Polysaccharide Hydrogel Combined with Mesenchymal Stem Cells Promotes the Healing of Corneal Alkali Burn in Rats

Yifeng Ke1☯, Yixiang Wu1☯, Xuan Cui1☯, Xun Liu1, Min Yu2, Chunbo Yang1*, Xiaorong Li1*

1 Tianjin Medical University Eye Hospital, The College of Optometry, Tianjin Medical University Eye Institute, Tianjin, China, 2 School of Public Health, Tianjin Medical University, Tianjin, China

☯ These authors contributed equally to this work.
* yangcbxh@gmail.com (CY); xiaorli@163.com (XL)

Abstract

Corneal chemical burns are common ophthalmic injuries that may result in permanent visual impairment. Although significant advances have been achieved on the treatment of such cases, the structural and functional restoration of a chemical burn-injured cornea remains challenging. The applications of polysaccharide hydrogel and subconjunctival injection of mesenchymal stem cells (MSCs) have been reported to promote the healing of corneal wounds. In this study, polysaccharide was extracted from Hardy Orchid and mesenchymal stem cells (MSCs) were derived from Sprague-Dawley rats. Supplementation of the polysaccharide significantly enhanced the migration rate of primarily cultured rat corneal epithelial cells. We examined the therapeutic effects of polysaccharide in conjunction with MSCs application on the healing of corneal alkali burns in rats. Compared with either treatment alone, the combination strategy resulted in significantly better recovery of corneal epithelium and reduction in inflammation, neovascularization and opacity of healed cornea. Polysaccharide and MSCs acted additively to increase the expression of anti-inflammatory cytokine (TGF-β), anti-angiogenic cytokine (TSP-1) and decrease those promoting inflammation (TNF-α), chemotaxis (MIP-1α and MCP-1) and angiogenesis (VEGF and MMP-2). This study provided evidence that Hardy Orchid derived polysaccharide and MSCs are safe and effective treatments for corneal alkali burns and that their benefits are additive when used in combination. We concluded that combination therapy with polysaccharide and MSCs is a promising clinical treatment for corneal alkali burns and may be applicable for other types of corneal disorder.

Introduction

Corneal chemical burns are common ophthalmic emergencies accounting for 7.7–18% of all ocular traumas, with alkali injuries being more common than those from acid [1]. In severe cases, the eye limbus and central epithelium can be lost leading to loss of vision [2]. Slow epithelialization, persistent ulceration, corneal perforation and angiogenesis are the main
complications and result from the processes of inflammation, neovascularization, and conjunctivalization of cornea [3–5].

Strategies to treat corneal chemical burns include antibiotics, tear substitutes, corticosteroids, ascorbic acid, collagenase inhibitors and surgical treatments such as penetrating keratoplasty and amniotic membrane transplantation [6]. However, the structural and functional restoration of alkali burn-injuries to the cornea remains a challenge, despite these therapies and thus prompting the search for novel treatment strategies including the use of polysaccharides and stem cell based therapies.

Polysaccharides are high molecular weight carbohydrates composed of long chains of monosaccharide units bound together by glycosidic bonds. At least five different types of polysaccharide exist in corneal stroma, i.e. keratin sulphate, chondroitin-4-sulfate chondroitin, dermatan sulfate and heparan sulfate which play important roles in maintaining the water content and transparency of the cornea [7]. It was reported that the levels of endogenous polysaccharides were reduced in the healing area of perforating cornea [8].

Treatment with sodium hyaluronate promoted corneal wound healing through the stimulation of epithelial cell proliferation, the promotion of epithelial cell adhesion to stroma fibronectin and the suppression of inflammatory cell infiltration [9–11]. Polysaccharides may also be beneficial in the treatment of corneal diseases involving neovascularization and inflammation [12,13]. The antinociceptive and anti-inflammatory activities of polysaccharides and their impacts on cellular proliferation and the immune response have been widely reported as well [14, 15].

Hardy Orchid has been shown to facilitate the treatment of canal mucosal damage, ulcers, bleeding, bruises and burns. Luo et al. found that Hardy Orchid polysaccharide controlled the inflammatory responses and accelerated the wound closure in a mouse model of cutaneous trauma [16]. Diao et al. reported that Hardy Orchid polysaccharide modulated the expression of pro-inflammatory cytokines including NO, TNF-α, and interleukin 1 beta (IL-1β) in a murine macrophage-like cell line [17].

Mesenchymal stem cells (MSC) are multipotent stromal cells derived from the mesoderm that can differentiate into a variety of cell types. MSCs are relatively easy to isolate and expand making them promising candidates for cell replacement therapy. Recently, MSCs have been studied for the treatment of corneal chemical burn with encouraging results [18,19]. Treatment with subconjunctival MSCs has been found to significantly enhance the recovery of the corneal epithelium and decrease the area of corneal neovascularization [20]. Transplanted MSCs are thought to promote corneal wound healing through secretion of anti-inflammatory and anti-angiogenic cytokines.

In the current study, we explored the effect of topical polysaccharide therapy in combination with subconjunctival administration of MSCs on the healing of corneal alkali burns.

**Materials and Methods**

**Extraction, purification and characterization of polysaccharides**

The plant Hardy Orchid was grounded and dispersed in 80°C distilled water for 4h and filtered to remove impurities. The filtrate was treated with 3x vol. of 95% ethanol overnight, and the precipitate was washed with 95% ethanol and resolved in distilled water. To exclude protein components, 1/3 vol. of chloroform/n-butanol (4:1 v/v) was added into the water solution and mixed by vigorous shaking. After chloroform water phase separation the upper aqueous phase was collected. The above procedure was repeated three times. The final aqueous phase was dialyzed at a cut-off of 3000–5000 Da and the concentrated solution was treated with 3x vol. of 95% ethanol overnight. The precipitate was lyophilized to give a crude extract. The crude extract was dissolved and applied onto Sephadex G-100 column for further purification and lyophilized to obtain polysaccharide powder.
To analyze glucosidic bonds and functional groups of the polysaccharide, 2 mg of the polysaccharide powder was mixed with KBr to obtain KBr pellet and subjected to infrared (IR) spectrum analysis using Nicolet 6700 FT-IR Spectrometer (PT.Contitus.Co.LTD) with a scan range of 4000–500 cm\(^{-1}\).

Monosaccharides composition of the polysaccharide was analyzed by PMP derivation high performance liquid chromatography (HPLC). Briefly, 2 mg polysaccharide powder was dissolved in 2 M trifluoroacetic acid (TFA) and completely hydrolyzed to monosaccharides at 105\(^\circ\) C for 4 h. The solution was derivated with 1-Phenyl-3-methyl-5-pyrazolone (PMP) and NaOH and subjected to analysis. 2 mg of individual standard monosaccharides including D-mannose (Man), L-rhamnose (Rha), D-glucuronic acid (GlcUA), D-galacturonic acid (GalUA), D-glucose (Glc), D-galactose (Gal), D-xylose (Xyl), L-arabinose (Ara), Fucose (Fuc) were derived in the same way and subjected to analysis.

The purified polysaccharide was thiolated by esterification with thioglycolic acid in the presence of hydrochloric acid. Polysaccharide was dissolved in distilled water at 100 mg/ml and 3.6 ml of thioglycolic acid and 2 ml of 7N HCl was added to allow reaction at 80\(^\circ\) C for 180 min. Thiolated polysaccharide was precipitated using methanol and lyophilized. The thiolated polysaccharide was dissolved in PBS at 20 mg/ml.

To generate polysaccharide hydrogel, we prepared maleimidized polylysine as crosslinker. In brief, polyllysine solution (20 mg/ml) was mixed with succinimide sulfate solution (5 mg/ml) with volume ratio of 1:10 and continually stirred at room temperature for 2 hours. After dialysis and lyophilisation, maleimidized polyllysine was obtained and dissolved in PBS at 20 mg/ml. When thiolated polysaccharide was mixed with maleimidized polyllysine at pH 7, the sulfhydryl groups reacted with maleimide to form stable thioether bonds to trigger the formation of polysaccharide hydrogel.

**Isolation and culture of bone marrow mesenchymal stem cells (BMSCs)**

Bone marrow mesenchymal stem cells (BMSCs) were derived from six-week-old female Sprague-Dawley rats weighing 180–220 g (Tianjin Medical University animal center, Tianjin, China). All rats were sacrificed with an overdose of 10% chloral hydrate. In brief, bone marrow was harvested from the long bones and layered onto Lymphoprep (1.077 g/mL; Nycomed, Birmingam, UK) for centrifugation at 850 g, 25 min. The isolated mononuclear cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2.5 ug/mL fungizone and 2 mM L-glutamine (Invitrogen, Paisley, UK) and incubated at 37\(^\circ\)C in a humidified atmosphere with 5% CO2. The BMSCs at passages 3 were used for all experiments described.

**Animals**

Six-week-old female Sprague-Dawley weighing 180–220 g were used (Tianjin Medical University animal center, Tianjin, China). At the end of the experiment, all rats were sacrificed with an overdose of 10% chloral hydrate. Only the right eye of each rat was used. All procedures used in this study were in accordance with the principles of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University Eye Hospital.

**Rat corneal epithelial cells (rCECs) culture and identification**

The corneas of the rats were harvested and washed twice with Hanks balanced salt solution buffer (Sigma Chemicals). After careful removal of excessive corneal stroma, the tissue was cut into small pieces, placed onto culture plate and cultured with medium containing Dulbecco’s...
modified Eagle medium (DMEM), 5 ng/ml of epidermal growth factor (EGF), 1% glutamax, 1% non-essential amino acids, 1% antibiotic solution and 5% FBS. The cultured CECs were identified by CK-3 immunofluorescence and the percentage of CK3-positive cells was examined by flow cytometry. CK3 antibody was from Biobyt (orb5866).

**MTT cell proliferation assay**

The proliferation of rat corneal epithelial cells (rCECs) was examined using 3-(4, 5)-dimethylthiazol-(-z-y1)-3, 5-di-phenyltetrazoliumromide (MTT) labeling assay. rCECs were incubated in a medium with 0, 50, 100, 200 μg/ml of Polysaccharide for 72 h followed by 4 h incubation with MTT. The MTT transformed crystals were dissolved in dimethyl sulfoxide, and absorbance at 490 nm was measured using a microplate reader (infinite M200PRO, Tecan, Switzerland).

**Wound healing assay**

The effect of polysaccharide on the migration of rat corneal epithelial cells (rCECs) was evaluated using a wound healing assay. Briefly, the cells were plated on 24 well culture plates coated with 1% gelatin. A scratch was made with a micropipette tip after confluence was reached. Cultures were then rinsed to remove detached cells and cultured with medium containing various concentrations of polysaccharide or with the solvent control. The cell migration rates were calculated 24h later with the formula \( m = (1-n/r) \times 100\% \) where \( n \) is the width of scratch at 24h, \( r \) is the initial width of scratch.

**Animal model of corneal alkali burn and treatments**

A corneal alkali burn was generated in the right eye of each rat (200 rats). The rats were anesthetized by an intramuscular injection of 40 mg/kg ketamine and 10 mg/kg xylazine hydrochloride. A piece of filter paper (6-mm diameter) soaked with 4 ul NaOH (1 mol/l) was applied to the center of the cornea for 30 seconds. The cornea was then rinsed with 30 ml of saline for 30 seconds. After the corneal alkali burn, animals were randomly divided into four groups: control group (n = 50), MSC group (n = 50), polysaccharide group (n = 50) and PM group (polysaccharide hydrogel combined with MSCs application, n = 50). Immediately and 2 days after the corneal alkali burn, various treatments were carried out twice separately. Animals in the MSC group received subconjunctival injections of 2x10^6 MSCs in 0.1 ml PBS. In the polysaccharide group, 50ul polysaccharide solution (200ug/ml) was applied onto the surface of the injured cornea and polylysine crosslinker solution was dropped on top subsequently to form hydrogel within 30 seconds. In the PM group, polysaccharide hydrogel was applied in conjunction with subconjunctival injection of MSCs. The control group was treated with subconjunctival injection and topical application of PBS. Levofloxacin was applied to the experimental eyes twice a day throughout the experiment.

Animal condition was monitored three times every day during the first 7 days post-injury. Thereafter animal condition was monitored once a day until the end of the experiment. On day 3 and 14 post-injury, 5 rats from each group were sacrificed and cornea excised for histological examination; on day 3, 7, 14 and 28 post-injury, RNAs were extracted from 5 corneas in each group for qRT-PCR examination; on day 3, 7 and 14 post-injury, total proteins were extracted from 5 corneas in each group for ELISA examination; on day 28 postinjury, ink perfusion via the aorta was performed with 5 rats in each group and cornea excised for observation of neovascularization.

**Observation and examination**

General observations were performed including corneal epithelial recovering, corneal clarity, and corneal neovascularization. All observations were performed by a single experienced
ophthalmologist who was blind to the allocation of the animals in each group. The corneal epithelial integrity was observed by corneal fluorescence staining at day 3 and day 7 after the corneal alkali burn. Corneal opacity was examined by slit-lamp (SL-120; Zeiss, Jena, Germany) at 3, 7 and 14 days after the corneal alkali burn and graded as described by Sonoda and Streilein [21]: grade 0, completely transparent cornea; grade 1, minimal corneal opacity, but iris clearly visible; grade 2, mild corneal opacity, iris vessels still visible; grade 3, moderate corneal opacity, pupil margin but not iris vessels visible; grade 4, complete corneal opacity, pupil not visible. Corneal neovascularization was quantified by calculating the wedge-shaped area of vessel growth using the following equation [22]: \[ A = \frac{C}{12} \times 3.1416 \times (r^2 - l^2) \], where \( A \) is the area, \( C \) is a fraction of circumference based on 12-h clock, \( l \) is the radius from the center to the border of vessel growth, and \( r \) is the radius of the cornea. The amount of neovascularization was compared between the groups using the ratio of the neovascularized area to the whole corneal area. An image analyzer (Image Pro Plus 6.0; Media Cybernetics) was used for measuring the radius. To evaluate the development of corneal neovascularization (CNV), ink perfusion via the aorta was performed on day 28 as described previously [23]. Thereafter, the eyes were fixed in 10% neutralized buffered formaldehyde overnight, and the corneas were dissected and flattened for imaging.

Hematoxylin and eosin staining histology

Eyeballs were fixed with formalin and paraffin-embedded. Serial sections (3μm) were deparaffinized by sequential washing with xylene followed by washing with descending series of ethanol and then processed for hematoxylin and eosin staining. The stained sections were observed under microscope (Olympus, Tokyo, Japan).

Quantitative Real-Time PCR (qRT-PCR)

Total RNAs were extracted from 5 corneas in each group with TRIzol (Invitrogen). 100 ng RNA was reverse transcribed into cDNA via Oligo (dT) with PrimeScript RT reagent kit (TaKaRa). Real-time quantitative PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). All experiments were carried out in triplicate with SYBR Green JumpStart Taq ReadyMix (Sigma). The sequences of the PCR primers for TNF-α, TGF-β, TSP-1, MIP-1α, MCP-1, MMP-2 and VEGF are listed in S1 Table.

Enzyme-linked immunosorbent assay (ELISA)

Corneas excised from the eyeballs were sliced and lysed with tissue extraction reagent (life technologies FNN0071). The total protein concentrations were measured using BCA protein assay kit (thermo scientific 23235). 20 μg total protein from each sample was applied for measurement of VEGF and TGF-β using ELISA kits (R&D Systems, Minneapolis, MN) at 490 nm. ELISA experiments were performed in triplicate.

Statistical analysis

Analysis of variance (single factor or two factors ANOVA with replication) was used to compare multiple samples (at one time or several time points). \( P \) value <0.05 was considered statistically significant (SPSS 12.0; SPSS, Chicago, IL).
Results

Hardy Orchid-derived polysaccharide showed compatibility with corneal epithelial cells and promoted cell migration

To analyze glucosidic bonds and functional groups of the polysaccharide, infrared (IR) spectrum analysis was carried out (Fig. 1A). According to the IR spectrum, Hardy Orchid—derived polysaccharide had typical polysaccharide absorption profile. The wide peak at 3600–3200 cm⁻¹ indicated O-H stretching vibration, the peak at 3000–2750 cm⁻¹ was C-H stretching vibration. The 1800–700 cm⁻¹ region characterized the types of glucoside, substituent and epimer. Peaks in the 1376–1028 cm⁻¹ band were characteristic hydrocarbon keys of pyranose. The characteristic absorption at 951–875 cm⁻¹ suggested that the polysaccharide contains β-glucosyl residues and 809 cm⁻¹ peak revealed the existence of mannose. Lack of absorption at 840 cm⁻¹ and presence of absorption at 873 cm⁻¹ indicated that monosaccharide residues were subject to β-glycosidic bond connection rather than α-type.

Monosaccharides composition of the polysaccharide was analyzed by PMP derivation high performance liquid chromatography (HPLC). By comparison of HPLC spectrum profile of polysaccharide with that of standard monosaccharides it was found that Hardy Orchid derived polysaccharide was mainly composed of D-mannose (Man) and D-glucose (Glc) with relative mole ratio of 6:1 according to their corresponding peak areas (S2 Fig.). Using BCA protein assay (thermo scientific 23235), a small amount of protein component was determined (0.04mg/ml) in the polysaccharide extract.

When mixed with polylysine crosslinker solution 1:1 at room temperature, the polysaccharide solution gelled within 30 seconds as a semi-transparent construct (Fig. 1B). We took advantage of this phenomenon to develop a polysaccharide treatment strategy in which this stable hydrogel was formed on the corneal surface, thereby ensuring intimate contact between the polysaccharide and the corneal epithelium.

To examine the compatibility and cytotoxicity of the polysaccharide with rat corneal epithelial cells (rCECs), polysaccharide solution was added into the culture of rCECs at concentrations of 50–200 μg/ml, after which their morphology, proliferation and migration characteristics were assessed.

In polysaccharide-supplemented culture, rCECs showed normal morphology identical to control rCECs cultured without polysaccharide supplementation. Immunostaining of cytokeratin-3 confirmed the corneal epithelial identity of the culture (Fig. 1C, D). There were no significant differences in the results of MTT assays at 24, 48 and 72 hours of culture between the polysaccharide and control groups, indicating that the polysaccharide treatment did not affect the proliferating rate of rCECs (Fig. 1E). A wound healing assay was performed to observe the effect of polysaccharide on rCECs migration. Compared to the control group, the 24 hour migration rates of rCECs were significantly increased in a dose-dependent manner when treated with polysaccharide, indicating that polysaccharide supplementation enhanced rCECs migration (Fig. 1F).

Polysaccharide hydrogel and mesenchymal stem cells promoted the recovery of corneal epithelium and enhanced corneal clarity

After the establishment of corneal alkali burn model, the animals were divided into four groups. The polysaccharide group was treated with topical application of polysaccharide hydrogel. The MSC group received subconjunctival injection of mesenchymal stem cells suspended in PBS. The PM group had both treatments simultaneously. The control group was treated with topical application and subconjunctival injection of PBS.
Fig 1. Characterization of Hardy Orchid-derived polysaccharide and examination of its impacts on migration and proliferation of rat corneal epithelial cells (rCECs). (A) Infrared spectrum analysis of Hardy Orchid-derived polysaccharide, (B) In vitro formed polysaccharide hydrogel composite, (C) Negative control for Cytokeratin-3 immunostaining of rCECs cultured with polysaccharide supplementation, (D) Cytokeratin-3 immunostaining of rCECs cultured with polysaccharide supplementation, (E) The migration rate of rCECs in control and polysaccharide treated groups 24h after wound. Polysaccharide treatment increased rCECs migration rate in a dose-dependent manner, (F) Proliferation analysis of rCECs in control and polysaccharide
To determine the effects of the polysaccharide and/or MSCs on the recovery of corneal epithelium, fluorescein staining was used to measure the epithelial defect areas in various treatment groups at 3 and 7 days after alkali burn. On day 3 post-injury, the corneal defect area in the control group accounted for 80% of the corneal surface, which was significantly decreased to around 65% in both polysaccharide and MSCs treatment groups. When combined, polysaccharide and MSCs treatments showed an additive effect, resulting in a defect area of around 50% of the corneal surface. On day 7, the corneal defect area in all the groups was notably reduced. In control group, a defect area of around 50% remained. The polysaccharide and MSCs groups showed a much better appearance than the control group with only 20–30% defect area left. Notably, in the polysaccharide plus MSCs group, defect area decreased to 10% and the wound re-epithelialised completely in some individuals (Fig. 2).

Corneal transparency in each group was evaluated using the slitlamp photography based on the principles described by Sonoda and Streilein [21]. From day 3 through day 14 post-injury, the control group showed severe corneal opacity graded as 3–3.6. Compared to the control group, polysaccharide and MSCs applications significantly reduced the grade of corneal opacification. Of note, after polysaccharide treatment, the corneal opacity grade decreased from 2.5 on day 3 to 1.5 on day 14. Furthermore, the polysaccharide and MSCs combination further improved corneal transparency, resulting in minimal opacity, graded at 2.2, 1.2 and 0.8 on day 3, day 7 and day 14 post-injury separately. Compared to single treatment groups, polysaccharide and MSCs combination showed significant additive effect on day 7 and day 14 post-injury (Fig. 3).

Polysaccharide hydrogel and mesenchymal stem cells suppressed corneal neovascularization

Neovascularization is one of the major complications of chemical corneal burns and is closely related to prognosis. The development of corneal neovascularization (CNV) in various groups was quantified by calculating the wedge-shaped area of vessel growth. From day 7 to day 28 post-injury, the control group showed gradually increased area of CNV, which is consistent with the pathological process of corneal chemical burn. In contrast, the areas of CNV in polysaccharide and MSCs treatment groups were significantly less than that in the control group at all three time points. On day 28 post-injury, the ratio of CNV area to cornea area was 0.51 in the control group, which was 0.37 and 0.30 in the MSC and polysaccharide group respectively. Of note, from day 7 through day 28 post-injury, the area of CNV remained at low level in the combined therapy group in contrast to the gradual increase observed over this time in other groups. On day 7, the CNV ratio was 0.10 in the PM group, which increased a bit to 0.17 on day 14 then decreased to 0.11 on day 28. Our study revealed that when used in isolation, polysaccharide and MSC therapy reduced the area of neovascularization to a similar degree and that their effects were additive when used in combination. In addition, the sustained effect of combination therapy on neovascularization was highlighted (Fig. 4A).

To further confirm the results by slit lamp and image analyzing, ink perfusion via the aorta was performed on day 28 post-injury to identify corneal neovascularization. The control group showed remarkable neovascularization and the new vessels extended into the central area of the cornea. MSCs and polysaccharide treatment groups showed significantly less new vessels compared to the control group, consistent with the CNV area calculation results. Notably, PM
Fig 2. Re-epithelialization examination of alkali burn injured cornea. (A-D) Fluorescence staining of corneal epithelium 3 days after injury in control, MSCs treatment, polysaccharide treatment and polysaccharide-MSCs combination (PM) groups, (E-H) Fluorescence staining of corneal epithelium 7 days after injury in various groups. (I) Statistics of corneal defect area ratios in various groups on day3 and day7 post-injury. Data are presented as mean ± standard deviation (n = 5). Two-way ANOVA analysis was performed to determine the significance of the difference between various treatment groups (* p<0.05, **p<0.01, ***p<0.001). At both time points, MSCs and polysaccharide treatments significantly enhanced the recovery of corneal epithelium. Polysaccharide-MSCs combination (PM) groups showed additive effects compared with single treatment groups.

doi:10.1371/journal.pone.0119725.g002
Fig 3. Corneal opacity evaluation of alkali burn injured corneas. (A-D) Corneal surface observation 3 days after injury in control, MSCs treatment, polysaccharide treatment and polysaccharide-MSCs combination (PM) groups. (E-H) Corneal surface observation 7 days after injury in various groups, (I-L) Corneal surface observation 14 days after injury in various groups, (M) statistics of corneal opacity grades in various groups on day 3, 7, 14 post-injury. Data are presented as mean ± standard deviation (n = 5). Two-way ANOVA analysis was performed to determine the significance of the difference between various treatment groups (*p < 0.05, **p < 0.01, ***p < 0.001). MSCs and polysaccharide treatments significantly decreased corneal opacity and Polysaccharide-MSCs combination (PM) groups showed additive effects compared with single treatment groups.

doi:10.1371/journal.pone.0119725.g003
Histological examination

Histological examination was performed on day 3 and day 14 after injury. On day 3, serious defects with bullous keratopathy in the corneal epithelium were identified in the control group. The defects were milder in the groups treated with either MSCs or polysaccharide and no bullous keratopathy was detected in the combination treatment group (Fig. 5).

On day 14 post-injury, histological examination of the control group revealed multiple layers of irregularly aligned, regenerating corneal epithelial cells, persistent bullous keratopathy, extensive inflammatory infiltrates and neovascularization within the stoma. In the polysaccharide and MSC groups the regenerating epithelial cells were more regularly aligned and there were fewer inflammatory infiltrates and less neovascularization in the stroma. In the PM group showed the least neovascularization confirming the additive suppression effect of MSCs and polysaccharide especially at late stages of corneal healing post-injury (Fig. 4B-E).

![Image](https://example.com/image.png)

**Fig 4.** Analysis of corneal neovascularization after corneal alkali burn. (A) Ratio of neovascularization area to corneal area in control, MSC, polysaccharide and PM groups at day 7, 14, 28 days post-injury. (B-E) Ink perfusion detection of corneal neovascularization on day 28 post-injury in control, MSC, and PM groups. Statistics data are presented as mean ± standard deviation (n = 5). Two-way ANOVA analysis was performed to determine the significance of the difference between various treatment groups (*p < 0.05, **p < 0.01, ***p < 0.001). MSCs and polysaccharide treatments significantly decreased corneal neovascularization and Polysaccharide-MSCs combination (PM) groups showed additive effects compared with single treatment groups.
group, no abnormalities were detected in the corneal epithelium. Few inflammatory cells were
detected and little neovascularization was observed in the stroma. In addition, the corneal
thickness was least in the PM suggesting the suppression of oedema (Fig. 5).

Thus, histological examination provided further evidence of the therapeutic effects of poly-
saccharide and MSC application on alkali burn-injured cornea.

Examination of cytokines related to inflammation and neovascularization
To explore the mechanisms by which the polysaccharide and MSC therapies promoted the
healing of corneal alkali burns, the expression level of cytokines related to inflammation, che-
motaxis and neovascularization were examined by real-time PCR. On day 3, 7, 14 and 28 days
post-injury, expression of the genes encoding the immunostimulatory cytokine TNF-α and the
chemotactic factors MIP-1α and MCP-1 were significantly lower in the polysaccharide and
MSCs groups than in the control group. PM group showed the lowest expression at all the time
points. Of note, the greatest reduction in expression of these genes was seen in the PM group
on day 3 post-injury. On the contrary, expression of the gene for the anti-inflammatory cyto-
kine TGF-β was significantly increased by treatment of polysaccharide and MSCs and the same
additive effect was observed when used in combination. VEGF and MMP-2 stimulate angio-
genesis following corneal chemical-burns. Expression of the VEGF gene increased in all groups
up until day 7 and remarkably decreased thereafter. The expression of the genes encoding both
VEGF and MMP-2 was significantly lower in the single treatment groups and was lowest in the
PM group compared to the control group. In contrast, expression of gene for the antiangi-
genic cytokine showed an opposite dynamics (Fig. 6).

To further confirm the effects of polysaccharide and MSCs on the expression level of cyto-
kines, ELISA assay was performed to detect the cellular protein content of VEGF and TGF-β.
The changes in TGF-β and VEGF genes expression were well mirrored by the results of ELISA
on day 3, 7, and 14 post-injury (Fig. 7). At each time point, polysaccharide and MSCs groups
showed decreased level of VEGF compared to the control group, and the PM group had the
lowest. In contrast to VEGF, TGF-β showed the opposite dynamic scenario, which was in-
creased by treatment of polysaccharide/MSCs alone or in combination additively.

Discussion
In recent years, polysaccharide has been widely reported for the experimental therapies of cor-
neal disorders such as corneal incisions [24], Corneal epithelial abrasion and alkali burn inju-
ries [25], dry eye syndrome [26] etc. In addition, numerous reports have described therapeutic
benefits of MSCs administration on corneal wound healing [18,19].

In the current study, polysaccharide hydrogel derived from Hardy Orchid and mesenchy-
mal stem cells were applied for the treatment of corneal alkali burn. Compared with control
group, single treatment of polysaccharide hydrogel or MSCs significantly promoted corneal ep-
ithelium recovery, suppressed inflammation, neovascularization, and greatly alleviated the
haze and opacity of injured cornea. When polysaccharide hydrogel and MSCs were applied
concurrently, additive effect was observed and the cornea showed the highest recovering rate
of epithelium, best clarity, least inflammatory infiltration and mildest neovascularization. To
our knowledge, this is the first report that combination of polysaccharide hydrogel and MSCs
additively promoted the healing of corneal alkali burn.

Hardy Orchid has the functions of hemostasia, detumescence and recovery enhancement,
therefore has been widely used to treat alimentary canal mucosal damage, ulcers, bleeding,
bruises and burns [17]. Our study provides evidence of biocompatibility and safety of Hardy
Orchid polysaccharide treatment for corneal injury. Co-culture of rCECs with polysaccharide
did not affect cellular morphology or proliferation rate and the application of polysaccharide to the corneal surface in-vivo did not stimulate inflammation. These findings are consistent with those of Wu et al who found no adverse effects following injection of the polysaccharide into the subconjunctival space and anterior chamber in rabbits [27].

We found that in vivo application of polysaccharide significantly promoted the recovery of alkali injured corneal epithelium and improved corneal clarity compared to control. Polysaccharide hydrogels are competent to moisture absorption, highly permeable to oxygen, nutrients and other water soluble metabolites [28]. These beneficial features are similar to those with hydrophilic bandage soft contact lenses whose high oxygen permeability is thought to be able to promote epithelial migration and enhance epithelial stromal adhesion [29]. When corneal epithelial cells were co-cultured with polysaccharide, the rate of cell migration was significantly increased, consistent with previous reports [30].

Therapies that rapidly and effectively suppress inflammation and angiogenesis are critical for the treatment of corneal chemical burns [31,32]. In this study we have shown that the application of polysaccharide significantly suppressed inflammation and neovascularization in alkali burn-injured corneal tissue in vivo, consistent with recent reports about inflammation- and neovascularization- suppression activities of polysaccharides [15,16,33,34]. Recent research have attributed corneal epithelium the functions of anti-inflammation and anti-angiogenesis [35], hence the recovery of corneal epithelium promoted by polysaccharide application would further contribute to the suppression of inflammation and neovascularization.

Additionally, our study provided evidence of the efficacy of MSC transplant in the treatment of corneal burns. Subconjunctival injection of MSCs improved the recovery of corneal epithelium, and suppressed inflammation and neovascularization to an extent that was comparable with polysaccharide therapy. These finding are consistent with previous reports that transplanted MSCs had immunoregulatory and anti-angiogenic properties and promoted the healing of corneal chemical burn [18,19,36].

When polysaccharide and MSCs were applied in combination, we found an additive benefit in all parameters examined in this study. Combination therapy resulted in the best corneal re-epithelialization and significantly less corneal opacity at 7 days post-injury compared to monotherapy with either treatment. Combination therapy resulted in sustained suppression of neovascularisation in contrast to the time-dependent increase observed in the monotherapy and control groups. Corneal bullous keratopathy, epithelium distortion, edema, and inflammatory infiltrates were minimal or absent in the combination treatment group in comparison with the more modest benefits observed in the monotherapy groups. These findings indicate
Fig 6. Quantitative Real-time PCR examination of inflammation, chemotaxis and neovascularization related genes. The data are presented as mean ± SD from three assays. Two-way ANOVA analysis was performed to determine the significance of the difference between various treatment groups (* p<0.05, ** p<0.01).

doi:10.1371/journal.pone.0119725.g006
that subconjunctival injection of MSCs in combination with topical application of polysaccharide hydrogel is more efficient for the treatment of corneal alkali burns.

With a cutaneous trauma mouse model, Luo et al. found that Hardy Orchid polysaccharide controlled the inflammatory responses and accelerated the wound closure [16]. Diao et al. reported that Hardy Orchid polysaccharide modulated the expression of pro-inflammatory cytokines including NO, TNF-α, and interleukin 1 beta (IL-1β) in a murine macrophage-like cell line [17].

In the current study, application of polysaccharide effectively decreased the expression of key proinflammatory cytokine TNF-α and increased the level of anti-inflammatory cytokine TGF-β, which was consistent with previous report [15]. Furthermore, application of polysaccharide remarkably suppressed the expression of MIP-1α and MCP-1, the major cytokines that regulate migration and infiltration of monocytes/macrophages, indicating that polysaccharide functioned through regulation of critical inflammation related cytokines as well as suppression of leukocytes infiltration.

It was reported that application of MSCs increased IL-10 and TGF-β, and reduced IL-2 and IFN-γ in the damaged cornea, which was accompanied by significantly improved clinical outcomes [36]. MSCs inhibited macrophage infiltration by suppressing the expression of the macrophage chemokine MIP-1α[20] and the proinflammatory cytokine TNF-α production by macrophages [37]. Consistently, our study proved that the application of MSCs increased TGF-β, and reduced the expression of TNF-α, MIP-1α and MCP-1 in alkali injured corneas.

When polysaccharide and MSCs were applied in conjunction, a greater magnitude of change in these parameters was observed, underpinning their additive effect on inflammation suppression.

The preservation of an avascular corneal stroma depends on the balance of pro and anti-angiogenic factors [38–40]. Our study indicates that the ability of polysaccharide and MSCs therapy to inhibit neovascularization is mediated (at least in part) by their suppression of VEGF and MMP-2 expression. As an inhibitor of VEGF and MMP2 expression, TSP-1 was additively up-regulated by polysaccharide and MSCs, which might counteract with VEGF and MMP2 to restore anti-angiogenic balance on the cornea [41,42].

Polysaccharide hydrogels have become attractive focus for clinical applications in recent years due to their efficacy and cost-efficiency in comparison to other therapies. Topical VEGF
inhibitors are safe and effective inhibitors of corneal neovascularization [43], however their cost implies financial burden to patients. Therefore, polysaccharide may provide an inexpensive alternative to such treatments. Furthermore, polysaccharide may also serve as a vehicle for drug delivery, facilitating localized administration and greater control of tissue concentrations. Although challenges remain in the clinical application of MSC therapy the field continues to advance. Problems of immunorejection, for example may be addressed by autotransplantation.

In summary, this study provides evidence that Hardy Orchid derived polysaccharide and MSCs are safe and effective treatments for corneal alkali burns and that their benefits are additive when used in combination. Although significant issues may remain in the development of this therapy for clinical application it represents a promising, novel strategy for the treatment of ocular surface injuries.

Supporting Information
S1 Fig. (TIF)
S2 Fig. (TIF)
S1 Table. (TIF)

Author Contributions
Conceived and designed the experiments: CY X. Li. Performed the experiments: YK YW XC X. Liu MY. Analyzed the data: YK YW XC CY. Contributed reagents/materials/analysis tools: YK YW XC CY. Wrote the paper: YK YW XC Y. Li.

References
1. Singh P, Tyagi M, Kumar Y, Gupta KK, Sharma PD. Ocular chemical injuries and their management. Oman J Ophthalmol. 2013; 6: 83–86. doi: 10.4103/0974-620X.116624 PMID: 24082664
2. Tseng SC. Regulation and clinical implications of corneal epithelial stem cells. Mol Biol Rep. 1996; 23: 47–58. PMID: 8983018
3. Wagoner MD. Chemical injuries of the eye: current concepts in pathophysiology and therapy. Surv Ophthalmol. 1997; 41: 275–313. PMID: 9104767
4. Brodovsky SC, McCarty CA, Snibson G, Loughnan M, Sullivan L, Daniell M, et al. Management of alkali burns: an 11-year retrospective review. Ophthalmology. 2000; 107: 1829–1835. PMID: 11013181
5. Adamis AP, Aiello LP, D’Amato RA. Angiogenesis and ophthalmic disease. Angiogenesis. 1999; 3: 9–14. PMID: 14517440
6. He J, Bazan NG, Bazan HE. Alkali-induced corneal stromal melting prevention by a novel platelet-activating factor receptor antagonist. Arch Ophthalmol. 2006; 124: 70–78. PMID: 16401787
7. Meyer K, Linker A, Davidson EA, Weissmann B. The mucopolysaccharides of bovine cornea. J Biol Chem. 1953; 205: 611–616. PMID: 13129238
8. Anseth A, Laurent TC. Polysaccharides in normal and pathologic corneas. Invest Ophthalmol. 1962; 1: 195–201. PMID: 13861724
9. Inoue M, Katakami C. The effect of hyaluronic acid on corneal epithelial cell proliferation. Invest Ophthalmol Vis Sci. 1993; 34: 2313–2315. PMID: 8505213
10. Nakamura M, Nishida T. Synergistic effects of hyaluronan and fibronectin on epithelial migration in rabbit cornea in vitro. Cornea. 1999; 18: 686–692. PMID: 10571300
11. Nakamura M, Mishima H, Nishida T, Otori T. Binding of hyaluronan to plasma fibronectin increases the attachment of corneal epithelial cells to a fibronectin matrix. J Cell Physiol. 1994; 159: 415–422. PMID: 8198759
12. Coura CO, de Araujo IW, Vanderlei ES, Rodrigues JA, Quindere AL, Fontes BP, et al. Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed Gracilaria cornea. Basic Clin Pharmacol Toxicol. 2012; 110: 335–341. doi: 10.1111/j.1742-7843.2011.00811.x PMID: 21985563

13. Yang L, Wang Y, Zhou Q, Chen P, Liu T, Xie L. Inhibitory effects of polysaccharide extract from Spirulina platensis on corneal neovascularization. Mol Vis. 2009; 15: 1951–1961. PMID: 19784394

14. Rodrigues JA, Vanderlei ES, Silva LM, Araujo IW, Queiroz IN, Paula GA, et al. Antinociceptive and anti-inflammatory activities of a sulfated polysaccharide isolated from the green seaweed Caulerpa cuppressoides. Pharmacol Rep. 2012; 64: 282–292. PMID: 22661177

15. Sheng X, Zhang N, Song S, Li M, Liang H, Zhang Y, et al. Morphological transformation and proliferation of rat astrocytes as induced by sulfated polysaccharides from the sea cucumber Stichopus japonicus. Neurosci Lett. 2011; 493: 37–42. doi: 10.1016/j.neulet.2011.08.003 PMID: 21855606

16. Luo Y, Diao H, Xia S, Dong L, Chen J, Zhang J. A physiologically active polysaccharide hydrogel promotes wound healing. J Biomed Mater Res A. 2010; 94: 193–204. doi: 10.1002/jbma.32711 PMID: 20128009

17. Diao H, Li X, Chen J, Luo Y, Chen X, Dong L, et al. Bletilla striata polysaccharide stimulates inducible nitric oxide synthase and proinflammatory cytokine expression in macrophages. J Biosci Bioeng. 2008; 105: 85–89. doi: 10.1263/jbb.105.85 PMID: 18343332

18. Jiang TS, Cai L, Ji WY, Hui YN, Wang YS, Hu D, et al. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. Mol Vis. 2010; 16: 1304–1316. PMID: 20664793

19. Ma Y, Xu Y, Xiao Z, Yang W, Zhang C, Song E, et al. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. Stem Cells. 2006; 24: 315–321. PMID: 16109757

20. Yao L, Li ZR, Su WR, Li YP, Lin ML, Liu Y, et al. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. PLoS One. 2012; 7: e30842. doi: 10.1371/journal.pone.0030842 PMID: 22363499

21. Sonoda Y, Streilein JW. Impaired cell-mediated immunity in mice bearing orthotopic corneal allografts. J Immunol. 1993; 150: 1727–1734. PMID: 8436812

22. Hou HY, Liang HL, Wang YS, Zhang ZX, Wang BR, Shi YY, et al. A therapeutic strategy for choroidal neovascularization based on recruitment of mesenchymal stem cells to the sites of lesions. Mol Ther. 2010; 18: 1837–1845. doi: 10.1038/mt.2010.144 PMID: 20647999

23. Su W, Li Z, Lin M, Li Y, He Z, Wu C, et al. The effect of doxycycline temperature-sensitive hydrogel on inhibiting the corneal neovascularization induced by BFGF in rats. Graefes Arch Clin Exp Ophthalmol. 2011; 249: 421–427. doi: 10.1007/s00417-010-1539-y PMID: 20953876

24. Bhatia SK, Arthur SD, Chenault HK, Figuly GD, Kodokian GK. Polysaccharide-based tissue adhesives for sealing corneal incisions. Curr Eye Res. 2007; 32: 1045–1050. PMID: 18085468

25. Yang G, Espandar L, Mamalis N, Prestwich GD. A cross-linked hyaluronan gel accelerates healing of corneal epithelial abrasion and alkali burn injuries in rabbits. Vet Ophthalmol. 2010; 13: 144–150. doi: 10.1111/j.1463-5224.2010.00771.x PMID: 20500713

26. Barabino S, Rolando M, Nardi M, Bonini S, Aragona P, Traverso CE. The effect of an artificial tear combining hyaluronic acid and tamarind seeds polysaccharide in patients with moderate dry eye syndrome: a new treatment for dry eye. Eur J Ophthalmol. 2014; 24: 173–178. doi: 10.5301/ejo.5000355 PMID: 24030535

27. Wu X, Yang J, Jiang H, Xu Y, Liu T, Zang X, et al. Safety evaluation of intracameral and subconjunctival injection of a novel mucocadhesive polysaccharide isolated from Bletilla striata in rabbit eye. J Ocul Pharmacol Ther. 2012; 28: 369–380. doi: 10.1089/jop.2011.0200 PMID: 22432946

28. Lee KY, Mooney DJ. Hydrogels for tissue engineering. Chem Rev. 2001; 101: 1869–1879. PMID: 11710233

29. Blackmore SJ. The use of contact lenses in the treatment of persistent epithelial defects. Cont Lens Anterior Eye. 2010; 33: 239–244. doi: 10.1016/j.clae.2010.06.004 PMID: 20638323

30. Sun LX, Chen LH, Lin ZB, Qin Y, Zhang-QJ, Yang J, et al. Effects of Ganoderma lucidum polysaccharides on IEC-6 cell proliferation, migration and morphology of differentiation benefiting intestinal epithelium healing in vitro. J Pharm Pharmacol. 2011; 63: 1595–1603. doi: 10.1111/j.2042-7158.2011.01367.x PMID: 22060291

31. Kuckelkorn R, Schrage N, Keller G, Redbrake C. Emergency treatment of chemical and thermal eye burns. Acta Ophthalmol Scand. 2002; 80: 4–10. PMID: 11906296

32. Fish R, Davidson RS. Management of ocular thermal and chemical injuries, including amniotic membrane therapy. Curr Opin Ophthalmol. 2010; 21: 317–321. doi: 10.1097/ICU.0b013e32833a8da2 PMID: 20467317
33. Park CM, Cho CW, Song YS. TOP 1 and 2, polysaccharides from Taraxacum officinale, inhibit NFκB-mediated inflammation and accelerate Nrf2-induced antioxidative potential through the modulation of PI3K-Akt signaling pathway in RAW 264.7 cells. Food Chem Toxicol. 2014; 66: 56–64. doi: 10.1016/j.fct.2014.01.019 PMID: 24447978

34. Yoon SY, Kim JY, Kim ES, Kim SY, Kim MJ, Tchah H. Subconjunctival injection of low-molecular-weight heparin-taurocholate 7 inhibits corneal neovascularization. Cornea. 2013; 32: 1488–1492. doi: 10.1097/ICO.0b013e3182a48009 PMID: 24055905

35. Daniels JT, Harris AR, Mason C. Corneal epithelial stem cells in health and disease. Stem Cell Rev. 2016; 2: 247–254.

36. Oh JY, Kim MK, Shin MS, Lee HJ, Ko JH, Wee WR, et al. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. Stem Cells. 2008; 26: 1047–1055. doi: 10.1634/stemcells.2007-0737 PMID: 18192235

37. Maggini J, Mirkin G, Bognanni I, Holmberg J, Piazzon IM, Nepomnaschy I, et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. PLoS One. 2010; 5: e9252. doi: 10.1371/journal.pone.0009252 PMID: 20169081

38. Sener E, Yuksel N, Yildiz DK, Yilmaz B, Ozdemir O, Caglar Y, et al. The impact of subconjunctivally injected EGF and VEGF inhibitors on experimental corneal neovascularization in rat model. Curr Eye Res. 2011; 36: 1005–1013. doi: 10.3109/02713683.2011.601840 PMID: 21999227

39. Zhang SX, Ma JX. Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy. Prog Retin Eye Res. 2007; 26: 1–37. PMID: 17074526

40. Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). Trans Am Ophthalmol Soc. 2006; 104: 264–302. PMID: 17471348

41. Zak S, Treven J, Nash N, Gutierrez LS. Lack of thrombospondin-1 increases angiogenesis in a model of chronic inflammatory bowel disease. Int J Colorectal Dis. 2008; 23: 297–304. PMID: 18043928

42. Zhou L, Isenberg JS, Cao Z, Roberts DD. Type I collagen is a molecular target for inhibition of angiogenesis by endogenous thrombospondin-1. Oncogene. 2006; 25: 536–545. PMID: 16247480

43. Stevenson W, Cheng SF, Dastjerdi MH, Ferrari G, Dana R. Corneal neovascularization and the utility of topical VEGF inhibition: ranibizumab (Lucentis) vs bevacizumab (Avastin). Ocul Surf. 2012; 10: 67–83. doi: 10.1016/j.jtos.2012.01.005 PMID: 22482468