injury-only group, followed by the MSC-only group. The group treated with MSCs encapsulated within PEG-collagen hydrogels presented the lowest vimentin expression and corneal haze levels, although compared to the MSC-only group, this difference was not statistically significant.

There are a number of limitations inherent to this study. The first limitation is that organ culture, due to the lack of vessels and lymphatic system, fails to model the host immune response after alkali burn of the cornea. However, Zhao *et al.* [46] showed that the response of *in vitro* organ culture models to severe alkali burn is similar to that observed by other groups in clinical and *in vivo* studies. Overt changes as well as ultrastructure of the corneal surface photographed over a period of 7 days after alkali burn can be seen in *ex vivo* corneas in a time-dependent manner. Although animal models are generally used for the study of pathologic changes after alkali burn, there has been a demand for the reduction of animal use in

research over the last few years. Even so, some of the benefits to using *in vitro* and *ex vivo* models include easier manipulation and monitoring and controlled applications of chemical agents and treatment modalities.

Conclusions

The authors found that PEG-collagen hydrogels are suitable encapsulating vehicles for MSCs and that MSCs can be reliably delivered to the corneal surface using this platform. MSCs in PEG-collagen hydrogels accelerated epithelial wound closure and reduced corneal scar formation to a significant extent compared with untreated corneal controls. Furthermore, immunohistochemical differences were observed in the gel-encapsulated MSC treatment group, including decreased vimentin expression and reduced corneal haze. Future work is merited to evaluate and validate these effects in *in vivo* models as well as to reduce batch-to-batch variability and avoid the risk of host immune rejection through the use of human-derived collagen rather than animal-derived collagen. The authors' data, together with our previous report demonstrating the mechanical and physical properties of PEG-collagen hydrogels, underscore that PEG-collagen hydrogels formed *in situ* may have potential as a delivery modality for MSCs in a clinical setting [45].

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Declaration of Competing Interest

DM, HJL and GMF have a patent application on the gel technology described in this article.

Author Contributions

Conception and design of the study: KN, GMF and DM. Acquisition of data: KN, GMF and IBV. Analysis and interpretation of data: KN, GMF, IBV and DM. Drafting or revising the manuscript: KN, GMF, IBV, HJL, YAS and DM. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.02.001.

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