

# The Effects of 2% Rebamipide Ophthalmic Solution on the Tear Functions and Ocular Surface of the Superoxide Dismutase-1 (*Sod1*) Knockout Mice

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**PURPOSE.** To investigate the efficacy of 2% rebamipide ophthalmic solution on the tear functions and ocular surface status of the superoxide dismutase-1 (*Sod1*<sup>-/-</sup>) mice.

**METHODS.** Two percent Rebamipide ophthalmic solution was applied to 40-week-old male *Sod1*<sup>-/-</sup> and wild-type (WT) mice four times a day for 2 weeks. We examined the cytokine concentrations in the tear fluid (by CytoBead assay), tear film break-up time, amount of tear production, and expressions of mucins 1, 4, and 5AC, by RT-PCR. We also performed vital staining of the ocular surface, PAS staining for muc5AC, and immunohistochemical stainings for 4-hydroxy-2-nonenal (4-HNE), 8-hydroxy-2'-deoxyguanosine (8-OHdG), in the conjunctiva to compare the results before and after rebamipide instillations.

**RESULTS.** The tear functions and ocular surface epithelial damage scores were significantly worse in the *Sod1*<sup>-/-</sup> than in the WT mice. Application of 2% rebamipide for 2 weeks significantly improved the tear film break-up time, the amount of tear production, and the corneal epithelial damage scores, which also significantly increased the conjunctival goblet cell density and muc5 mRNA expression, in the *Sod1*<sup>-/-</sup> mice. The mean IL-6, IL-17, TNF- $\alpha$ , and IFN- $\gamma$  levels in the tear fluid were reduced significantly along with a significant decrease in the density of cells positive for 4-HNE and 8-OHdG in the conjunctiva.

**CONCLUSIONS.** Two percent Rebamipide ophthalmic solution significantly improved the tear stability and corneal epithelial damage, and enhanced the expression of muc5 mRNA on the ocular surface. We also observed anti-inflammatory effects in the tear film together with antioxidative effects in the conjunctiva, suggesting the efficacy of rebamipide in age-related dry eye disease attributable to SOD1 knockout.

**Keywords:** dry eyes, inflammation, secretagogue

Dry eye is a multifactorial disease of the tears and the ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. Increased osmolarity of the tear film and inflammation in the lacrimal system and ocular surface are important operational factors in the genesis of some dry eye disorders.<sup>1</sup> It also has been reported that aging and oxidant stress are among the other causes of dry eye disease (DED).<sup>2</sup>

DED is encountered in many parts of the world. The largest epidemiological studies of dry eye to date, namely the Women's Health Study,<sup>3</sup> the Physicians Health Study,<sup>3</sup> and others (Miljanovic B, et al. *IOVS* 2007;48:ARVO E-Abstract 4293),<sup>4-13</sup> estimated that in the United States, approximately 3.23 million females and 1.68 million males 50 years or older have DED (Miljanovic B, et al. *IOVS* 2007;48:ARVO E-Abstract 4293 and Ref. 3).

In relation to the treatment of DED, the 2007 International Dry Eye Workshop (DEWS) report suggested selecting treat-

ments from a menu of therapies for which evidence of therapeutic efficacy has been presented.<sup>14</sup> The menu contained treatment options, including artificial tear substitutes, gels/ointments, moisture chamber spectacles, anti-inflammatory agents (topical cyclosporine A, corticosteroids, and omega-3 fatty acids), tetracyclines, plugs, tear and mucin secretagogues, serum, contact lenses, systemic immunosuppressives, and surgical alternatives.<sup>14</sup>

Among these options, OPC-12759, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4yl]-propionic acid, known as rebamipide (Mucosta; and classified in the DEWS report as a mucin secretagogue) has long been used as an antigestric ulcer drug.<sup>15</sup> OPC-12759 stimulates the synthesis of prostaglandin E2 to increase gastric mucus glycoprotein secretion,<sup>15</sup> stimulates migration and proliferation of wounded epithelial cell monolayers,<sup>16,17</sup> increases expression of epidermal growth factor (EGF) and its receptor, and scavenges active oxygen radicals.<sup>18-21</sup> All these actions have been suggested to contribute to

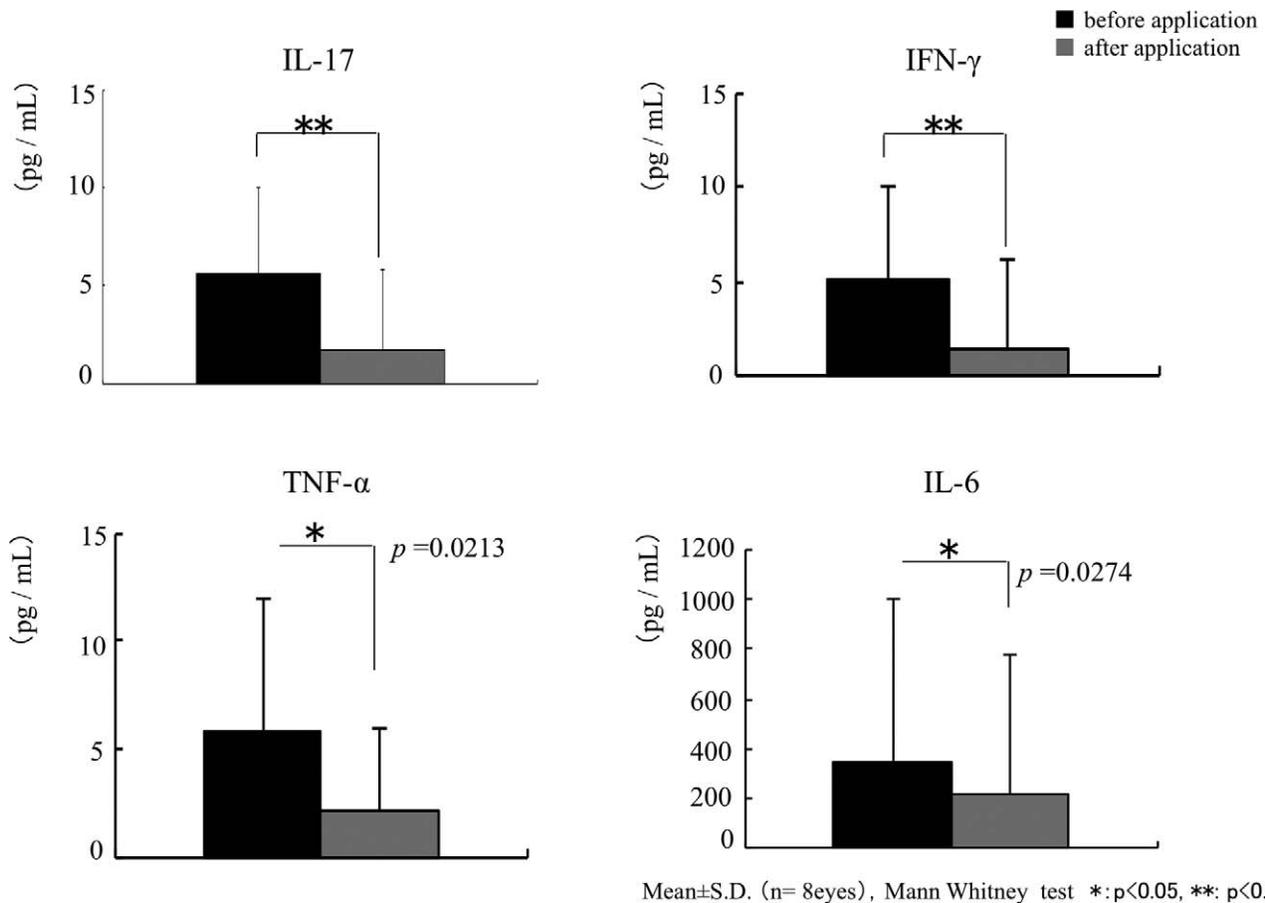


FIGURE 1. Tear fluid cytokine changes with 2% rebamipide eye drop application. Note the significant decreases in the mean concentrations of IL-17, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  after 2 weeks of rebamipide instillation.

the healing of gastric ulcers. Recent studies in several experimental animal models have demonstrated that topical application of OPC-12759 improved the ocular surface by increasing the amount of mucin-like substances in the cornea and conjunctiva,<sup>22</sup> and by attenuating the UV-B-induced oxidative damage to the cornea.<sup>23</sup> We previously reported that the lack of SOD1 led to increased oxidative lipid and DNA damage, and increased CD4<sup>+</sup> T-cell inflammation and epithelial mesenchymal transition (EMT) in the lacrimal glands of the SOD-1 knockout mouse (*Sod1*<sup>-/-</sup>) model, with perturbation of glandular secretory functions, which resulted in dry eyes and translated into an ocular surface disease.<sup>24</sup> We had then suggested that the *Sod1*<sup>-/-</sup> mice may be a good model system in which to study the mechanism of reactive oxygen species-mediated lacrimal gland alterations and age related dry eye disease.<sup>24</sup>

In this study, we examined the effect of 2% rebamipide ophthalmic solution on the tear functions, ocular surface status, inflammatory tear film cytokines, muc5AC expression, and oxidative stress markers in the conjunctiva of the *Sod1*<sup>-/-</sup> mice.

## MATERIALS AND METHODS

### Animals

Eight eyes of four *Sod1*<sup>-/-</sup> male mice with C57BL/background and eight eyes of four C57BL6 strain wild-type (WT) male mice

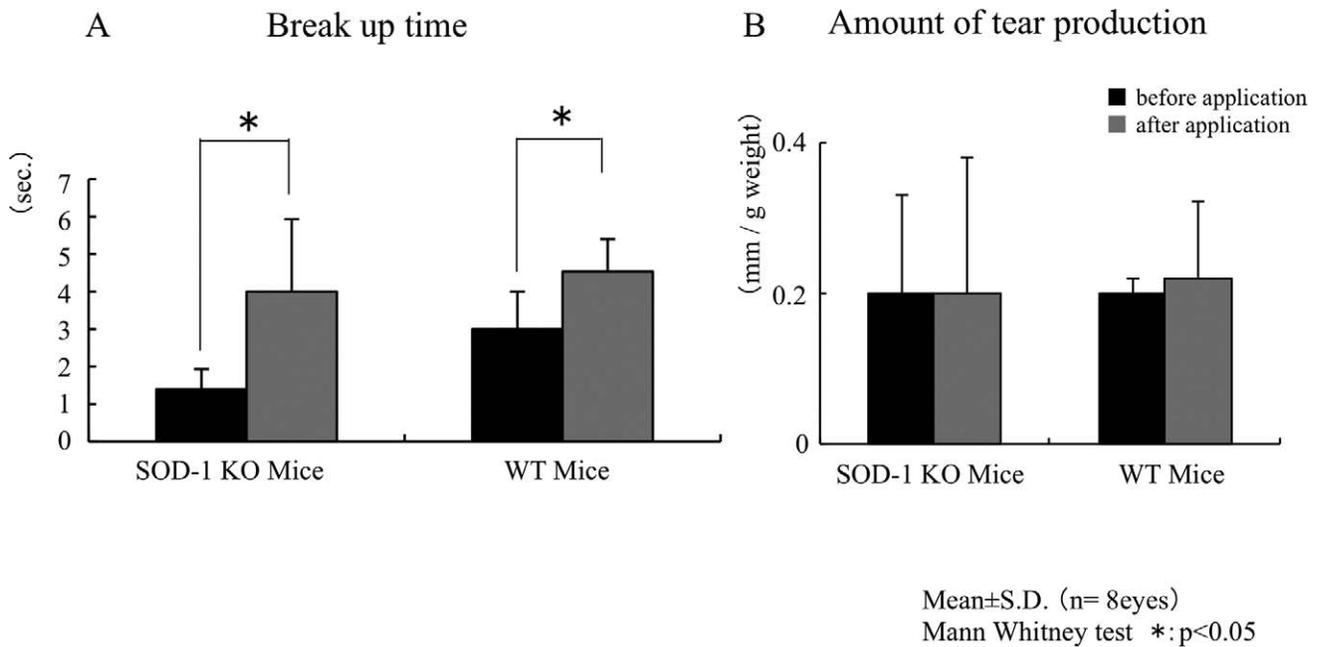
were examined at 40 weeks in this study. The *Sod1*<sup>-/-</sup> mice were received from the Chiba University Graduate School of Medicine and the WT C57BL/6 mice were purchased from Japan Clea (Osaka, Japan). *Sod1*<sup>-/-</sup> mice were backcrossed to *Sod2*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> for two generations to obtain the *Sod1*<sup>-/-</sup>, *Sod2*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>. All mice had application of 2% rebamipide ophthalmic solution four times a day for 2 weeks. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

### Tear Fluid Collections

An aliquot of 10  $\mu$ L of 0.1M PBS was introduced onto the ocular surface by a micropipette and then was collected with a 10- $\mu$ L glass capillary tube (Hirschmann Laborgerate GmbH & Co., Eberstadt, Germany) by capillary action from the tear meniscus in the lateral canthus. Collected tears were stored at -80°C until tear cytokine concentration assessments.

### Cytometric Bead Array for Assessment of Inflammatory Cytokines in Tears

The Becton Dickinson Cytometric Bead Array system was used to investigate the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analyses in particle-based immunoassay. Each bead provides a capture surface for a specific protein and is analogous to an individual coated well in an ELISA plate. The testing allows the detection of multiple



**FIGURE 2.** Tear function changes with 2% rebamipide eye drop application. (A) Note the significant improvement of the tear film breakup time in the SOD-1 knockout and WT mice after 2 weeks of rebamipide application. (B) Tear quantity showed no significant change in the SOD-1 knockout and WT mice after 2 weeks of rebamipide application

analyses in a small volume sample. We quantitatively measured IL-6, IL-10, IL-17, monocyte chemoattractant protein-1 (MCP-1), INF- $\gamma$ , TNF, and IL-12p70 protein levels in tears and serum samples using the Mouse Inflammation Kit (BD Bioscience, Franklin Lakes, NJ).

After reconstituting the mouse inflammation standards, the cytokine standard mixture (20  $\mu$ L) and the tear and serum (20  $\mu$ L) samples were diluted with 30  $\mu$ L of the assay diluent. Each standard and samples were added to a mixture of 50  $\mu$ L capture Ab-bead reagent and detector Ab-PE reagent. The mixture (150  $\mu$ L) was subsequently incubated for 2 hours at room temperature, and washed with 1 mL wash buffer (from the kit) to remove unbound detector Ab-PE reagent. After washing, the samples and standards were centrifuged at 200g for 5 minutes and then the supernatant was carefully removed. The bead pellet was resuspended with 300  $\mu$ L wash buffer before data acquisition using flow cytometry.

Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were acquired and analyzed using the Becton Dickinson (BD) Cytometric Bead Array software (BD Bioscience, Franklin Lakes, NJ).

### Ocular Surface Epithelial Damage Assessment

Corneal fluorescein staining was evaluated with slit lamp biomicroscopy using cobalt blue light after instillation of 2  $\mu$ L 0.5% sodium fluorescein. Excess of fluorescein was wiped from the lateral tear meniscus. Tear breakup time was recorded after a natural blink with a handheld slit lamp biomicroscope (Kowa, Nagoya, Japan). Punctuate staining was recorded using a grading system of 0 to 3 points for superior, central, and inferior corneal areas. The fluorescein staining scores ranged from a minimum of 0 to a maximum of 9 points. The corneas were then stained with 1% Rose Bengal solution. Punctuate staining was recorded using a grading system of 0 to 3 points for superior, central, and inferior corneal areas. The Rose

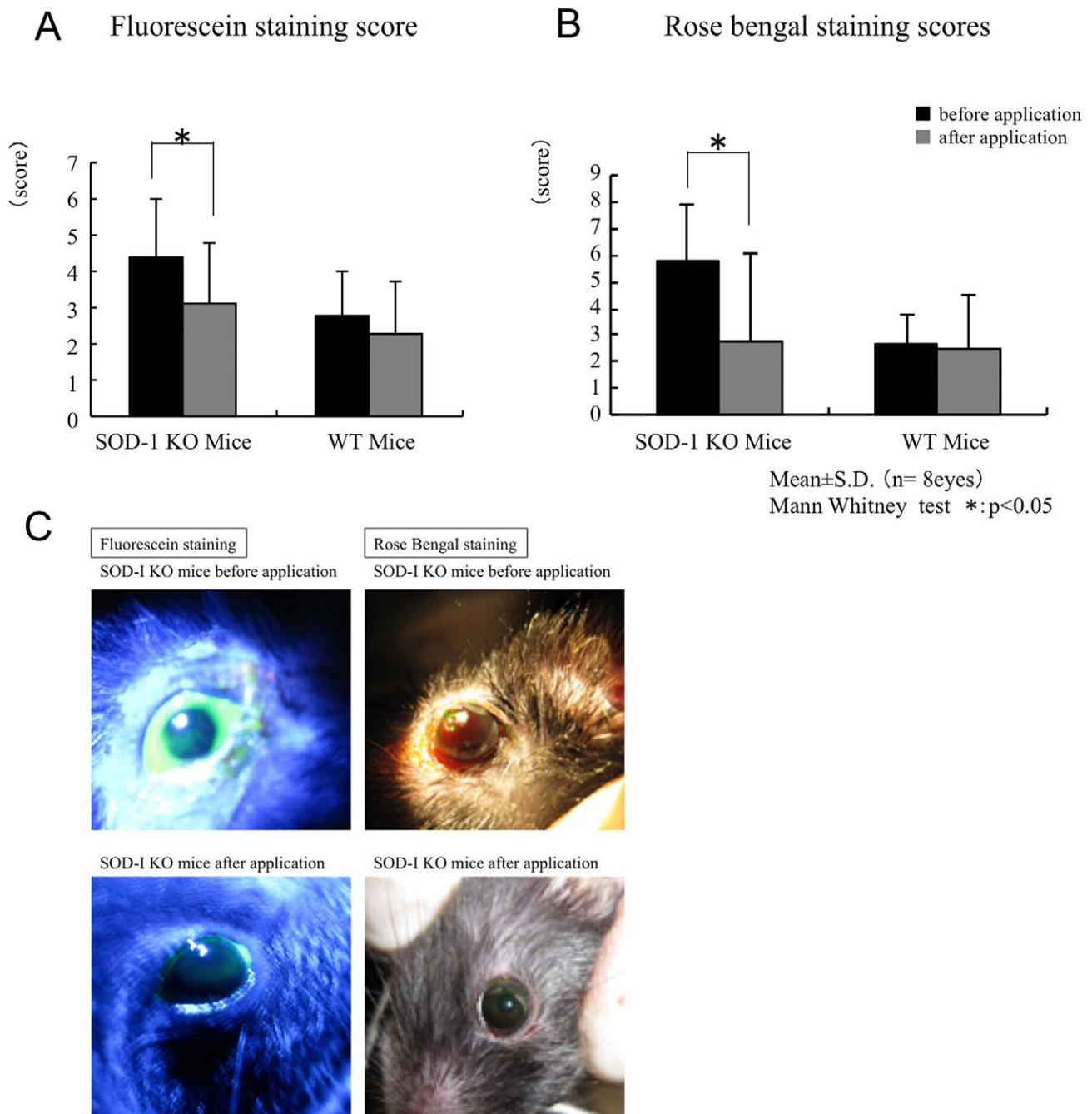
Bengal staining scores ranged from a minimum of 0 to maximum of 9 points.

### Conjunctival Specimen Collections and Histopathological Assessment of Specimens

Animals were anesthetized intraperitoneally and were killed using a combination of 6 mg/mL ketamine and 4 mg/mL xylazine. The whole globes were rapidly removed after animals were killed. Samples were immediately fixed in 4% buffered paraformaldehyde, embedded in paraffin wax, sliced in 4- $\mu$ m-thick paraffin sections, and processed according to conventional histologic techniques for Periodic Acid Schiff (PAS) stainings. The goblet cell densities before and after rebamipide instillations were also calculated.

### Immunohistochemistry Staining for Oxidative Stress Markers

Oxidative stress-induced lipid peroxidation was assessed by immunohistochemical detection of 4-hydroxy-2-nonenal (4-HNE). Oxidative DNA damage was investigated by immunohistochemical staining with anti 8-hydroxy-2'-deoxyguanosine (8-OHdG) antibodies. The avidin-biotin-peroxidase complex method was used in immunostainings. Tissues were fixed overnight in a 4% buffered paraformaldehyde solution and processed for paraffin embedding; 4- $\mu$ m sections were cut from paraffin wax blocks, mounted on precoated glass slides, deparaffinized, and rehydrated. To block nonspecific background staining, lacrimal gland sections were treated with normal horse serum (Vector Laboratories, Burlingame, CA) for 2 hours at room temperature. The tissues were then treated with mouse anti-8-OHdG monoclonal antibody at a concentration of 10  $\mu$ g/mL diluted with horse-blocking serum (Japan Institute for the Control of Aging [JaICA], Shizuoka, Japan), anti-4-HNE monoclonal antibody at a concentration of 25  $\mu$ g/mL diluted with horse blocking serum (JaICA) for 2 hours at



**FIGURE 3.** Corneal vital staining changes with 2% rebamipide eye drop application. **(A)** Note the significant improvement of the fluorescein staining score in the SOD-1 knockout mice with rebamipide eye drop instillation for 2 weeks. **(B)** Note the significant improvement of the Rose Bengal in staining score in the SOD-1 knockout mice with rebamipide eye drop instillation for 2 weeks. **(C)** Note the representative *anterior segment photos* showing improvement of corneal fluorescein and Rose Bengal staining scores after rebamipide application.

room temperature. For the negative controls, the primary antibody was replaced with mouse IgG1 Isotype control (MOPC-21; Sigma, St. Louis, MO). Endogenous peroxidase activity was blocked using 3.0% H<sub>2</sub>O<sub>2</sub> in methanol for 3 minutes. The sections were incubated for 30 minutes with biotin-labeled horse anti-mouse IgG serum (Vector Laboratories), followed by avidin-biotin-alkaline phosphatase complex treatment (Vector Laboratories) for 30 minutes. The sections were washed in PBS buffer, developed in prepared 3,3'-diaminobenzidine (DAB) chromogen solution (Vector Laboratories), lightly counterstained with hematoxylin for 4 minutes

at room temperature, washed with tap water, dehydrated, and mounted. To quantitatively assess the area of staining by the primary antibodies for the oxidative stress markers, ImageJ software was used (National Institutes of Health, Bethesda, MD).

#### Quantitative Real-Time PCR for muc1 and muc4 and 5AC mRNA Expression

Conjunctival samples were homogenized and RNA was extracted from Isogen samples. Quantitative real-time PCR

was performed according to the manufacturer's instructions (Applied Biosystems, Weiterstadt, Germany). cDNA (10 ng) was amplified in 25  $\mu$ L final volume in the presence of 1.25  $\mu$ L of the following "Assay by Design" oligonucleotides (muc5AC and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]; Applied Biosystems). Test gene primer and probe sets were optimized for concentration, amplification efficiency, and faithful coamplification with housekeeper gene primer and probe sets, the latter including GAPDH. Real-time quantitative RT-PCR was set up in 96-well plates using the above reagents and Taq Man master mix (Applied Biosystems) and as indicated by optimization data and it was run on 7700 ABI thermal cyclers (Applied Biosystems). The thermal profile consisted of 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Real-time data were acquired and analyzed using Sequence Detection System Software (Applied Biosystems) with manual adjustment of the baseline and threshold parameters. The expression levels of mRNA were normalized by the median expression of a housekeeping gene (GAPDH). The primer sequences were as follows: GAPDH (sense 5'TGA CGT GCC GCC TGG AGA AA3', antisense, 3'AGT GTA GCC CAA GAT GCC CTT CAG5'); muc1 (sense 5'TGG AAA GGC CTT CTC TAG GC3', antisense 3'CTT CAC ATC CGA GTG GGT TT5'); muc4 (sense 5'TGG AAA GGC CTT CTC TAG GC3', antisense 3'CTT CAC ATC CGA GTG GGT TT5'); muc5AC (sense 5'TGG AAA GGC CTT CTC TAG GC3', antisense 3'CTT CAC ATC CGA GTG GGT TT5').

### Statistical Analysis

Data were processed using Graph Pad software (InStat, San Diego, CA). Unpaired *t*-test with Welch correction was used for the analyses of nonparametric values. A probability level less than 5% was considered statistically significant.

## RESULTS

### Tear Fluid Cytokine Concentration Changes

After application of 2% Rebamipide ophthalmic solution, the mean tear IL-6, IL-17, TNF- $\alpha$ , and INF- $\gamma$  concentrations decreased significantly, as shown in Figure 1. There were no statistically significant differences between the mean IL-10, MCP-1, and IL-12p70 concentrations before and after rebamipide instillations (data not shown).

### Tear Function Changes

The mean tear film breakup times were  $1.5 \pm 0.25$  seconds before application and  $3.5 \pm 2.0$  seconds after application in the *Sod1*<sup>-/-</sup> mice, whereas these values were  $3.0 \pm 1.0$  seconds to  $4.5 \pm 0.75$  seconds before and after rebamipide application in the WT mice. The differences were statistically significant (Fig. 2A) ( $P < 0.05$ ). No significant changes were observed in the amount of tear production in either the *Sod1*<sup>-/-</sup> or the WT mice before and after rebamipide instillations as shown in Figure 2B ( $P > 0.05$ ).

### Fluorescein and Rose Bengal Staining Score Changes

The mean fluorescein staining score improved significantly from  $5.5 \pm 2.5$  points to  $2.5 \pm 3.5$  points after rebamipide application in the *Sod1*<sup>-/-</sup> mice ( $P < 0.05$ ), whereas these scores did not change significantly in the WT mice ( $P > 0.05$ ) as shown in Figure 3A ( $3.0 \pm 1.0$  points before and  $2.5 \pm 1.5$  points after rebamipide application). Likewise, the mean Rose

Bengal staining score improved significantly from  $4.5 \pm 1.5$  to  $3.0 \pm 1.5$  points after rebamipide application in the *Sod1*<sup>-/-</sup> mice ( $P < 0.05$ ), whereas these scores did not change significantly in the WT mice ( $P > 0.05$ ) as shown in Figure 3B ( $3.0 \pm 1.0$  points before and  $2.5 \pm 2.0$  points after rebamipide application). Representative corneal fluorescein and Rose Bengal stainings in the *Sod1*<sup>-/-</sup> mice before and after rebamipide applications are shown in Figure 3C.

### Goblet Cell Density and Mucin mRNA Expression Changes

The mean number of conjunctival goblet cells significantly increased in both *Sod1*<sup>-/-</sup> and WT mice after rebamipide ophthalmic solution application, as shown in Figure 4A ( $P < 0.05$ ). muc5AC mRNA expression increased 2.48-fold after rebamipide application. The increase was statistically significant, as shown in Figure 4B ( $P < 0.05$ ). Representative PAS stainings before and after rebamipide application in the *Sod1*<sup>-/-</sup> and WT mice are shown in Figure 4C. Although muc1 and muc4 mRNA expressions showed a tendency to increase after rebamipide application in both the *Sod1*<sup>-/-</sup> and WT mice, the differences were not significant, as shown in Figure 5 ( $P > 0.05$ ).

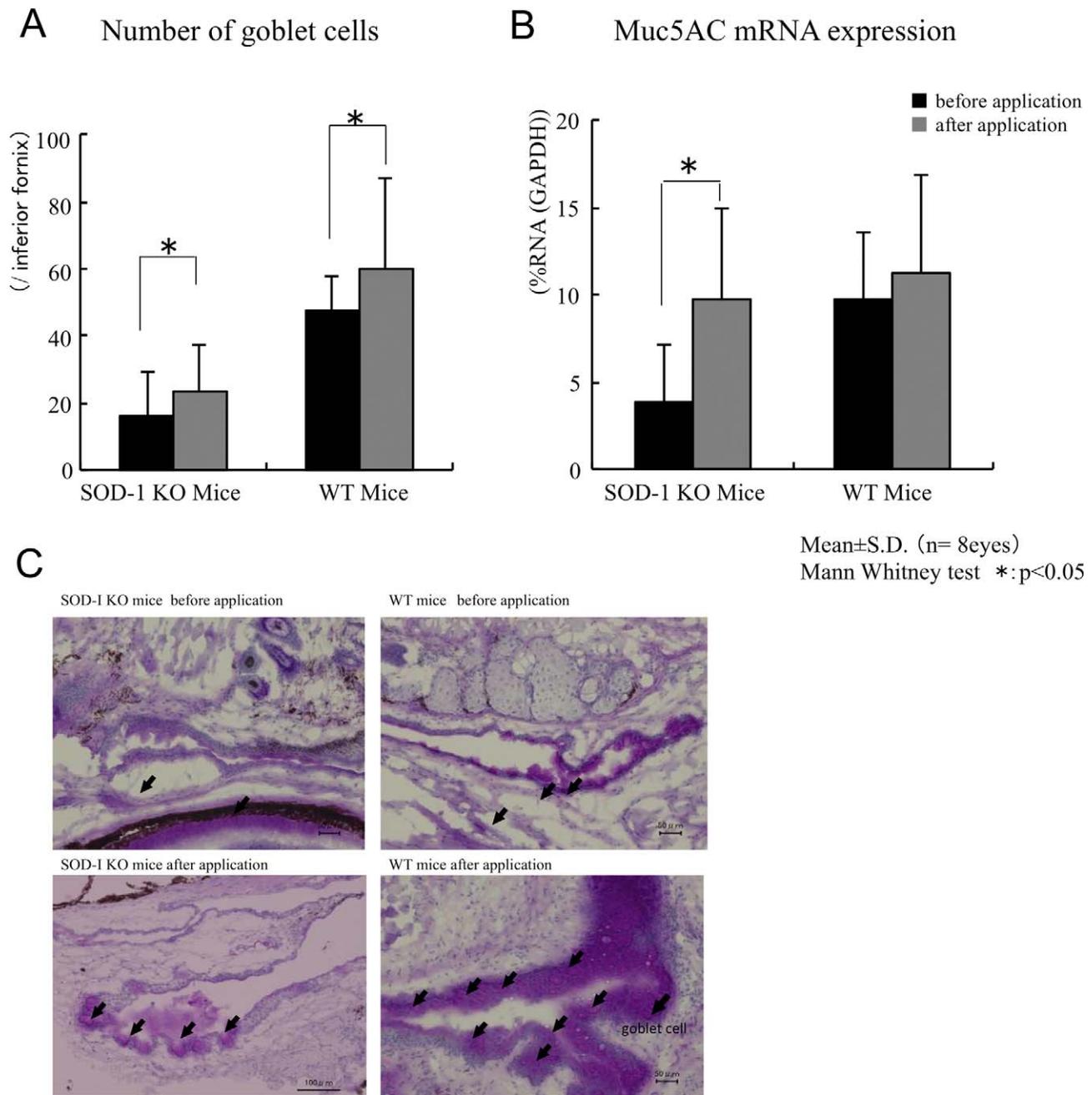
### Quantitative Staining Intensity Changes for Oxidative Stress Markers in the Conjunctival Tissues

The staining intensities for 4HNE and 8-OHdG appeared to decrease in the *Sod1*<sup>-/-</sup> mice after rebamipide application, as shown in Figure 6A. The mean area of epithelial cells that stained positive for 4-HNE decreased significantly from  $450 \pm 370 \mu\text{m}^2$  before application to  $200 \pm 10 \mu\text{m}^2$  after rebamipide application in the *Sod1*<sup>-/-</sup> mice ( $P < 0.05$ ), whereas no significant changes were observed in staining area calculations before versus after rebamipide application in the WT mice (Fig. 6B) ( $P > 0.05$ ). The mean area of epithelial cells that stained positively for 8-OHdG decreased significantly from  $35 \pm 13 \mu\text{m}^2$  before application to  $10 \pm 5 \mu\text{m}^2$  after rebamipide application in the *Sod1*<sup>-/-</sup> mice ( $P < 0.05$ ). No significant changes were observed in the WT mice (Fig. 6C) ( $P > 0.05$ ).

## DISCUSSION

Kojima et al.<sup>24</sup> previously demonstrated that the *Sod1*<sup>-/-</sup> mouse is a good model for the study of oxidative lipid and DNA damage, aging induced lacrimal gland inflammation, epithelial-mesenchymal transition in the lacrimal glands, age-related dry eye, and related ocular surface disease. Kojima et al.<sup>24</sup> also reported that the ocular surface disease in the *Sod1*<sup>-/-</sup> mice was associated with tear instability, ocular surface epithelial damage evidenced by increased fluorescein and Rose Bengal staining scores, decrease of goblet cells, decreased conjunctival muc5AC mRNA expression, increased apoptosis of the conjunctival epithelium, and increase of subconjunctival inflammatory cell infiltration (Kojima T, Ibrahim OMA, Wakamatsu TH, reported at the Gordon Conference, March 7-12, 2010, Ventura, CA).

Currently available treatment modalities for dry eye disease include artificial tears substitutes, gels/ointments, moisture chamber spectacles, anti-inflammatory agents (topical CsA and corticosteroids, omega-3 fatty acids), tetracyclines, punctal plugs, secretagogues, autologous serum drops, contact lenses, systemic immunosuppressives, and surgery.<sup>14</sup> There has been a commensurate increase in the knowledge regarding the pathophysiology of dry eye. This has led to a paradigm shift in dry eye management from simply lubricating and hydrating



**FIGURE 4.** Goblet cell density and muc5AC mRNA expression changes with 2% rebamipide eye drop application. **(A)** Note the significant improvement of the goblet cell density in the SOD-1 knockout and WT mice after 2 weeks of rebamipide application. **(B)** muc5AC mRNA expression improved significantly with 2 weeks of rebamipide application. **(C)** PAS staining of representative specimens before and after rebamipide application in the SOD-1 knockout and the WT mice. Note that goblet cells, depicted by *black arrows*, appear to increase with rebamipide instillation for 2 weeks.

the ocular surface with artificial tears to strategies that stimulate natural production of tear constituents through administration of secretagogues, maintain ocular surface epithelial health and barrier function, and inhibit the inflammatory factors that adversely affect the ability of ocular surface and glandular epithelia to produce tears.<sup>14</sup>

The 2007 international workshop report included rebamipide into the category of mucin secretagogues. A number of basic research studies have been performed to clarify the mechanisms of rebamipide's action, which demonstrated that it increases gastric mucus glycoprotein components, stimulates

migration and proliferation of wounded epithelial cell monolayers, increases expression of EGF, and its receptor in normal and ulcerated gastric mucosa, and scavenges active oxygen radicals.<sup>22</sup> Based on these observations, we investigated the effects of commercially available 2% rebamipide eye drops on the tear functions, tear film inflammatory cytokines, conjunctival mucin expression, and lipid/DNA oxidative stress markers in the *Sod1*<sup>-/-</sup> and WT mice in the current study.

We detected the presence of IL-17, IL-6, IFN- $\gamma$ , and TNF- $\alpha$  in the tears of the SOD-1 KO mice at 40 weeks but not in the WT mice (data not shown) before rebamipide application in this

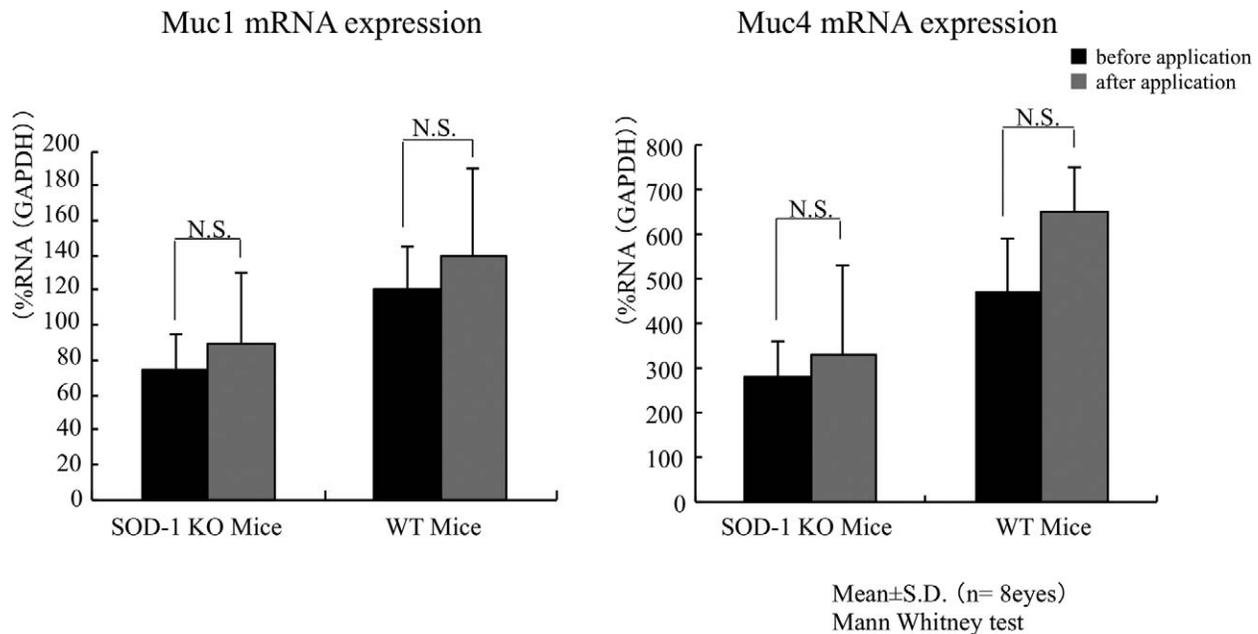
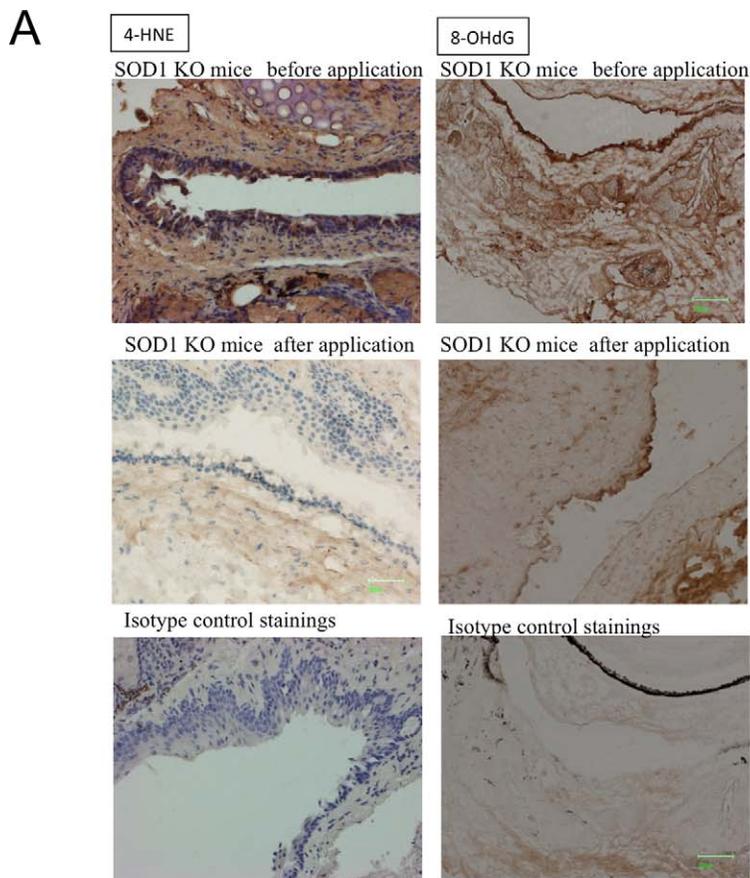


FIGURE 5. muc1 and muc4 mRNA expression changes with 2% rebamipide eye drop application. Note that while there was a tendency for some increase in mRNA expressions of conjunctival muc1 and muc4, no significant differences were observed before and after rebamipide instillation.

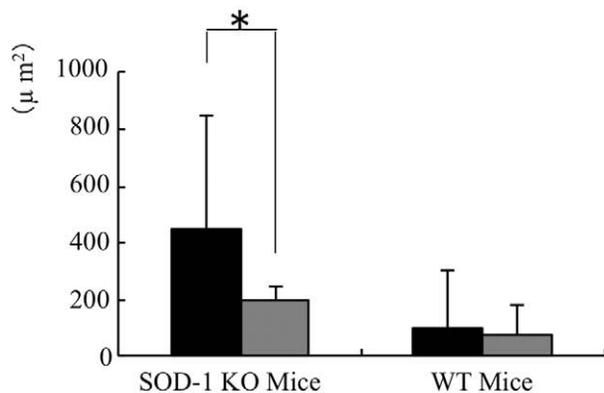
study. Lacrimal gland epithelial cells in an inflamed environment have been shown to express cytokines in dry eye disease and with aging.<sup>25</sup> TNF- $\alpha$  has indeed been demonstrated in increased amounts in the lacrimal glands of old but not young mice.<sup>26</sup> Increased IL-17, IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 concentrations have also been shown in dry eye syndromes in previous studies.<sup>26–31</sup> The reported roles for TNF- $\alpha$  include induction of inflammation and cell death, and those for IL-6 include induction of inflammation and fibrosis.<sup>25,32</sup> IL-17 and IFN- $\gamma$  are also known to induce inflammation.<sup>33,34</sup> We noted with interest that rebamipide application could decrease the aforementioned cytokines in the tears of the SOD-1 knockout mice effectively with 2 weeks of application. This observation was consistent with previous studies that rebamipide could inhibit proinflammatory cytokines in the gastric mucosa, such as TNF $\alpha$  and IL-8, and reduce the concentrations of TNF- $\alpha$  and IL-1 $\beta$  in serum, as well as suppressed gastric inflammation in mice infected with *Helicobacter pylori*.<sup>35</sup> The drug has been shown to decrease cigarette-induced TNF- $\alpha$  release by the bronchial epithelium.<sup>22</sup> Recently, rebamipide has been shown to protect corneal epithelial cells from the TNF- $\alpha$ -induced disruption of barrier function by maintaining the distribution and expression of ZO-1, as well as the organization of the actin cytoskeleton.<sup>36</sup> Although we did not investigate the changes in the conjunctival epithelial inflammatory cells in detail this time, it will be interesting to study the inflammatory pathways involved in the pathogenesis of the conjunctival dry eye disease in this mouse model and rebamipide effects in each single inflammatory cell subtype in the future. Of interest in this study was the decrease of tear IL-17 concentration with rebamipide application. Pflugfelder et al.<sup>37</sup> reported in animal models that desiccating stress upregulates the expression of Th-17 inducer cytokines, including IL-23, IL-17, and IFN- $\gamma$ , which results in upregulation of MMP-3 and -9, which are known to be associated with disruption of corneal epithelial barrier function and epithelial damage. Th-17 induction involves inflammatory pathways activating dendritic cells.<sup>38</sup> Conversely, De Paiva et al.<sup>35</sup> also reported that antibody neutralization of IL-17 decreases MMP-3 and -9 mRNA in the corneal epithelium of experimental dry eye murine and

improves corneal epithelial status. We believe that the reduction of IL-17 concentration in tears with rebamipide treatment could also explain the improvement in corneal epithelial damage scores. Whether this improvement occurred concurrently with reductions in tear concentrations of MMP-3 and -9 needs to be clarified in further studies.

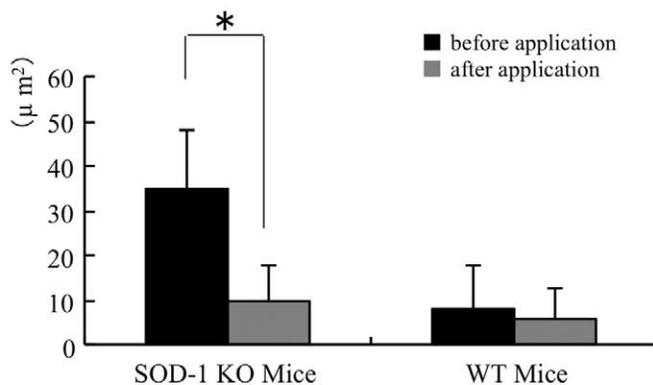
We re-observed in this current batch of SOD-1 knockout mice that there was a decrease in goblet cells and muc5AC expression in the conjunctival epithelium, which may all be held responsible for the tear instability and the ocular surface epithelial disease evidenced by presence of marked fluorescein and Rose Bengal staining before rebamipide application. To clarify whether the diseased ocular surface could be salvaged by a mucin secretagogue, we applied 2% rebamipide ophthalmic solution for 2 weeks four times a day. Interestingly, we observed that the tear stability, fluorescein, and Rose Bengal staining (which also is known to indicate mucin-secreting cells and assess disorders of mucin secretion) scores and muc5 mRNA expression improved significantly with rebamipide treatment in our knockout mice, which was consistent with the in vitro or in vivo observations made in other animal models. Indeed, in N-acetylcysteine-treated rabbit eyes, OPC-12759 has been shown to increase the level of mucin-like substances on the cornea and conjunctiva that correlated with an improvement in Rose Bengal scores of N-acetylcysteine-treated eyes.<sup>39</sup> OPC-12759 also has been reported to induce mucin-like secretion from rat conjunctival goblet cells in culture by activation of the EGF receptor (EGFR) and the MAPK pathways. Our results imply that OPC-12759 affected goblet cell numbers and mucin production and may play at least two roles in the conjunctiva by stimulating goblet cell mucin secretion and/or by inducing goblet cell proliferation in vivo. It should be noted that we have not determined if OPC-12759 increased goblet cell mucin synthesis and relevant future studies investigating the pathways involved and clarifying these issues should provide very interesting information. There is some evidence from other studies that have shown that EGF and the cholinergic agonist carbachol both phosphorylate the EGFR and activate p44/p42 MAPK in conjunctival pieces and in cultured goblet cells.<sup>40,41</sup> Activation



**B** Area of epithelial cells that stained positive for 4-HNE



**C** Area of epithelial cells that stained positive for 8-OHdG



Mean±S.D. (n= 8eyes)  
Mann Whitney test \*:p<0.05

**FIGURE 6.** The 4-HNE and 8-OHdG staining and staining area alterations with 2% rebamipide eye drop application. (A) Note the decrease in 4-HNE staining intensity with rebamipide instillation (*left upper insert* and *left middle insert*). *Left lower insert* shows isotype staining. Note also that 8-OHdG staining intensity decreased with rebamipide instillation (*right upper insert* and *right middle insert*). *Right lower insert* shows isotype staining. (B) Note that the quantitative assessment of the staining area showed a significant decrease in the mean area of staining for the lipid oxidative stress marker 4-HNE. (C) Note that the quantitative assessment of the staining area showed a significant decrease in the mean area of staining for the DNA oxidative stress damage marker 8-OHdG.

of the EGFR then serves as a scaffold for the Shc-Grb2 complex, leading to the activation of Ras and Raf and the downstream activation of p44/p42 MAPK.<sup>42</sup> Increases in extracellular Ca<sup>2+</sup>, as well as activation of PKC, were also shown to activate p44/p42 MAPK in conjunctival goblet cells.<sup>40,41</sup> p44/p42 MAPK has been implicated in a variety of cellular processes including “long-term” events, such as cell proliferation and differentiation and gene expression<sup>43</sup> and “short-term” events, such as secretion.<sup>40,41</sup> In rat conjunctival goblet cells, OPC-12759-activated p44/p42 MAPK is responsible for both a “long-term” event (goblet cell proliferation) and a “short-term” event (mucin secretion).<sup>22</sup> Rebamipide has been demonstrated to stimulate MUC 1 and MUC4 in human corneal epithelial cell cultures.<sup>39</sup> Rebamipide application was associated with an insignificant increase from the conjunctiva of both the SOD-1 knockout and WT mice in this study. To what extent these mucin expression alterations along with an increase in muc5AC mRNA expression influenced tear stability remains to be further investigated.

We also were able to confirm antioxidative effects through decreased staining for lipid and DNA oxidative stress damage markers, 4-HNE and 8-OHdG, after 2 weeks of rebamipide application. Other studies have shown consistent evidence that OPC-12759 appears to directly inhibit the production of superoxide (O<sub>2</sub><sup>-</sup>) and inhibit neutrophil recruitment, and activation of the EGFR in airway epithelium.<sup>18–20,22</sup> Whether the antioxidant effects observed in our study were due to direct interactions with the nuclear factor-κB pathway or not, needs to be clarified in further studies.

Because we observed no changes in the tear quantity, we suggest that the improvement in tear stability and corneal epithelial damage may have resulted from decreased tear inflammation, decrease in lipid and DNA oxidative stress-induced damage, increase in goblet cell density, and increased mucin expression after rebamipide application. Future investigation into the relationships between changes of mucin expression, goblet cell differentiation, and role of and type of inflammatory pathway involvement with rebamipide application should provide very interesting information.

In conclusion, this study revealed that the ocular surface disease in the *Sod1*<sup>-/-</sup> mice was associated with tear film inflammation, increased conjunctival lipid and DNA oxidative damage, tear instability, ocular surface epithelial damage evidenced by increased fluorescein and Rose Bengal staining scores, loss of goblet cells and decreased muc5 expression. The 2% rebamipide ophthalmic solution was effective in the treatment of the ocular surface disease in this knockout mouse model, the mechanisms of action of which need to be studied in the future.

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