The Role of 2% Rebamipide Eye Drops Related to Conjunctival Differentiation in Superoxide Dismutase-1 (Sod1) Knockout Mice

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PURPOSE. The superoxide dismutase-1 knockout (Sod1−/−) mouse is an age-related dry eye mouse model. We evaluated the role of 2% rebamipide opthalmic solution on the conjunctiva and ocular surface alterations in Sod1−/− mice.

METHODS. Rebamipide eye drops (2%) were instilled in six 50-week-old male Sod1−/− mice and six C57BL/6 strain wild-type (WT) male mice four times a day for 2 weeks. Aqueous tear secretion quantity and tear film breakup time measurements as well as vital stainings were performed. Immunohistochemistry staining of the conjunctiva was performed using SAM pointed domain-containing ETS transcription factor (SPDEF), transglutaminase-1, and involucrin antibodies. Quantitative RT-PCR was carried out to study mRNA expression of the same markers.

RESULTS. The mean tear quantities showed no significant changes in both mice strains after treatment (P = 0.24). The mean tear film breakup time (P = 0.003) and vital staining scores significantly improved in the Sod1−/− mice after treatment. Treatment with 2% rebamipide eye drops significantly decreased the corneal fluorescein (P = 0.0093) and Rose Bengal (P = 0.002) staining scores in the Sod1−/− mice. We showed a notable increase in SPDEF and a marked decrease in transglutaminase-1 and involucrin immunohistochemistry stainings, together with a significant increase in SPDEF (P = 0.0003) and a significant decline in transglutaminase-1 (P = 0.0072) and involucrin (P = 0.0009) mRNA expression after treatment in the Sod1−/− mice.

CONCLUSIONS. Topical use of 2% rebamipide drops was observed to improve conjunctival epithelial differentiation and suppress keratinization in the Sod1−/− mice.

Keywords: dry eye, keratinization, conjunctiva, rebamipide, secretagogue

Dry eye is a multifactorial disease due to tear film instability associated with symptoms and/or visual impairment and keratoconjunctival epithelial damage. It has been reported that tear instability is a core mechanism in dry eye causing symptoms and/or visual impairment.1

Dry eye disease (DED) affects between 7.8% and 14.6% of the United States population.2,5 According to the Women’s Health Study and other studies, approximately 3.23 million women and 1.68 million men older than 50 years have DED in the United States.4,5 Based on a large population study of DED in Japan, the prevalence of clinical dry eye diagnosis was 2.1% in men and 7.9% in women.6

Recently, the Japanese Dry Eye Research Society proposed a new dry eye treatment strategy called “tear film oriented therapy.”1 According to this concept, when an abnormality occurs in a tear film layer, the tear film becomes unstable, which can cause DED. According to the tear film oriented therapy guidelines, if a patient has mucin deficiency, first the mucin secretion should be increased, and eye drops containing diquafosol sodium or rebamipide, which increases mucin secretion, should be prescribed.7

Rebamipide is a mucin secretagogue that was first released for gastric ulcer therapy in 1990 (Otsuka Pharmaceutical Co, Ltd., Tokyo, Japan).8-10 Rebamipide stimulates gastric endogenous prostaglandin E2 and I2 production and inhibits mucosal injury10,11 through its antioxidant properties and through suppression of myeloperoxidase activity.12-15 Rebamipide has also been shown to be effective in healing of wounds and cytoprotection.8 Rebamipide is an amino acid analogue of 2(1H)-quinolone, which can also induce mucin secretion and has been available as an eye drop for dry eyes since 2012 in Japan. Topical rebamipide reportedly reduces UV-B-induced corneal oxidative damage and surface irregularity in mice16 and improves the ocular surface by inducing mucin-like secretion from the rat keratoconjunctival epithelium.17

The conjunctival epithelium is a nonkeratinized, stratified, squamous epithelium that also harbors mucin-secreting goblet cells.17-19 Goblet cell loss has been reported to be associated with many types of dry eyes, especially in keratoconjunctivitis sicca.20,21

SAM pointed domain-containing ETS transcription factor (SPDEF) is a member of the ETS family of transcription factors...
that is expressed in epithelial cells in various tissues and has suggested roles in cell differentiation. SPDEF provides the differentiation of goblet cells in the conjunctival epithelium of mice. In this context, SPDEF may also have an effect on conjunctival goblet cell differentiation and pathogenesis of dry eyes in humans. On the other hand, involucrin is a soluble, highly reactive, structural protein that is specifically expressed in epidermal keratinocytes and other stratified squamous epithelia. Transglutaminases crosslink involucrin to cell membrane proteins forming an insoluble envelope. Both involucrin and transglutaminase-1 are known markers of keratinization that are upregulated in the keratoconjunctival epithelium with desiccating stress. Sod1 mice have been reported to develop dry eye associated with a decline in muc5AC and SPDEF mRNA expression in the conjunctiva. The 2007 International Dry Eye Workshop report recommends use of tear and mucin stimulants to maintain the ocular surface health and epithelial barrier-related functions and inhibition of inflammation in addition to use of simple lubricants.

The purpose of this work was to study the effect of 2% rebamipide eye drops on the conjunctival epithelial status by analyzing the changes in SPDEF and keratinization markers including transglutaminase-1 (TGase-1) and involucrin in Sod1 and wild-type (WT) mice.

**Materials and Methods**

**Animals**

Twelve eyes of six Sod1 male mice with C57BL/6 background and 12 eyes of six C57BL6 strain WT male mice were examined at 50 and 52 weeks. We determined the sample size according to a previous report using software G Power by Faul et al. We adjusted the power value as 80% and Type 1 error as 0.05 in our study. The mice underwent a 2-week 2% rebamipide instillation four times a day. All mice underwent examinations before and 2 weeks after rebamipide instillations.

All experiments followed the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol using animals was approved by the ethical committee at the laboratory animal center at Keio University School of Medicine (Approval No. 080067-7).

**Tear Quantity Measurement**

Phenol red test was used to measure tear quantity (Zone-Quick; Showa Yakuhin Kako Co., Ltd., Tokyo, Japan). The cotton threads were applied to the lateral canthus for 60 seconds and the wetting length was measured in millimeters.

**Ocular Surface Epithelial Damage and Tear Stability Assessment**

We used the tear film breakup time (BUT) test to assess tear film stability. The SL-15 (Kowa, Tokyo, Japan) portable slit-lamp microscope was used to evaluate the tear BUT. First, 1 μL 2% fluorescein solution was instilled. Then, one researcher applied an air puff with a 1-mL syringe to induce a blink, and another researcher observed the tear BUT with the handheld slit lamp after the blink response. After a natural blink, the BUT was assessed thrice and the mean of these measurements was then calculated. Corneal epithelial cell damage was evaluated 2 minutes after fluorescein dye application. The staining in the upper, middle, and lower corneal zones was then allocated a staining score ranking between 0 and 3 points (total score: min: 0 points, max: 9 points). Distilled water (2 μL) was used for corneal wash before the same procedure was applied for 1% Rose Bengal dye. Vital stainings before and 2 weeks following rebamipide application were recorded through photographs using the same camera with the same settings for each mouse.

**Conjunctival Specimen Collection and Goblet Cell Quantification**

Mice were killed by cervical neck dislocation following sedation using 6 mg/mL ketamine and 4 mg/mL xylazine before and after 2% rebamipide instillation. The tissue samples were obtained from different mice before treatment at 50 weeks and after treatment at 52 weeks. Twelve eyes were enucleated, including eyelids and palpebral conjunctiva. After enucleation, the conjunctiva including subconjunctival connective tissue was trimmed from the eyeball. Then, for real-time RT-PCR experiments, tissues were immediately submerged into RNAlater (Applied Biosystems, Carlsbad, CA, USA) and stored in a –80°C freezer. For immunohistochemical analysis, tissue was submerged in transparent racks containing optimum cutting temperature (OCT) compound and frozen with liquid nitrogen, then stored in a –80°C freezer. Paraaffin-embedded conjunctival tissue was also reserved for goblet cell quantification with periodic acid-Schiff (PAS) staining. Briefly, after deparaffinization, slides were immersed in PAS solution for 5 minutes, rinsed in three changes of distilled water, and immersed in Schiff solution for 15 minutes. The slides were rinsed with distilled water and underwent hematoxylin staining for 1 minute. The slides were dehydrated with ascending grades of alcohol and mounted with resin and finally coverslipped. The quantitative studies of goblet cells were performed by taking photographs under a calibrated grid using a light microscope. Three nonoverlapping areas of each sample were photographed and the outcomes were averaged for a final mean specimen score.

** Immunohistochemistry Staining for Conjunctival Epithelial Differentiation**

To assess the epithelial alterations of the conjunctiva in the 50- and 52-week-old mice, SPDEF, transglutaminase-1 (TGase-1), and involucrin immunohistochemistry staining was used. Five-micrometer-thickness conjunctival cryosections were collected over glass slides. The cut specimens were dried for 10 minutes and washed with distilled water. Tissues were fixed for 10 minutes in a 4% buffered paraformaldehyde solution and rewarshed with distilled water. Endogenous peroxidase activity was blocked using 3% H2O2 in methanol for 30 minutes at 25°C. Tissue sections were incubated with normal rabbit serum ( Vectastain Elite IgG ABC kit; Vector Laboratories, Burlingame, CA, USA) for 2 hours at 25°C to block background staining. First, mouse anti-SPDEF polyclonal antibody diluted with goat blocking serum (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied to the tissues. This SPDEF antibody is a purified goat polyclonal antibody. The anti-TGase-1 and anti-involucrin monoclonal antibodies with rabbit blocking serum (dilution 1:100; Santa Cruz Biotechnology) were applied overnight at 4°C. For the SPDEF negative controls, the primary antibody was replaced with goat IgG isotype at the same concentration of the primary antibody. For the TGase-1 and involucrin negative controls, the primary antibody was replaced with rabbit IgG isotype control. PAS staining was used for the study of goblet cells. The sections were studied and digitally photographed with an Axiosplan2 Imaging microscope (Carl Zeiss, Jena, Germany). Five non-overlapping images from both mice strains were randomly selected and used for conjunctival staining density.
Assessment of SPDEF, TGase-1, and Involucrin mRNA Expression by Quantitative Real-Time PCR

Quantitative real-time PCR was performed according to a previously described protocol.\textsuperscript{19} Real-time data were acquired and analyzed using Sequence Detection System Software (Applied Biosystems) with manual adjustment of the baseline and threshold parameters. The levels of mRNA expression were normalized by the median expression of a housekeeping gene (\textit{GAPDH}). The primer sequences included GAPDH (sense 5\textsuperscript{'}– TGA CGT GCC TGG AGA AA – 3\textsuperscript{'}, antisense, 3\textsuperscript{'}– AGT GTA GCC CAA GAT GCC CTT CAG – 5\textsuperscript{'}); SPDEF (sense 5\textsuperscript{'}– TTG GAT GAG CTC TCG CTA GA – 3\textsuperscript{'}, antisense, 3\textsuperscript{'}– TTG GAT GAG GCT CTC TCG AAG AA – 5\textsuperscript{'}); TGase-1 (sense 5\textsuperscript{'}– TCA GAT GCT GGA GGT GAC AG – 3\textsuperscript{'}, antisense, 3\textsuperscript{'}– CTC GTG TGC CTA CTC AA – 5\textsuperscript{'}); involucrin (sense 5\textsuperscript{'}– TAA GGT CCT CAG CTG GCC TA – 3\textsuperscript{'}, antisense, 3\textsuperscript{'}– GTT GAA GGA CTC CGT CC – 5\textsuperscript{'}).

Statistical Analysis

GraphPad software (InStat, San Diego, CA, USA) was used for studying the data. The Wilcoxon signed rank test and Mann-Whitney \textit{U} tests were applied for statistical analyses. A \textit{P} value less than 0.05 was considered statistically significant. Five nonoverlapping images from both mice strains were randomly selected and used for conjunctival staining density. The statistician was masked to any information about the mice.

RESULTS

Aqueous Tear Secretion Quantity Alterations

The mean tear quantities showed no significant changes in both mice strains with 2 weeks of 2% rebamipide instillation (\textit{P} > 0.05) (Fig. 1).

Tear Function Changes

The mean BUT of 2.75 ± 0.75 seconds before eye drop instillation increased significantly to 4.50 ± 1.45 seconds after 2 weeks of 2% rebamipide instillation in the \textit{Sod1}\textsuperscript{+/−} mice (\textit{P} < 0.01). Tear film stability showed no significant change in the WT mice (Fig. 2).

Vital Staining Alterations

The mean fluorescein staining score decreased with significance from 5.0 ± 2.1 to 2.5 ± 1.7 points after 2 weeks of 2% rebamipide instillation in the \textit{Sod1}\textsuperscript{+/−} mice (\textit{P} = 0.003). No significant score changes were observed in the WT mice (Fig. 3).

FIGURE 1. Aqueous tear production assessment. The mean aqueous tear production quantity did not reveal a significant difference in both mice strains after 2 weeks of 2% rebamipide instillation (\textit{P} > 0.05).

FIGURE 2. Tear film stability assessment. The mean tear breakup time values increased significantly with 2% topical rebamipide instillation in the \textit{Sod1}\textsuperscript{+/−} mice (\textit{P} < 0.01). Tear film stability showed no significant change in the WT mice.

FIGURE 3. Timewise change of vital staining scores before and after rebamipide instillation. (A) Alterations of fluorescein staining scores with 2% rebamipide instillation in the \textit{Sod1}\textsuperscript{+/−} and WT mice. Note the significant decrease in the fluorescein scores in the \textit{Sod1}\textsuperscript{+/−} mice with 2 weeks of rebamipide treatment. *\textit{P} < 0.05. Fluorescein staining scores showed no difference in the WT mice. (B) Alteration of Rose Bengal scores with 2% topical rebamipide instillation in the \textit{Sod1}\textsuperscript{+/−} and WT mice. Note significant decrease in the Rose Bengal scores in the \textit{Sod1}\textsuperscript{+/−} mice with 2 weeks of rebamipide treatment. *\textit{P} < 0.05. Rose Bengal staining scores showed no difference in the WT mice.
rebamipide eye drops in the Sod1−/− mice (P = 0.0093). The scores did not show significant changes in the WT mice (P = 0.5) (Fig. 3A). The mean Rose Bengal score increased significantly from 4.5 ± 2.0 to 1.8 ± 1.8 points following rebamipide eye drops in the Sod1−/− mice (P = 0.002). These scores showed no significant changes in the WT mice (Fig. 3B) after instillation (P = 0.15).

**PAS Staining for Goblet Cells**

PAS staining for goblet cells showed a significant increase in goblet cell density from 500 ± 50 to 2652 ± 250 cells/mm² after rebamipide treatment in the Sod1−/− mice. Figure 4 shows representative conjunctival PAS staining in the Sod1−/− mice before and after rebamipide treatment. Markedly increased goblet cells were observed after rebamipide treatment.

**Immunohistochemistry Staining Intensity Changes**

SPDEF expression was noted in the superficial conjunctival epithelial layers and in the goblet cells. The conjunctival staining intensity for SPDEF appeared to increase in the superficial layer of conjunctiva epithelium following rebamipide instillations in both mice strains (Fig. 5). In contrast, the conjunctival staining intensity for TGase-1 and involucrin appeared to decrease for both markers in all layers of conjunctiva following rebamipide instillations in both groups of mice (Figs. 6A, 6B).

**Quantification of SPDEF, TGase-1, and Involucrin mRNA Expression Level**

Assessment of mRNA expression for SPDEF in the conjunctiva revealed a significant increase following 2% rebamipide eye drops in the Sod1−/− mice (Fig. 7A) (P = 0.0003). SPDEF mRNA levels did not show any significant differences before and 2 weeks after rebamipide eye drop treatment in the WT mice (P = 0.8). The mRNA expression level of TGase-1 decreased significantly in both mice strains with 2 weeks of rebamipide eye drop use (Fig. 7B) (P = 0.0072 and P = 0.0357). Additionally, mRNA expression of involucrin decreased significantly in the Sod1−/− mice after 2 weeks of rebamipide eye drop use (Fig. 7C) (P = 0.009). Conjunctival involucrin mRNA expression values showed no statistically significant difference in the WT mice after 2 weeks of rebamipide eye drop application (P = 0.78). Likewise, conjunctival TGase-1 and involucrin mRNA expression values showed no statistically significant difference between the 50-week Sod1−/− and WT mice before rebamipide eye drop application (P = 0.53).

**DISCUSSION**

Oxidative stress results from perturbation of the balance between free radicals and radical cleaning systems, which may eventually lead to cellular damage. The superoxide dismutase
The (SOD) family containing SOD1, SOD2, and SOD3 enzymes are responsible for the removal of reactive oxygen species (ROS) from cells. The accumulation of ROS in ocular tissues may lead to DED. A previous report by us showed that Sod1 knockout caused increased oxidative lipid and DNA damage, increased tissue inflammation, changes in the lacrimal gland phenotype, and deterioration of glandular secretory functions.

Currently, there are few animal models in which the link between oxidative stress and DED can be studied. However, the Sod1 knockout mouse model has recently become popular for evaluating the role of oxidative stress, related tissue damage, age-related tissue inflammation, epithelial–mesenchymal transition, and aging-induced DED. Additionally, the Sod1 mouse is a good model for studying potential treatment modalities for dry eyes. We previously demonstrated that the lacrimal glands of Sod1−/− mice harbored oxidative stress–induced tissue damage and inflammatory alterations with a decline in aqueous secretions. The present study is the first to evaluate the role of 2% rebamipide eye drops in the conjunctiva in the Sod1−/− mouse model.

In the current study, we observed a significant shortening of tear BUT and an increase in vital staining scores in 50-week-old Sod1−/− mice in comparison to WT mice. These observations were consistent with the findings of our prior research. We demonstrated that tear BUT, vital staining scores, and conjunctival staining intensity for SPDEF revealed a significant improvement in the Sod1−/− mice after 2 weeks of 2% rebamipide eye drop instillations. We presume that normalization of tear stability might have resulted in part from an increase of SPDEF mRNA expression on the conjunctival glands of Sod1−/− mice harbored oxidative stress–induced tissue damage and inflammatory alterations with a decline in aqueous secretions. The present study is the first to evaluate the role of 2% rebamipide eye drops in the conjunctiva in the Sod1−/− mouse model.

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epithelium that might have had a positive influence on goblet cell density or increase in mucin expression.22

Indeed, the goblet cell numbers increased significantly with rebamipide treatment in this study. The presence of goblet cells has been considered one of the criteria to use to describe conjunctival differentiation and a healthy ocular surface.20 A previous study showed that 2% rebamipide treatment increased both the goblet cell density and Muc5AC mRNA expression in Sod1-/- mice with a concomitant decrease in tear TNF-α and INF-γ levels.29 Although mRNA expression of Muc5AC was not studied in the current investigation, future studies looking into the relation between conjunctival SPDEF and Muc5AC expression with rebamipide treatment would provide invaluable information. Solomon et al.31 recently reported a significant increase in INF-γ and IL-12 concentrations in conjunctival culture supernatants from SPDEF KO mice. While tear levels of these cytokines, with suggested roles in epithelial differentiation, were not looked at in this study and warrant investigation, future studies linking these with goblet cell loss and SPDEF expression changes in the SOD-1 KO mice would be very interesting.

A reduction in goblet cell density has a role in Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and Sjögren syndrome associated dry eye.2 Previous studies have identified that SPDEF expression is necessary for goblet cell differentiation and maturation in the respiratory and intestinal epithelium.32 Recently, Marko et al.22 reported that loss of SPDEF induced goblet cell dropout in human DED. Kojima et al.33 demonstrated that decline in goblet cell density and expression of MUC5AC with an increase in conjunctival inflammation in Sod1-/- mice could be responsible for the perturbation of tear film and conjunctival epithelial functions. In our study, conjunctival staining of SPDEF became more intense and the mRNA expression of SPDEF increased significantly following 2 weeks of rebamipide drop instillation in the Sod1-/- mouse. It should be noted that localization of SPDEF staining in the current report is different from that in a previous report by Marko et al.,34 where SPDEF staining was mainly located in the apical conjunctival epithelium and the epithelial nuclei. Marko et al.35 reported SPDEF proteins to be localized in the human conjunctival epithelium in goblet cells using immunofluorescence microscopy, suggesting a regulatory role for SPDEF in goblet cell differentiation.54 Park et al.36 and Chen et al.22 described similar roles for SPDEF in goblet cell differentiation in the airway epithelium and showed SPDEF staining not only in goblet cells but also in the nuclei of airway epithelium and the apical cells. The differences in SPDEF staining localization warrant further investigation.

TGase-1 is a representative marker of keratinization and an enzyme responsible for keratinocyte terminal differentiation to form the highly insoluble keratins in cornified cells.30 Nishida et al.37 studied immunohistochemical data to assess the pathologic keratinization of keratinized conjunctival epithelium and demonstrated a role for TGase-1 gene expression and cellular keratinization in severe Stevens-Johnson syndrome. They reported TGase-1 upregulation in pathologic keratoconjunctival epithelium, with no expression in the normal conjunctival epithelium. In an earlier report, Nakamura et al.38 showed a correlation between the overexpression of TGase-1 and involucrin in DED, where they reported an upregulation of both TGase-1 and involucrin pathologic keratinization of the ocular surface. Our study looked into histopathologic assessment and real-time RT-PCR for TGase-1 and involucrin to evaluate keratinization in the conjunctiva. Our results showed a marked decrease of TGase-1 and involucrin staining intensity and a significant decrease in TGase-1 and involucrin mRNA expression after 2 weeks of rebamipide application in Sod1-/- mice. The current report revealed an improvement of keratinization in conjunctiva using rebamipide eye drops in the Sod1 knockout mouse for the first time in the literature based on PubMed and Medline database research using the keywords dry eye, rebamipide and tear stability.

In conclusion, instillation of 2% rebamipide eye drops for 2 weeks improved the tear film BUT, ocular surface, epithelial health status, and differentiation with a decrease in keratinization in this age-related dry eye mouse model.

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