

Efficacy of Sodium Hyaluronate in Murine Diabetic Ocular Surface Diseases

Guohu Di, PhD, Xia Qi, Xiaowen Zhao, Songmei Zhang, and Qingjun Zhou, PhD

Purpose: To evaluate the efficacy of sodium hyaluronate (HA) eye drops for the treatment of diabetic ocular surface diseases in mice.

Methods: Male 6- to 8-week-old C57BL/6 mice underwent induction of type 1 diabetes with intraperitoneal injections of streptozotocin, with normal mice as the control. Topical 0.3% HA, 0.1% HA, 0.4% polyethylene glycol eye drops, and normal saline were administered to diabetic mice with an intact or debrided corneal epithelium. Normal saline was applied in the controls. Corneal epithelial wound healing rate, corneal sensation, nerve fiber density, conjunctival goblet cell number, and MUC-5AC content were measured and compared.

Results: Compared with the controls, topical 0.3% HA use in diabetic mice showed significant improvements in the corneal epithelial wound healing rate (48 hours: $91.5\% \pm 4.8\%$ vs. $79.8\% \pm 6.1\%$; $P < 0.05$), corneal sensitivity (4.1 ± 0.3 cm vs. 3.5 ± 0.3 cm; $P < 0.05$), nerve fiber density ($12.9\% \pm 2.3\%$ vs. $6.6\% \pm 2.4\%$; $P < 0.05$), conjunctival goblet cell number ($31.0 \pm 8.4/100 \mu\text{m}$ vs. $19.6 \pm 7.1/100 \mu\text{m}$; $P < 0.05$), and MUC-5AC content (12.5 ± 1.4 ng/mg vs. 7.8 ± 1.5 ng/mg protein; $P < 0.05$). The beneficial effects of 0.3% HA were better than those of 0.1% HA and 0.4% polyethylene glycol.

Conclusions: Topical 0.3% HA treatment promoted corneal epithelial regeneration, improved corneal sensation, and increased density of corneal nerve fibers and conjunctival goblet cells in mice with diabetic ocular surface diseases.

Key Words: sodium hyaluronate, diabetes, corneal epithelial wound healing

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The epidemic of diabetes mellitus has soared globally in recent decades. Diabetes is associated with a number of ocular complications, many of which can even lead to permanent loss of vision.¹ Although ocular manifestations such as diabetic retinopathy, glaucoma, and cataract have been studied extensively, the awareness of ocular surface manifestations during the course of diabetes mellitus has increased in recent years.^{2,3} It has been reported that 47% to 64% of patients with diabetes develop primary corneal lesions.⁴ In addition, with a long-term duration, hyperglycemia can affect most structures of the patients' ocular surface, including reduced goblet cell counts, superficial punctate keratopathy, persistent epithelial defects, neurotrophic corneal ulceration, disorder of tear quality and quantity, and dry eye.^{2,5–7} The causes of these alterations, however, are not fully elucidated.

Sodium hyaluronate (HA) is a high molecular weight, naturally occurring glycosaminoglycan composed of repeating alternating sequences of N-acetylglucosamine and glucuronate in linear chains. Because of the sponge-like structure of polysaccharide chains, HA has a huge capacity to bind water and acts as a reservoir of slowly releasing water molecules.⁸ Thus, HA eye drops are commonly used to treat dry eye disease.⁹ In addition, recent studies showed that HA eye drops can protect the ocular surface epithelium by facilitating corneal epithelial healing.^{10–12} However, whether HA eye drops have an effect on diabetic ocular surface disease remains unclear.

The purpose of this study was to evaluate and compare the efficacy of 0.3% HA, 0.1% HA, and 0.4% polyethylene glycol (PEG) eye drops for the management of diabetic ocular disease using a streptozotocin (STZ)-induced diabetic mouse model, by evaluating the changes of ocular surface irregularities, corneal epithelial wound healing rate, corneal sensitivity, nerve fiber density, conjunctival goblet cell number, and MUC-5AC content.

MATERIALS AND METHODS

Preparation of Diabetic Mice and Experimental Procedure

Adult C57BL/6 mice (6–8 weeks old; Institute of Laboratory Animal Sciences, Beijing, China) were maintained in the animal facility of Shandong Eye Institute, and the procedures were performed in accordance with the Association

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From the Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong Academy of Medical Sciences, Qingdao, China.

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Guohu Di and Xia Qi contributed equally to this study.

Q. Zhou: conception and design, data analysis, manuscript writing, and final approval of the manuscript. G. Di and X. Qi: animal experiment conduction and data collection and interpretation. X. Zhao and S. Zhang: animal experiment conduction and data collection.

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Reprints: Qingjun Zhou, PhD, Shandong Eye Institute, 5 Yan'erdao Rd, Qingdao, 266071, China (e-mail: qjzhou2000@hotmail.com).

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for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes was induced by intraperitoneal injection of STZ (50 mg/kg body weight; Sigma, St Louis, MO) in 0.01 M citrate buffer for 5 consecutive days. Control mice were injected with citrate buffer solution. Blood glucose levels were measured from the tail vein with the OneTouch Basic glucometer (LifeScan; Johnson & Johnson, Milpitas, CA) starting from 4 weeks after the final STZ injection. A diabetic condition was confirmed as hyperglycemia over 300 mg/dL. Twelve weeks after STZ injection, the mice were randomly divided into 5 groups (n = 20 per group): normal mice treated with phosphate-buffered saline (PBS), diabetic mice treated with PBS, diabetic mice treated with 0.3% HA (containing 0.3% HA, edetate disodium, aminocaproic acid, and 0.03 mg/mL benzalkonium chloride, Santen, Osaka, Japan), diabetic mice treated with 0.1% HA (containing 0.1% HA, other components are same as 0.3% HA, Santen), diabetic mice treated with 0.4% PEG (containing 0.4% PEG, 0.3% propylene glycol, and 0.001% polyquaternium-1; Alcon, Fort Worth, TX) eye drops. All treatment groups received 3 μ L of eye drops 4 times a day.

Corneal Epithelial Debridement

At 10 days after treatment, all the mice were anesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (70 mg/kg) followed by topical application of 2% xylocaine. Central corneal epithelium (2 mm) was debrided with the Algerbrush II corneal rust ring remover (Alger Co, Lago Vista, TX). The experimental groups unceasingly received eye drops 4 times a day. The epithelial defects were stained with 2% fluorescein sodium and photographed immediately after debridement and at 24, 36, 48 hours, and 14 days thereafter. The staining area was analyzed using ImageJ software and calculated as the percentage of the wound healing rate according to the following formula: (original defect area – residual defect area)/original defect area \times 100%.

Corneal Sensitivity Assay

At 7 days after corneal epithelial debridement, corneal sensation restoration was monitored using a Cochet–Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France). Testing began with the maximal length (6 cm) of nylon filament and shortened by 0.5 cm each time until the eye-blinking response was found. The longest filament length resulting in a positive response was considered as the corneal sensitivity threshold, which was verified 4 times.

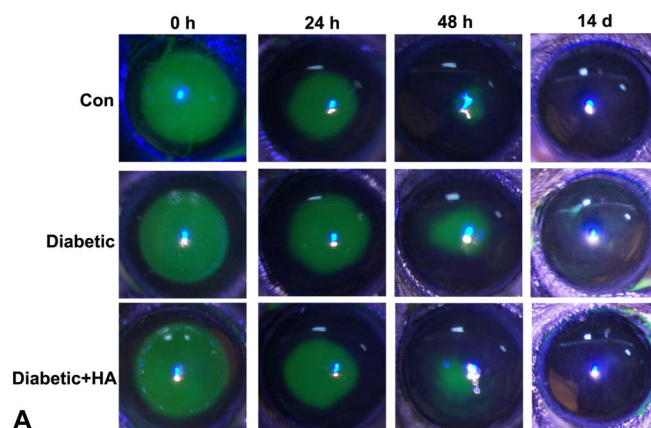
Immunostaining Assay

Corneal whole-mount staining was performed as previously described 7 days after epithelial debridement.¹³ In brief, full-thickness corneal flat mounts were fixed for 1 hour in Zamboni fixative, incubated with 1% bovine serum albumin (Sigma-Aldrich), 2% goat serum, and 0.2% Triton X-100 in PBS for 1 hour to block nonspecific staining. Subsequently, all samples were incubated with a fluorescein isothiocyanate–conjugated mouse anti- β -III tubulin antibody (1:100; Merck

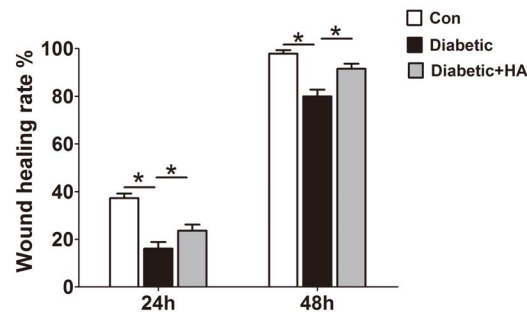
Millipore, Darmstadt, Germany) for 24 hours at 4°C. Finally, the flat mounts were examined under an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan). Quantification of corneal innervation was calculated as the percentage of the area positive for β -tubulin staining as previously described.^{14,15} For corneal section staining, eyeballs were excised and snap-frozen in Tissue-Tek optimum cutting temperature compound (Sakura Finetek, Tokyo, Japan) at 10 days after treatment (n = 4 per group). The sections were stained with MUC-5AC (1:100; Santa Cruz, Dallas, TX), CD44 (1:200; R&D, Minneapolis, MN), ICAM-1 (1:200; Abcam, Cambridge, United Kingdom), VCAM-1 (1:200; Abcam), ZO-1 (1:200; Abcam), and ZO-2 (1:200; Abcam) followed by the respective secondary antibodies. The stainings were examined and photographed with an Eclipse TE2000-U microscope.

Periodic Acid–Schiff Staining Assay

At 10 days after treatment, eyeballs with adnexa were excised, fixed in 4% formaldehyde solution, and embedded in paraffin (n = 5 per group). The sections were stained with the periodic acid–Schiff reagent. Sections from each group were



A



B

FIGURE 1. Effects of 0.3% HA on corneal epithelial wound healing of diabetic mice. A, Topical 0.3% HA application for 10 days was performed to evaluate the wound healing rate in the diabetic corneal epithelium. Corneal epithelium was removed and stained with fluorescein sodium at 24 hours, 48 hours, and 14 days after corneal epithelium scrape, with continuous daily 0.3% HA administration in the diabetic + HA group. B, Histogram of the wound healing rate is presented as the percentage of the original wound. * $P < 0.05$.

examined and photographed with a microscope. Goblet cells in the conjunctiva were counted in 3 sections from each eye and expressed as the number of goblet cells per 100 μm .

MUC-5AC Enzyme-Linked Immunosorbent Assay

Total protein was extracted from the conjunctiva of 3 eyes of different groups. The supernatants were centrifuged and subjected to quantitative sandwich immunoassay with enzyme-linked immunosorbent assay (ELISA) detection kits for mouse MUC-5AC (USCN, Wuhan, China) according to the manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

A commercial statistical/analytical software program (SPSS 17.0 for Windows; SPSS, Inc, Chicago, IL) was used for statistical analysis. Statistical analysis was performed by an unpaired *t* test for comparison of 2 groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

0.3% HA Promotes Corneal Epithelial Wound Healing in Diabetic Mice

To evaluate the effects of 0.3% HA on diabetic corneal epithelial wound healing, central corneal epithelium was scraped from both normal and diabetic mice with or without

topical HA application for 10 days. The healing rate of corneal epithelium in HA-treated diabetic mice (24 hours: $22.6 \pm 9.5\%$; 48 hours: $91.5 \pm 4.8\%$, $n = 10$) was significantly improved compared with that of vehicle-treated diabetic mice (24 hours: $16.2 \pm 7.4\%$; 48 hours: $79.8 \pm 6.1\%$, $n = 10$), whereas it did not reach that of normal mice (24 hours: $36.0 \pm 6.7\%$; 48 hours: $96.9 \pm 3.2\%$, $n = 10$) (Figs. 1A, B). Furthermore, 0.3% HA-treated diabetic mice showed reduced punctate fluorescein staining compared to vehicle-treated diabetic mice at 14 days after epithelial debridement (Fig. 1A). In addition, the immunostaining results showed that HA's main receptor, CD44, exhibited more intense staining in 0.3% HA-treated diabetic mice, whereas either cell adhesion molecules (ICAM-1 and VCAM-1) or tight junction markers (ZO-1 and ZO-2) demonstrated no obvious changes after HA treatment (see Figure, Supplemental Digital Content, <http://links.lww.com/ICO/A531>).

0.3% HA Promotes Corneal Nerve Regeneration and Sensation Recovery in Diabetic Mice

Previous studies have confirmed the delayed corneal nerve regeneration and sensation recovery in diabetic mice.^{13,16,17} In this study, we examined the effects of 0.3% HA on diabetic corneal nerve regeneration and sensation recovery after 7-day treatment. As shown in Figure 2A, B, 0.3% HA treatment increased the corneal subbasal nerve fiber density (n = 5 per group) and improved corneal sensation (n = 6 per group) in diabetic mice, although it was still lower than that of age-matched control mice.

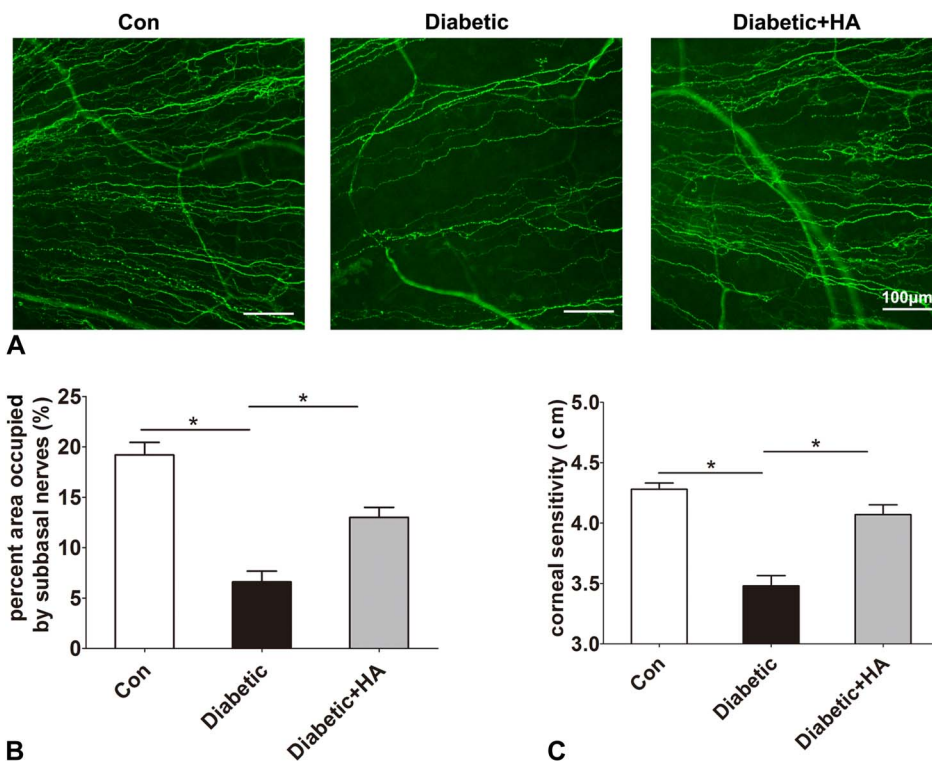


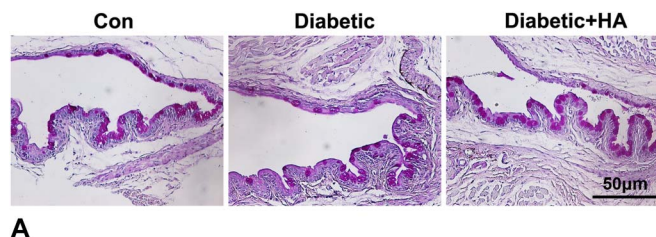
FIGURE 2. Effects of 0.3% HA on corneal nerve regeneration and sensation restoration of diabetic mice. A, Corneas were harvested, flat mounted, and stained with β III-tubulin antibody 7 days after injury. Representative images of the subbasal nerve plexus were taken. B, Histogram of regenerated subbasal nerve plexus density is presented as the ratio of control mice. C, Corneal sensitivity was measured in unanesthetized control, diabetic, and HA-treated diabetic mice 7 days after injury. * $P < 0.05$.

0.3% HA Promotes Conjunctival Goblet Cell Recovery in Diabetic Mice

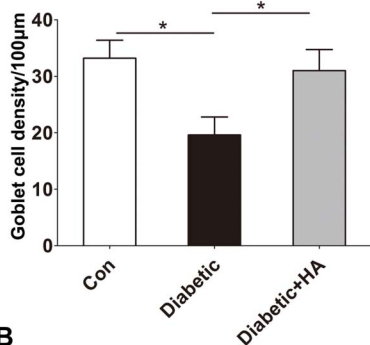
Conjunctival goblet cells were evaluated with periodic acid–Schiff staining. As shown in Figure 3A, B, the goblet cell density decreased in diabetic mice compared with normal mice, whereas the mean number significantly increased in 0.3% HA-treated diabetic mice and reached a similar level to normal mice ($n = 5$ per group). Moreover, both immunofluorescence staining and ELISA analysis of MUC-5AC showed a significantly lower staining intensity and content in the conjunctival epithelium of diabetic mice than that of control mice. However, the MUC-5AC expression level was significantly upregulated in 0.3% HA-treated diabetic mice compared to the vehicle-treated diabetic mice for 10 days (Fig. 4A, B, $n = 4$ per group).

Comparison of 0.3% HA With 0.1% HA and 0.4% PEG in Treating Diabetic Ocular Surface Complications

To investigate whether the effect of HA depended on the concentration of HA, and whether other lubricant eye drops have similar effects, we compared 0.3% HA with 0.1% HA and 0.4% PEG ($n = 6$ per group). As shown in Figures 5A, D, the corneal epithelial wound healing rate was significantly faster in 0.3% HA-treated diabetic mice than that of either 0.1% HA- or 0.4% PEG-treated diabetic mice. In addition, the corneal nerve fiber density (Figs. 5B, E), corneal sensation recovery (Fig. 5F), and goblet cell density

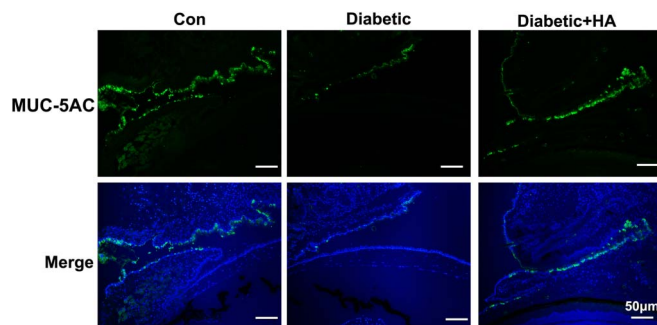


A

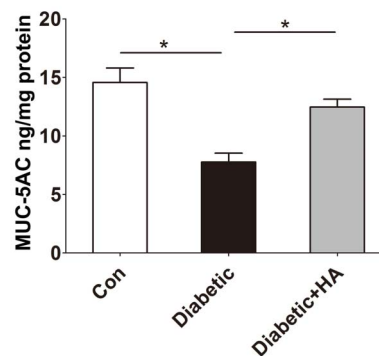


B

FIGURE 3. Effects of 0.3% HA on goblet cells in the conjunctival epithelium of diabetic mice. A, Representative PAS-stained conjunctival specimens showed that PAS-positive goblet cells were scanty in diabetic mice, while restored by 0.3% HA application. B, The mean number of goblet cells in the conjunctival epithelium ($n = 5$ per group). $*P < 0.05$. PAS, periodic acid–Schiff.



A



B

FIGURE 4. Effects of 0.3% HA on MUC-5AC expression in the conjunctival epithelium of diabetic mice. A, Immunofluorescence staining of representative specimens before and after 0.3% HA application in diabetic mice. Note that the density of positive cells seems to increase with 0.3% HA treatment for 10 days. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. B, The mean MUC-5AC protein expression in the 0.3% HA-treated mice was significantly higher than the diabetic mice. $*P < 0.05$.

(Fig. 5C, G) showed similar effects when comparing 0.3% HA treatment with 0.1% HA or 0.4% PEG treatment.

DISCUSSION

Patients with diabetes mellitus are at high risk of developing ocular surface disorders. The ocular surface disease in diabetes is mainly characterized by decreased corneal sensitivity, persistent epithelial fragility, irregular tear function, and goblet cell loss. It has been suggested that these abnormalities might be responsible for the clinical features of diabetic keratopathy.¹⁸ In this study, we evaluated the effect of 0.3% HA eye drops on treating ocular surface irregularity using a type 1 diabetic mice model. The results revealed that the corneal epithelial wound healing rate, nerve fiber regeneration, sensation recovery, as well as goblet cell counts and MUC-5AC expression in the conjunctiva significantly improved with topical application of 0.3% HA eye drops, which is better than either 0.1% HA or 0.4% PEG eye drops.

Corneal complications in diabetic mouse models share similarities with patients with diabetes, particularly with the delayed corneal epithelial wound healing.¹⁹ Previous studies have reported that hyaluronate stimulates human corneal epithelial cell migration in vitro and facilitates corneal

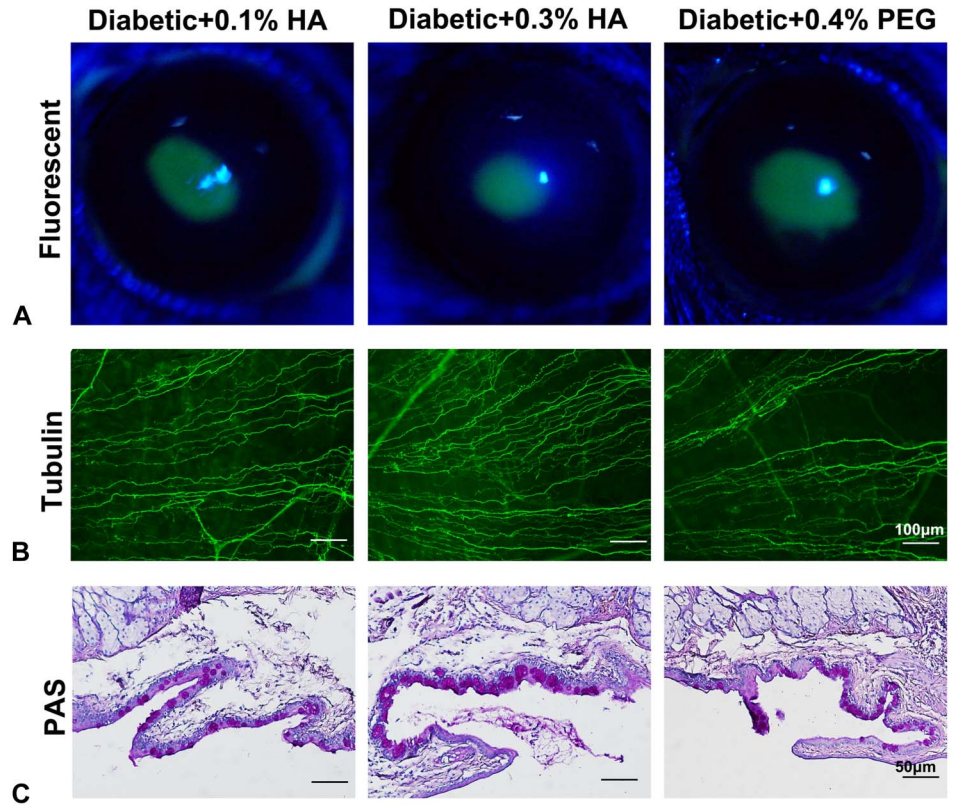
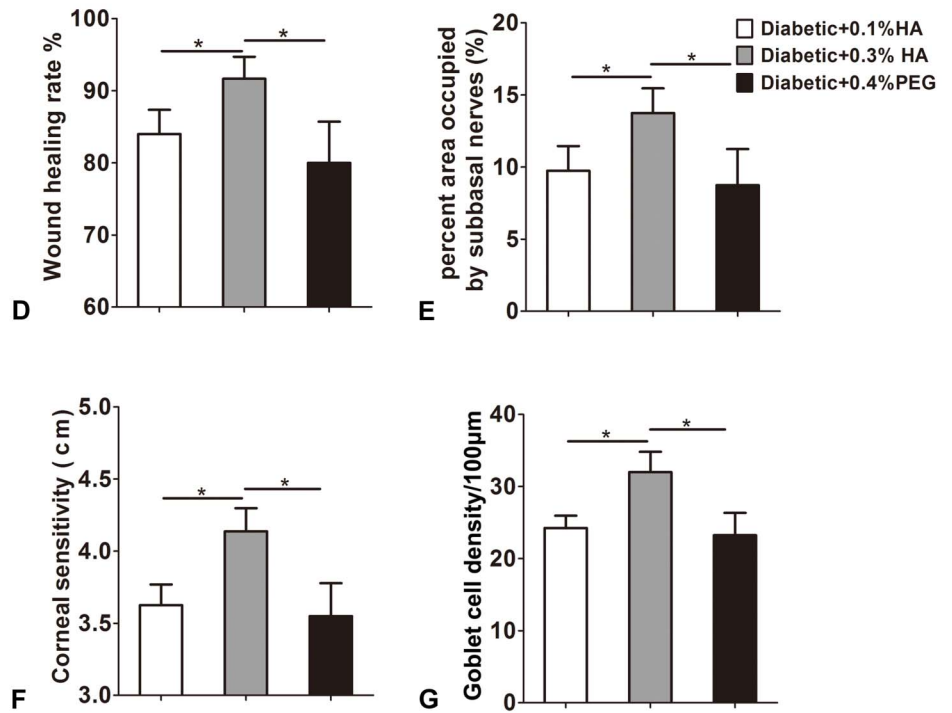


FIGURE 5. Comparison of 0.3% HA with 0.1% HA and 0.4% PEG in treating diabetic ocular surface complications. A, After topical application of 0.3% HA, 0.1% HA, or 0.4% PEG for 10 days, the corneal epithelium in diabetic mice was debrided and stained with fluorescein sodium at 48 hours after injury. B, Representative images of the sub-basal nerve plexus. C, Representative images of PAS-stained conjunctival specimens. D, Histogram of the wound healing rate is presented as the percentage of the original wound. E, Histogram of regenerated subbasal nerve plexus density. F, Histogram of corneal sensitivity. G, Density of goblet cells in the conjunctival epithelium. * $P < 0.05$. PAS, periodic acid–Schiff.



epithelial wound healing in both normal and diabetic animal models in vivo.^{20,21} In this study, we showed an enhancement of corneal epithelial regeneration in diabetic mice when pretreated with 0.3% HA. Moreover, we confirmed that expression of either cell adhesion molecule or tight junction marker was not significantly changed, whereas the expression of the HA receptor, CD44, improved with the 0.3% HA treatment in the diabetic mice. The results suggested that the promotion of corneal epithelial wound healing by 0.3% HA in diabetic mice may be mediated through the upregulation of HA receptor and the interaction with fibronectin.²²

We also found that corneal nerve regeneration in diabetic mice is likely to benefit with the 0.3% HA treatment regarding the improvement of corneal nerve fiber density and corneal sensation. In fact, the loss or decrease of corneal innervation and subsequent neurotrophic deficiency of trigeminal sensory nerves on the cornea and conjunctiva may be responsible for those ocular surface disorders.²³ Moreover, other factors, such as age and duration of diabetes mellitus, which are closely correlated with impairment of corneal sensitivity, can also adversely affect the goblet cell counts.

Goblet cell counts in the conjunctival epithelium are known to reflect the health status of the ocular surface. Goblet cells are the major source of mucin in the tear film, albeit other sources of mucin are present in the ocular surface.²⁴ Reduction in goblet cell numbers may account for the instability of the tear film, decreased mucin production, and high incidence of dry eye in patients with diabetes.²⁵ Eye drops containing hyaluronate are typically used as the first-line management of dry eye. It is supposed to improve tear stability and corneal surface irregularity by prolonging the retention time of the tear film.²⁶ In this study, the 0.3% HA showed a significant improvement of goblet cell counts and MUC-5AC expression in diabetic mice. This result indicates that 0.3% HA application might be useful for ameliorating chronic damage of the conjunctival surface in patients with diabetes.

In summary, we conclude in this study that topical application of 0.3% HA eye drops had beneficial effects in the treatment of diabetic ocular surface diseases in mice, including the improvement of corneal epithelial and nerve regeneration, corneal sensation, and goblet cell functional recovery.

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