Desiccation Stress-Mediated Dry Eye Disease

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Submitted: May 31, 2018
Accepted: January 30, 2019

Citation: Lee H, Jeon S, Kim CE, Park YJ, Yang J. A new ophthalmic pharmaceutical formulation, topical sulglycotide, enhances the ocular mucin secretion in desiccation stress-mediated dry eye disease. 2019;60:1076–1087. https://doi.org/10.1167/iovs.18-24935

PURPOSE. The aim of this study was the investigation of the effect of sulglycotide (SOS), a polysulfated glycopeptide derived from porcine duodenal mucin, for the treatment of dry eye disease.

METHODS. NOD.B10.H2b mice were exposed to an air draft for 10 days, and, simultaneously, scopolamine hydrobromide was injected subcutaneously. The mice were randomly divided into nine groups as follows: four kinds of SOS formulations and three kinds of commercial solutions. After 10 days of treatment, we estimated the effect of treatment on tear production, epithelium stabilization, mucin secretion, and inflammation.

RESULTS. The desiccation stress significantly decreased tear production and corneal epithelium stabilization, as well as markedly decreased the numbers of goblet cells and mucin-stained cells in conjunctiva. However, the topical 4% SOS eye drops markedly increased tear production and corneal stabilization, which recovered to baseline levels. In addition, topical 4% SOS significantly induced an increase in the numbers of goblet cells and the expression of membrane-associated mucins including MUC1, MUC4, and MUC16, as well as the gel-forming mucin, MUC5AC. Furthermore, SOS formulations provided anti-inflammatory improvement in a dose-dependent manner.

CONCLUSIONS. In summary, we suggest that a new ophthalmic pharmaceutical formulation, topical sulglycotide, enhances the ocular mucin secretion in dry eye disease and can be used as a new ophthalmic pharmaceutical material to treat dry eye disease.

Keywords: dry eye disease, sulglycotide (SOS), mucin, conjunctival goblet cell, inflammation

Dry eye (DE) syndrome is a heterogeneous disorder of the ocular surface that results in symptoms of discomfort, visual disturbance, tear film instability, and inflammation, with potential damage to the ocular surface.1,2 Tear instability is accompanied by increased tear osmolarity, which leads to an inflammation of the ocular surface that initiates a vicious cycle.3 The tear film is composed of many substances, including lipids, proteins, mucins, and electrolytes.4 The lipid layer is derived from meibum secreted from the lid margins to prevent the loss of the aqueous layer through overspill and evaporation.5 The aqueous layer protects the exposed ocular epithelium by providing lubrication, some nutrients, antimicrobial proteins, and appropriate osmolarity. The mucin layer covers the ocular surface and lowers the supposed hydrophobicity of the epithelial cells.6,7

Mucins, as part of the tear film and glycocalyx, contribute to homeostasis on the ocular surface, maintaining clarity of the cornea and tear film to allow light to pass through the anterior segment of the eye. Moreover, disruption of this homeostasis, including alteration in expression and/or glycosylation of the mucins, can occur in various ocular surface disease states, such as DE.8,9 Recently, several studies have reviewed that the role of mucins in ocular surface health, and DE is a subject of great interest.10,11 Diquafosol stimulates the secretion of sialic acid, which is a mucin-like substance, as well as mucin from the conjunctiva, directly onto the ocular surface.12,13 Several studies demonstrated that diquafosol promotes tear fluid and a good safety profile with clinical improvement of the ocular surface in patients with severe DE.14–16 However, some studies have reported that diquafosol may not act on the lacrimal glands directly or not provide a major improvement in symptoms related to DE.16–18 It has previously been demonstrated that the topical administration of rebamipide, an antiulcer agent, increases the mucin level of the tear film and improves the conditions of the ocular surface in DE.19 In addition, rebamipide ophthalmic solution (Mucosta ophthalmic suspension UD 2%; Otsuka Pharmaceutical, Tokyo, Japan) has recently entered the market and is limited to Japan.20 Sulglycotide, a polysulfated glycopeptide derived from porcine duodenal mucin that is unabsorbable from the gastrointestinal tract,21,22 is used as a potent gastroprotective antiulcer agent.23,24 Although several studies demonstrated that SOS has anti-inflammatory and anticancer effects, the inhibitory effect of SOS on dry eye disease has not been reported; its mucin regulative effect and anti-inflammatory effects have not been investigated in DE disease. SOS, in common with
SOS Enhances the Ocular Mucin Secretion for Dry Eye

MATERIALS AND METHODS

Materials

The SOS used in our study was obtained from Samil Pharm. Co. Ltd. (Ansan, Korea). The topical cyclosporine A (CsA; Cyporin N 0.05%) and sodium hyaluronate (HA; Hyaluni 0.1%) were obtained from Taejoon Pharm Co., Ltd. (Seoul, Korea), and the diquafosol was obtained from Santen Pharmaceutical Co., Ltd. (DQS; Diquas ophthalmic solution 3%; Osaka, Japan). All other excipients were of chemical grade and purchased from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea).

Preparation of SOS Solution

The four formulations with different quantities of SOS were prepared by dissolving in 160 mL, pH 7.8, phosphate buffer, 0.58 g potassium phosphate monobasic, and 8.86 g sodium phosphate dibasic in 1000 mL of purified water, adjusted to pH 7.8 with phosphoric acid. The SOS 1, SOS 2, SOS 3, and SOS 4 formulations contained 1% SOS, 2% SOS, 3% SOS, and 4% SOS in pH 7.8 phosphate buffer, respectively. The osmolarity of the SOS formulations ranged from 280 to 320 mOsm/kg. The pH of the SOS formulations was adjusted to 8 by titration using 0.2 N sodium hydroxide solution. Finally, the SOS formulations were filtered through a 0.22-μm polyvinyl diisopropyl fluoride (PVDF) membrane for sterilization.

Animals and Experimental Procedures

This study was conducted in accordance with the Guidelines for Animal Experimentation of Inje University Busan Paik Hospital with approval of the Institutional Animal Care and Use Committee (No. IJUBPH_2016-005-02) for the use of animals in ophthalmic and vision research. This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We purchased 44 NOD.B10. H2b/H2b (6 weeks old) mice (6 weeks old) from Jackson Laboratory (Bar Harbor, ME, USA). The mice were acclimatized for 6 to 10 weeks. Twelve-week-old or more mice were used for all experiments. Four mice were used for histopathologic analysis at baseline. Forty-six mice were subjected with approval of the Institutional Animal Care and Use Committee. We randomly divided 46 mice into seven groups as follows: SOS 1 group (n = 4, 5 μL SOS 1%), SOS 2 group (n = 4, 5 μL SOS 2%), SOS 3 group (n = 4, 5 μL SOS 3%), SOS 4 group (n = 6, 5 μL SOS 4%), cyclosporine A group (n = 6, 5 μL CsA; Cyporin N 0.05%), the diquafosol group (n = 6, 5 μL Diquas ophthalmic solution 3%), and the sodium hyaluronate group (n = 6, 5 μL Hyaluni 0.1%). SOS solutions and sodium hyaluronate were administered four times per day for 10 days. Cyclosporine A and diquafosol were administered two and six times per day for 10 days, respectively. The tear amount, corneal irregularity score, and fluorescein staining score were measured at baseline, at DS 10d, and after treatment for 3, 5, 7, and 10 days. After treatment for 10 days, the mice were euthanized.

Measurement of Tear Volume

The tear volume was measured as previously described.²⁶,²⁷ Briefly, the tear amount was evaluated with phenol red-impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA) for 20 seconds. The tear volume was measured at 2 hours after the last scopolamine injection and at 1 hour after the last treatment in both eyes and calculated with a standard curve of response to a stock basic solution (1.5 L 0.9% saline and 5 mL 5 N NaOH).

Evaluation of Corneal Irregularity

Corneal irregularity was evaluated as the corneal irregularity score according to the extent of the distortion of the white ring in digital images.²⁸ The white ring images of the corneal surface were acquired from a fiberoptic circle illuminator with a microscope immediately after the mice were euthanized.

Corneal Fluorescein Staining

Corneal fluorescein staining was performed according to the National Eye Institute (NEI) grading system.²⁹ The eyes were examined for fluorescein staining with a slit-lamp biomicroscope (SLD7; Topcon Medical Systems, Inc., Oakland, NJ, USA) under a cobalt blue light. Punctate staining was recorded in a masked fashion using the standard NEI grading system, giving a score from 0 to 3 (0 = normal and 3 = severe) for each of the five areas (superior, nasal, central, inferior, temporal) of the cornea. Grade 0 is specified when no staining is present, and the maximum score is 15.

Histology

The orbit of mice was surgically extracted and fixed in 10% formalin. The tissues were embedded in paraffin and were cut to 5-μm-thick sections with a microtome (RM2245; Leica Biosystems, Nussloch, Germany). For the evaluation of the detaching epithelium, the sections were stained with hematoxylin and cosin (H&E).³⁰ For evaluating the density of conjunctival goblet cells, the conjunctival sections were stained with periodic acid Schiff (PAS), and the test was performed using a commercial kit (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. The sections were photographed with a virtual microscope (NanoZoomer 2.0 RS; Hamatsu Photonics, Shizuoka Prefecture, Japan). Goblet cell density in the superior and inferior conjunctiva was measured in three sections of each eye and was indicated as the number of goblet cells per 100 μm.³⁰

Mucin Staining

Staining of 6-μm sections of the eyes and adnexa for mucin was performed with Alcian blue, pH 2.5, using a kit (Abcam, Inc., Cambridge, MA, USA) according to the manufacturer’s
instructions. Sections were deparaffinized if necessary and hydrated in distilled water. They were next incubated in acetic acid solution for 3 minutes, followed by 30 minutes at room temperature or 15 minutes at 37°C incubation in Alcian blue (pH 2.5) solution. The stained sections were rinsed briefly in acetic acid solution to remove excess Alcian blue and were then given a 2-minute rinse in running tap water, followed by two changes of distilled water. The sections were next stained with safranin O solution for 5 minutes and rinsed for 2 minutes in running tap water followed by two changes of distilled water. After dehydrating through graded alcohols, the sections were cleared and mounted in synthetic resin. The eye sections from each group were assessed in a 0.1-mm² area of the cornea or inferior fornices of the conjunctiva. The stained sections were then examined and imaged using a Virtual Microscope (NanoZoomer 2.0 RS, Hamamatsu, Japan).

**Immunofluorescence**

The eyes and adnexa fixed in formalin for 3 days were cut into 6-μm-thick sections using a microtome and prepared for immunofluorescence. The sections were rehydrated in PBS (Tech & Innovation, Gangwon, Korea), followed by incubation in 0.5% Triton X-100 for 20 minutes. After three rinses with PBS for 5 minutes each, the sections were incubated in 3% BSA for 1 hour to block nonspecific staining. After blocking, the sections were incubated with primary antibodies such as MUC1 (1:250; catalog no. ab15481; Abcam, Inc.), MUC4 (1:250; catalog no. bs-4772R; Bioss, Inc., Woburn, MA, USA), MUC5AC (1:250; catalog no. MA5-12178; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and MUC16 (1:250; catalog no. 250566; Abbiotec, Inc., San Diego, CA, USA) for 1 day. After three washes with PBS for 5 minutes each, the sections were next incubated with an FITC-conjugated donkey anti-mouse immunoglobulin G (IgG) or donkey anti-rabbit (1:500; Thermo Fisher Scientific, Inc.) secondary antibody for 1 hour. The stained sections were washed three times (5 minutes each) with PBS and then counterstained and mounted with mounting medium containing 4,6-diamino-2-phenylindole (DAPI; Southern Biotech, Inc., Birmingham, AL, USA). The sections were observed under a fluorescence microscope (Leica DM2500; Leica Microsystems GmbH, Wetzlar, Germany).

**Immunohistochemistry**

The lacrimal gland of mice was surgically extracted, fixed in 10% formalin, and embedded in paraffin. Five-micrometer-thick sections were cut with a microtome (RM2245). Immunohistochemical analysis of the lacrimal glands was performed by the method according to Kim et al.33 The primary antibodies for TNFα and matrix metalloproteinase (MMP)-2 were obtained from Abcam, Inc. (catalog no. ab6671, ab57150). The intercellular adhesion molecule (ICAM)-1 antibody and vascular cell adhesion molecule (VCAM)-1 antibody were purchased from Bioss, Inc. (catalog no. bs-4615R, bs0920R). MMP-9 was obtained from LifeSpan BioSciences, Inc. (catalog no. LS-B2486; Seattle, WA, USA). Images of the sections were photographed with a virtual microscope (NanoZoomer 2.0 RS).

**RNA Isolation and Quantitative Reverse Transcriptase PCR**

The total RNA from the cornea or conjunctiva was collected through four eyes per group and RNA was isolated according to the manufacturer's protocol using a Purelink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA). The RNA yield and quality were then analyzed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One microgram of the total RNA was synthesized with cDNAs using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR was performed using the SYBR Green PCR Core Reagents System (Applied Biosystems, Paisley, UK) and Applied Biosystems Viia 7 Real-Time PCR System (Applied Biosystems). The primers sequences used product sizes were as follows34: TNFα forward 5′-CTGAACTCTGC GGGTAGTCCG-3′, reverse 5′-GGCTTTGTCACTC GAATTITAGA-3′; MMP-2 forward 5′-ACCTGAACACTTT TATGCGGT-3′, reverse 5′-CTTCCGCATGTCTCGATG-3′; MMP-9 forward 5′-GGACC CGGAAGCCGATTG-3′, reverse 5′- CGTCGTCGAAATGTCGCCAT-3′; ICAM-1 forward 5′-TG CCTCTCTGAAGTCGCGATATAC-3′, reverse 5′- TCTTGCGAACCTCCTCAGTCAC-3′; VCAM-1 forward 5′-AGTGGGGAATGTGGTGTCC-3′, reverse 5′-CCCCCTACCTCT TACCACCC-3′; GAPDH forward 5′-AGGTTCCG TGGTGAAGC GATTTG-3′, reverse 5′-TGTAGACCATGTAGGAGGTCA-3′.

The analysis was performed in duplicate and repeated three times. The quantitative RT-PCR results normalized GAPDH on an endogenous reference.

**Tear Fluid Washings and ELISA**

Tear fluid washes were collected according to previously reported methods. Tear fluid washes were collected according to previously reported methods.32 PBS (1.5 μL) containing 0.1% BSA was instilled into the conjunctival sac and collected in a 1-μL glass capillary tube (Drummond Scientific Co., Broomhall, PA, USA). Samples of tear wash (2 μL) were collected in both eyes of each mouse and stored at −80°C until ELISA was performed. The concentrations of inflammatory cytokines (TNFα, MMP-2, MMP-9, ICAM-1, and VCAM-1) in the tear collection were analyzed by ELISA kit for mice (R&D Systems, Minneapolis, MN, USA). The ELISA kit was performed according to the manufacturer’s protocol. ELISA was performed using the electrochemical analysis equipment WizECM-1200 premium (WizMAC, Daejeon, Korea).

**Statistical Analyses**

The data were analyzed with SPSS version 23.0 (SPSS, Chicago, IL, USA) and were indicated as means ± SDs. Comparison among the three groups for tear production, corneal irregularity, and corneal fluorescein staining was studied by Kruskal-Wallis 1-way ANOVA on ranks test, followed by Dunn’s multiple comparison tests. The differences between the groups were analyzed using 1-way ANOVA, and statistical significance was defined at P < 0.05 by Tukey’s test.

**Results**

**Effect of Topical Sulglycotide on Tear Production in Desiccation Stress-Mediated DE Disease**

Figure 1 shows the change of tear volume in NOD.B10.H2b mice. The mean tear volume was 0.27 ± 0.01 μL at baseline. However, the tear volume was gradually suppressed to 0.03 ± 0.01 μL by desiccation stress in all mice, with no significant difference between the groups. The tear production of the SOS 1 group, SOS 2 group, and SOS 4 group was also slightly improved to 0.13 ± 0.02, 0.13 ± 0.01, and 0.13 ± 0.01 μL, respectively, at 3 days after treatment. The topical SOS 4 eye drops markedly increased tear production to 0.21 ± 0.04 μL from 5 days after treatment, similar to medicines for DE disease including CsA. Furthermore, the tear volume of the SOS 4
group (0.25 ± 0.02 µL) recovered to baseline level at 10 days after treatment. From 3 days after treatment, the CsA group and the DQS group had significantly increased tear production to 0.22 ± 0.01 and 0.21 ± 0.02 µL, respectively. Especially, the topical DQS group had significantly increased tear production to the baseline level from 5 days after treatment, and the effect was maintained to the endpoint. However, although the tear volume of the CsA group (0.26 ± 0.01 µL) was markedly increased at 10 days after treatment, the levels did not recover to the baseline level nor to the levels of the SOS 4 and DQS groups. The tear volume of the HA group was slightly increased for treatment, but the level was similar to that of the SOS 1 and SOS 2 groups.

**Effect of Topical Sulglycotide on the Corneal Circular Ring in Desiccation Stress-Mediated DE Disease**

The desiccation stress markedly increased breaking of the corneal circular ring in all mice (Fig. 2A), and the irregularity score was not significantly different between the groups (3.61 ± 0.10; Fig. 2B). At 7 days after treatment, the corneal irregularity score was significantly suppressed by SOS 4 treatment (2.08 ± 0.20). In addition, the breaking of the corneal circular white ring and the score of corneal irregularity were markedly decreased in the SOS 4 group (0.67 ± 0.41), and the score recovered to the baseline score at 10 days after treatment. The effectiveness of SOS 4 was better than that of DQS (1.33 ± 0.41) at 10 days after treatment. The distorted corneal circular ring of the CsA and HA groups did not improve over the 10 days of treatment.

**Effect of Topical Sulglycotide on the Corneal Fluorescein Grading Score in Desiccation Stress-Mediated DE Disease**

The desiccation stress induced the increase of the corneal fluorescein score in all mice (Figs. 3A, 3B; DS 10D). Topical sulglycotide formulation eye drops decreased the corneal fluorescein scores to statistically significant differences at 5 days after treatment. At 7 days after treatment, the corneal fluorescein scores of the SOS 4 group were significantly decreased to 6.83 ± 0.75. Similar to the results from the corneal irregularity score, the result of corneal fluorescein also showed a marked decrease in SOS 4 group (3.17 ± 0.75), and its effectiveness was superior to that of the DQS group (7.00 ± 1.73) at 10 days after treatment. The CsA and HA topical eye drops did not change the score of corneal fluorescein staining at each time point.

**Effect of Topical Sulglycotide on the Detachment of Corneal Epithelium in Desiccation Stress-Mediated DE Disease**

We showed the results of detaching corneal epithelium by H&E staining (Fig. 4A) and indicated the quantitative data of the
results as number per 0.1 mm² (Fig. 4B). Desiccation stress markedly induced the detachment of epithelium to 8.56-fold of control (0.19 ± 0.14/0.1 mm²), but this increase in the detachment of corneal epithelium by desiccation stress was gradually suppressed to 76.64% of DS 10d by SOS 4 treatment, and it was improved to the control group level (0.38 ± 0.23/0.1 mm²). Topical DQS eye drops also significantly reduced detachment of corneal epithelium (0.38 ± 0.16/0.1 mm²) and were similar to the results from SOS 4. Although topical SOS 2 and SOS 3 eye drops slightly decreased the detachment of corneal epithelium, there was no statistical significance compared with SOS 4 and DQS groups. The detachment of the corneal epithelium was significantly decreased to approximately 58.39% by CsA and HA but did not recover to the levels of the control groups.

Effect of Topical Sulglycotide on Conjunctival Goblet Cells in Desiccation Stress-Mediated DE Disease

We performed PAS staining to observe of goblet cells that secrete gel-forming mucins in the inferior fornix conjunctiva, and goblet cells were stained a deep purple color (Fig. 5A). In the control mice, the number of conjunctival goblet cells was 16.29 ± 1.52/0.1 mm², but it gradually decreased to 44.74% due to desiccation stress (7.29 ± 0.94/0.1 mm²; Fig. 5B). Nevertheless, the topical eye drops of SOS 4 significantly increased conjunctival goblet cells, which was similar to that in control mice. The number of conjunctival goblet cells was slightly increased to 65.74% and 74.27% of control by the SOS 3 and CsA groups, respectively. However, these alterations did not improve to the levels in control mice. The topical eye drops of SOS 1, SOS 2, DQS, and HA did not upregulate over the 10 days of treatment.

Effect of Topical Sulglycotide on Conjunctival Mucins in Desiccation Stress-Mediated DE Disease

Based on the previous results, we selected the most effective treatment as SOS 4, the 4% sulglycotide formulation, and evaluated that the correlation with goblet cells and mucin using Alcian blue staining. As shown in Figure 6A, mucin was stained a deep blue color in the inferior fornix conjunctiva of control mice (16.76 ± 1.07/0.1 mm²; Fig. 6A), and it was similar to the result of PAS staining (Fig. 5A). The desiccation stress markedly decreased the numbers of stained mucin to 41.89% of the control group. In contrast, the conjunctival mucin was significantly increased to 95.02%...
of control by SOS 4 topical eye drops, and the efficacy was better than CsA treatment (68.84% of control). The eye drops of DQS and HA did not change the amount of conjunctival mucin for 10 days.

Effect of Topical Sulglycotide on the Expression of Subtypes of Mucin in Desiccation Stress-Mediated DE Disease

We performed immunofluorescence staining for subtypes of mucin in the inferior fornix conjunctiva. In the control mice, the expression of membrane-associated mucins including MUC1, MUC4, and MUC16 was observed as a bright red signal in the conjunctiva (Fig. 7). However, these expression levels were gradually suppressed by desiccation stress. In contrast, the topical eye drops of SOS 4 and CsA markedly upregulated the expression of MUC1, MUC4, and MUC16. Figure 7B shows that the gel-forming mucin, MUC5AC, was evenly spread among the conjunctiva of the control mice, and the expression of MUC5AC was green in color. The desiccation stress downregulated the expression of MUC5AC in the conjunctiva, whereas it was upregulated by SOS 4 and CsA treatment. The topical eye drops of DQS, and HA did not increase the expression of all subtypes of mucin in the conjunctiva.

Effect of Topical Sulglycotide on the Expression of Inflammatory Markers in Desiccation Stress-Mediated DE Disease

We assessed the efficacy of SOS 4 on the expression of inflammatory markers including TNFα, ICAM-1, VCAM-1, MMP-2, and MMP-9 in the lacrimal gland (Fig. 8B), tears (Fig. 8C), and corneas and conjunctivae (Fig. 8D) after desiccation stress. We performed immunohistochemistry for specific antibodies of inflammation in the lacrimal gland (Fig. 8B). In the lacrimal gland, the expression of TNFα was gradually increased by desiccation stress, but it was significantly decreased to the control level by SOS 4. Although the expression of TNFα was markedly suppressed by CsA, its efficacy was lower than that of SOS 4. The expressions of adhesion molecules including ICAM-1 and VCAM-1 were also significantly upregulated by desiccation stress. However, the topical eye drops of SOS 4 and CsA markedly decreased the expression of adhesion molecules, and the efficacy was similar between SOS 4 and CsA. The expression of MMP-2 and MMP-9 also significantly increased to the control level due to desiccation stress, whereas the expression of MMP-2 and MMP-9 was decreased by SOS 4 and CsA. Nevertheless, the DQS and HA topical eye drops did not change the expression of inflammatory markers, except for DQS treatment, which induces a slight inhibition of MMP-9 expression. We performed ELISA for inflammatory factors in...
the tears (Fig. 8C). In the tears, the production of TNF-α increased to 2.1-fold the control level due to desiccation stress. However, the topical eye drops of SOS 4 significantly reduced the production of TNF-α by 46.6% compared with desiccation stress. The production of MMP-2, MMP-9, ICAM-1, and VCAM-1 did not show increased by desiccation stress in the tears (data not shown). In addition, we performed quantitative RT-PCR for mRNA of inflammatory factors in the corneas and conjunctivae (Fig. 8D). In the corneas, the mRNA expression of VCAM-1 increased to 1.5-fold the control level due to desiccation stress. However, the topical eye drops of SOS 4, CsA, DQS, and HA significantly reduced the mRNA expression of VCAM-1 by 39.6%, 19.0%, 26.2%, and 36.9% compared with desiccation stress, respectively, and the efficacy was similar between SOS 4 and HA. The mRNA expression of TNF-α, MMP-2, MMP-9, and ICAM-1 did not show increased by desiccation stress in the corneas (data not shown). In the conjunctivae, the mRNA expression of VCAM-1 increased to 5.3-fold the control level due to desiccation stress. However, the topical eye drops of SOS 4, CsA, and DQS significantly reduced the mRNA expression of VCAM-1 by 63.6%, 12.6%, and 25.6% compared with desiccation stress, respectively. The mRNA expression of TNF-α, MMP-9, and VCAM-1 did not show increased by desiccation stress in the conjunctivae (data not shown).

**DISCUSSION**

DE is a multifactorial disease associated with aqueous tear deficiency, excessive evaporation, and inflammation of the ocular surface related to lacrimal film instability, with potential damage to the corneal epithelium. Currently, the aim of new medications is to affect the various pathogenetic factors involved in the onset of DE, such as tear film instability and inflammation. With this background, we hypothesized that SOS, a glycopeptide derived from mucin, can be used as a new ophthalmic pharmaceutical material for DE through its activity in promoting mucin production and its anti-inflammatory effects.

To date, the application of an artificial tear supplement is the most popular treatment for DE, although it only remedies tear deficiency and lubricates the ocular surface, without focusing on the pathophysiologic fundamentals. Based on the concept, improvement of tear volume (quantity) and quality should be considered the priority for the development of new medications for DE. In this study, we evaluated the effects of SOS on symptoms of DE, including tear production in NOD.B10.H2<sup>−/−</sup> mice, but the tear volume was significantly improved by topical 4% SOS from 3 days after...
treatment (Fig. 1). Indeed, topical 4% SOS eye drops recovered tear production to the baseline levels at 10 days after treatment, which is similar to DQS.

As previously mentioned, tear instability is accompanied by increased tear osmolarity, which leads to damage of corneal epithelium. Therefore, we estimated the degree of the damage to the ocular surface by corneal irregularity score, corneal fluorescein score, and detachment of corneal epithelium. Several studies suggested that the detachment of corneal epithelium is one of the ways of assessing damage of the ocular surface in DE. In the present study, 4% SOS gradually suppressed the detachment of epithelium by desiccation stress, and it was similar to results from the control group and the DQS group (Fig. 4). Furthermore, topical 4% SOS eye drops significantly decreased the corneal irregularity score and fluorescein score to control levels (Figs. 2, 3). Conversely, the CsA and HA topical eye drops did not improve the damage on the ocular surface including corneal irregularity, corneal fluorescein score, and detachment of the corneal epithelium. These results suggest that the benefit of 4% SOS on tear production and corneal stabilization was similar to that of DQS and similar and/or superior to those of CsA and HA.

Observation of the fluorescein-stained tear film indicates that the mucmucous layer of the precorneal film is freshly deposited with each blink and has the physical properties of a gel, due to the presence of the goblet cell mucin. The conjunctival goblet cells are highly specialized epithelial cells present in mucosal tissues of the body. The main function of these cells is to produce and secrete mucins, which hydrate and lubricate mucosal surfaces. Our results show that 4% SOS eye drops significantly decreased the corneal fluorescein score (Fig. 4); thus, we expected an improvement of mucin production by SOS. Therefore, we evaluated whether SOS stimulated conjunctival goblet cells and mucin expression. PAS staining and Alcian blue staining showed that the numbers of goblet cells was markedly increased by 4% SOS, and stained goblet cells are correlated with mucin (Figs. 5, 6). Interestingly, the efficacy of topical 4% SOS on conjunctival goblet cell and mucin expression was better than that of CsA treatment. There are various mucins found in tears, with the major soluble mucin being MUC5AC and the transmembrane mucins being MUC1, MUC4, and MUC16. On the ocular surface, the goblet cells in the conjunctiva synthesize MUC5AC and secrete it into the tear film as a scaffold. MUC1, MUC4, and MUC16 each have a soluble form that has been found in the gel layer of the tear film and assist in tear film stabilization. Although the function of the soluble form of MUC1 found in the tear film is unknown, MUC1 contributes to providing a hydrophilic surface for the tear film to spread evenly and to protect against inflammation through increasing MUC1 mRNA and protein levels. However, there are changes to the concentration of MUC5AC, MUC1, MUC4, and MUC16 in the tears of DE subjects, but not all studies agree on the magnitude or direction of the changes. Nevertheless, decreased MUC5AC secretion and alteration in mucin glycosylation in DE is a consistent finding among most studies. In this study, we evaluated the alterations of MUC5AC, MUC1, MUC4, and MUC16 in conjunctival tissue in the DE condition. Our results show that 4% SOS eye drops gradually increased the expressions of mucin in the inferior fornix conjunctiva (Fig. 7). Interestingly, the efficacy of topical 4% SOS on conjunctival mucin expression was similar to that of CsA treatment,
FIGURE 6. Effect of topical sulglycotide on conjunctival mucins in desiccation stress-mediated DE disease. (A) The sections of conjunctiva were stained with mucin and photographed with a virtual microscope. The conjunctival mucins were stained a deep blue color in the inferior fornix conjunctiva and were measured in the three sections of each eye (n = 4). Scale bar denotes 100 μm. (B) The numbers of stained mucin are indicated as the number per 100 μm. **Bars with different letters are significantly different at P < 0.05 by Tukey’s test. Control, before desiccation stress; DS 10d, immediately after desiccation stress for 10 days; CsA, cyclosporine A; DQS, diquafosol; HA, sodium hyaluronate.

FIGURE 7. Topical sulglycotide enhances the expression of mucin subtypes in desiccation stress-mediated DE disease. (A) Mouse IgG or rabbit IgG was used as a negative control (without primary antibody). (B) The expression of Muc1 (red), Muc4 (red), Muc5AC (green), and Muc16 (red) in the conjunctiva. The stained cells were counterstained with DAPI (blue) and viewed under a fluorescence microscope (Leica DM2500; Leica Microsystems GmbH). Scale bar denotes 50 μm. Control, before desiccation stress; DS 10d, immediately after desiccation stress for 10 days; CsA, cyclosporine A; DQS, diquafosol; HA, sodium hyaluronate.
whereas DQS and HA did not increase the expression of all subtypes of mucin.

Tear instability is accompanied by increased tear osmolarity, which activates stress signaling pathways in the ocular surface epithelium and resident immune cells. Increasing tear osmolarity also triggers an inflammatory response that initiates a vicious cycle that may lead to further decrease in tear function and worsening of symptoms. The lacrimal gland is richly supplied by immune cells that occupy the interstitial space. Our previous studies suggested that desiccation stress upregulated the expression of inflammatory markers including TNF-α, ICAM-1, VCAM-1, MMP-2, and MMP-9 in the lacrimal gland. Likewise, the present study shows the desiccation stress-mediated overexpression of inflammatory factors such as cytokine, adhesion molecules, and MMPs, whereas these factors were markedly decreased by topical SOS treatment in a dose-dependent manner (Fig. 8). The efficacy of 4% SOS eye drops on inflammation was similar to and/or superior to that of CsA.

Overall, our results showed that the administration of topical 4% SOS, a glycopeptide derived from porcine duodenal mucin, affects the various pathogenic factors involved in the onset of DE, including tear production, epithelium stabilization, goblet cell mucin, and inflammation. The benefit of topical 4% SOS was more similar to DQS with regard to tear production and stabilization of the ocular surface. Furthermore, 4% SOS eye drops enhanced the population of goblet cells and secretion of mucins in the conjunctiva, and its efficacy was similar to CsA. The efficacy of 4% SOS eye drops on inflammation in the lacrimal gland was similar to and/or superior to that of CsA. Consequently, we suggested that administration of topical 4% SOS lead to tear stability by mucin increasing and anti-inflammatory effects and can be used as a new ophthalmic pharmaceutical formulation to treat DE. The limitation of our study is that we observed the efficacy of SOS
for a short-term administration of 10 days in the DE mouse model, and the efficacy for long-term administration of SOS should proceed to the next study. In addition, our study is a preclinical study using a DE mouse model, and the efficacy of SOS revealed in our study should be accurate clinical study.

Acknowledgments

The authors thank Kyuhong Lee (National Center for Efficacy Evaluation of Respiratory Disease Product, Korea Institute of Toxicology, Republic of Korea) for providing support for the ELISA test measurement of tear samples.

Supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant HI15C1142).

Disclosure: H. Lee, None; S. Jeon, None; C.E. Kim, None; Y.-J. Park, None; J. Yang, None

References


