Molecular Vision 2014; 20:929-938 <http://www.molvis.org/molvis/v20/929>
Received 13 August 2012 | Accepted 26 June 2014 | Published 28 June 2014 © 2014 Molecular Vision

The effects of 3% diquafosol sodium application on the tear functions and ocular surface of the Cu,Zn-superoxide dismutase-1 (*Sod1*)–knockout mice

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Purpose: To investigate the role of a water and mucin secretagogue (3% diquafosol sodium eye drops) on the tear function and conjunctival ocular surface changes in *Sod1*$^{-/-}$ in comparison to the wild-type (WT) mice.
Methods: Fourteen eyes of 7 *Sod1*$^{-/-}$ male mice with C57BL/background and 14 eyes of 7 C57BL6 strain wild-type male mice were examined at 40 weeks in this study. All mice had application of 3% diquafosol ophthalmic solution six times a day for 2 weeks. Tear film stability and corneal epithelial damage was evaluated by fluorescein and Rose Bengal stainings. Anterior segment photography was performed before and after eye drop instillations. Aqueous tear quantity was measured with phenol red–impregnated cotton threads without anesthesia. Animals were sacrificed at 42 weeks after diquafosol treatment and the whole globe specimens were subjected to periodic acid Schiff staining. Goblet cell density was quantified by J Image software. Quantitative real-time PCR for conjunctival muc 5AC messenger RNA expression was also performed.
Results: *Sod1*$^{-/-}$ mice had significantly higher fluorescein staining scores compared to the WT mice before eye drop instillation. The mean tear film breakup time, Rose Bengal staining scores, and muc5 messenger RNA expression improved significantly with diquafosol treatment in both the WT and the knockout mice. The mean fluorescein staining score and aqueous tear quantity significantly improved in the *Sod1*$^{-/-}$ mice with treatment. A notable and consistent increase in goblet cells and decrease in inflammatory cell infiltrates could be confirmed in all specimens after 2 weeks of diquafosol eye drop application.
Conclusions: Three percent diquafosol ophthalmic solution appears to be effective in the treatment of ocular surface disease in this age-related dry eye disease mouse model.

According to the 2000 general population census statistics of the Japanese Ministry of Health, Labour and Welfare website, there were approximately 22.7 million elderly people above 65 years of age at this time, corresponding to 17.3% of the overall population in Japan. In comparison, approximately 34.8 million people in the United States (12.4% of the overall population) were seniors aged 65 years or older [1].

Aging brings about inflammation, age-related chronic disease, and disability. In the field of ophthalmology, age-related diseases include cataracts, age-related macular degeneration, glaucoma, diabetes-related retinal disease, and dry eye. Dry eye symptoms are common in the elderly population. In particular, the short tear film break-up time (BUT) type of dry eyes has been reported in 70% of the Japanese elderly [1-8]. Population-based dry eye studies in the elderly have shown that dry eye symptoms are common in White, Asian, Hispanic, and Danish people [9-14].

An imbalance between the generation of free radicals and radical scavenging antioxidant systems results in oxidative stress, a condition that has been associated with inflammatory cell injury observed in many age-related diseases and is also considered a major factor in the process of senescence [15]. One of the well-known antioxidant defense systems is superoxide dismutase 1, which is widely distributed in the tissues and represents 90% of the total SOD activity [16,17].

It has been reported that the *Sod1*$^{-/-}$ mice had oxidative stress–related damage and inflammation in the lacrimal glands with a decrease in tear secretion ability [18]. Recently, Kojima et al. reported signs of increasing oxidative stress and conjunctival inflammatory cell infiltrates from 10 to 40 weeks in a *Sod1*$^{-/-}$ mouse model had (unpublished data, presented at 2010 Gordon Conference, March 7–12, Ventura). Currently available treatment modalities for dry eye disease

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include artificial tears substitutes, gels/ointments, moisture chamber spectacles, anti-inflammatory agents (topical CsA and corticosteroids, omega-3 fatty acids), tetracyclines, punctual plugs, secretagogues, autologous serum drops, contact lenses, systemic immunosuppressives, and surgery [19]. There has been a commensurate increase in the knowledge regarding the pathophysiology of dry eye in recent years. This has led to a paradigm shift in dry eye management from simply lubricating and hydrating the ocular surface with artificial tears to strategies that stimulate natural production of tear constituents through the administration of secretagogues, maintain ocular surface epithelial health and barrier function, and inhibit the inflammatory factors that adversely impact the ability of ocular surface and glandular epithelia to produce tears [19].

Diquafosol is a uridine triphosphate–related compound, an agonist of the purinergic P_2Y_2 receptor, which contributes to water transfer and mucin secretion and is expressed in several ocular (including the conjunctival epithelium, meibomian glands, and goblet cells) and pulmonary tissues [20-22]. In this study, we investigated tear functions, muc5 AC messenger RNA (mRNA) expression, and conjunctival histopathological alterations in *Sod1*$^{-/-}$ mice, comparing the results with wild-type (WT) mice and the changes in the results associated with 2 weeks of diquafosol sodium eye drop instillation.

METHODS

Animals: Fourteen eyes of seven *Sod1*$^{-/-}$ male mice with C57BL/background and 14 eyes of seven C57BL6 strain WT male mice were examined at 40 weeks in this study. The *Sod1*$^{-/-}$ mice were received from the Department of Advanced Aging Medicine, Chiba University Graduate School of Medicine (Chiba, Japan) and the WT C57BL/6 mice were purchased from Japan Clea (Osaka, Japan). All mice were subject to application of 3% diquafosol ophthalmic eye drops for 2 weeks, six times a day. The diquafosol eye drops used in this study contained benzalkonium chloride, potassium chloride, sodium chloride, and dibasic sodium phosphate. On day 15, tear functions were reevaluated and mice were sacrificed for histopathological examinations 14 h after termination of eye drop use. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular surface epithelial damage and tear film breakup time assessment: Corneal fluorescein staining was evaluated with slit-lamp biomicroscopy using cobalt blue light after instillation of 2 µl of 0.5% sodium fluorescein. Excess of fluorescein was wiped from the lateral tear meniscus. The cornea was examined with a handheld slit lamp 2 min after fluorescein instillation. The tear film breakup time was initially evaluated by waiting for the natural blink response of the mouse, which was recorded three times; the mean of the measurements was then calculated. Punctuate staining was recorded using a grading system of 0–3 points for superior, central, and inferior corneal areas after breakup time examination. The fluorescein staining scores ranged from a minimum of 0 to a maximum of 9 points. Fluorescein staining was followed by introduction of 2 µl of 1% Rose Bengal solution by a micropipette. The Rose Bengal staining scores ranged from a minimum of 0 to maximum of 9 points. Corneal statuses before and after staining were recorded through photographs for each mouse on days 1 and 15 before and at the end of topical diquafosol applications. After Rose Bengal staining, the mouse corneas were washed with 2 µl of distilled water. Tear quantity measurements were performed 3 h after Rose Bengal staining.

Tear quantity measurements: Aqueous tear quantity was measured with phenol red–impregnated cotton threads (Zone-Quick, Showa Yakuhin Kako co., Ltd., Tokyo, Japan) without anesthesia. The threads were held with jeweler forceps and immersed into the tear meniscus in the lateral canthus for 60 s. The length of wetting of the thread was measured in millimeters. Aqueous tear production was weight adjusted by dividing the amount of total aqueous tear produced over 60 s by the animal's weight.

Conjunctival specimen collections and histopathological assessment of specimens: Animals were anesthetized intraperitoneally for conjunctival biopsies measuring 2×2 mm from the inferior temporal conjunctiva at 40 weeks. The same animals were sacrificed using a combination of 6 mg/ml of ketamine and 4 mg/ml of xylazine at 42 weeks after 2 weeks of 3% diquafosol application. The whole globes were rapidly removed after sacrifice. Samples were immediately fixed in 4% buffered paraformaldehyde at 4 degrees for 24 h, embedded in paraffin wax, sliced in 4 µm thick paraffin sections, and processed according to conventional histological techniques for periodic acid Schiff staining.

Periodic acid Schiff staining and goblet cell density evaluations: Samples were deparaffinized in xylene for 10 min, hydrated in descending grades of alcohol (100%, 90%, 80%, 70%; 3 min in each solution), treated with periodic acid for 10 min, and washed in distilled water three times (5 min each wash). The specimens were then immersed in Schiff reagent for 30 min, washed with distilled water for 5 min, stained with hematoxylin for 2 min, and rewashed with distilled water for another 5 min. The specimens were dehydrated in

ascending grades of alcohol (70%, 80%, 90%, 100%; 2 min in each solution), treated with xylene for 6 min, and finally coverslipped after mounting. Five randomly selected nonoverlapping areas in each specimen in 890 x 705 µm frames were digitally photographed (Axioplan2imaging, Carl Zeiss, Jena, Germany). The photographer was masked to the mouse group information. Goblet cell densities were given as cells/mm^2. Using image capturing software (Adobe Photoshop, San Jose, CA), a subset of color that indicated the goblet cells was selected from the raw pictures, and using another analysis program (Image J, NIH) that checked the intensity of staining for each picture, the number of goblet cells was calculated and expressed in cells/mm^2.

Quantitative real-time polymerase chain reaction for mucin 5AC glycoprotein (Muc5AC) messenger RNA expression: Mouse conjunctival tissues were preserved overnight in RNA laterR (Applied Biosystems, Carlsbad, CA) after prompt excision. Tissues were then transferred into ISOGEN (NIPPON GENE, Tokyo, Japan) and homogenized well. Total RNA was extracted, cleaned up and treated with DNase using RNeasy mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). cDNA (10 ng) was amplified in 25 µl final volume in the presence of 1.25 µl of the following "Assay by Design" oligonucleotides (muc5AC and 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time quantitative reverse transcriptase PCR (RT-PCR) was performed according to the manufacturer's instructions (Applied Biosystems, Weiterstadt, Germany). The thermal profile consisted of 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C 1 min. 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 AA-3′, antisense, 3′-AGT GTA GCC CAA GAT GCC CTT CAG-5′) and Muc5AC (sense 5′-TGG AAA GGC CTT CTC TAG GC-3′, antisense, 3′ –CTT CAC ATC CGA GTG GGT TT -5′).

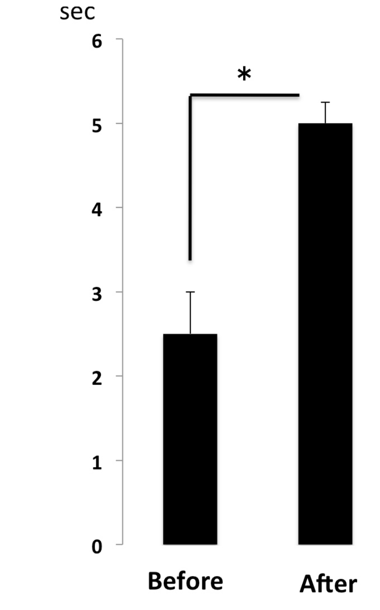
Statistical analysis: Data were processed using Graph Pad software (InStat, San Diego, CA). The Wilcoxon matched pairs test was used for the analyses of nonparametric values. A probability level less than 1% was considered statistically significant.

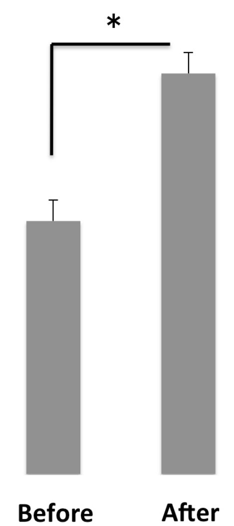
RESULTS

Changes in tear film breakup time and corneal epithelial staining scores: The mean tear film breakup time values were significantly higher in both the S*od1*$^{-/-}$ mice and the WT mice after diquafosol eye drop application (5±0.25 s and 4.75±0.25 s, respectively) compared to preinstillation values (2.5±0.5 s and 3.0±0.25 s, respectively; $p<0.01$; Figure 1). The anterior segment photographs showed improvement of fluorescein and Rose Bengal stainings both in the *Sod1*$^{-/-}$ (Figure 2) and the WT mice (Figure 3) after 2 weeks of diquafosol eye drop instillation. The mean fluorescein scores showed a significant decrease after 2 weeks of diquafosol eye drop instillation in the S*od1*$^{-/-}$ mice (postinstillation fluorescein score: 2.0±0.5 points; preinstillation fluorescein score: 7.0±0.5 points; $p<0.01$; Figure 4). The mean Rose Bengal scores showed a significant decrease after 2 weeks of diquafosol eye drop instillation both in the S*od1*$^{-/-}$ and the WT mice (0.5±0.25 points and 0.5±0.25 points, respectively) compared to the preinstillation scores (6.5±0.5 points and 3.5±0.25 points, respectively; $p<0.01$; Figure 5).

Changes in tear quantity: We measured aqueous tear production using the cotton thread test and divided the values by the respective mouse weights. The mean weight-adjusted aqueous tear production was slightly but insignificantly lower in the *Sod1*$^{-/-}$ mice compared with the age- and sex-matched WT mice before administration of diquafosol eye drops, as shown in Figure 6. A significant increase in tear quantity after 2 weeks of topical diquafosol application was observed in both the *Sod1*$^{-/-}$ and the WT mice (0.23±0.04 mm/g and 0.25±0.01 mm/g, respectively) compared to preinstillation values (0.12±0.04 mm/g and 0.15±0.03 mm/g, respectively; Figure 6).

Conjunctival histopathological alterations: Periodic acid Schiff staining showed an apparently normal conjunctival architecture in the WT mice before and after diquafosol eye drop instillation (data not shown). Conjunctival specimens in the *Sod1*$^{-/-}$ mice consistently showed lack of goblet cells and infiltration with inflammatory cells before diquafosol eye drop instillation (Figure 7A). A notable and consistent increase in goblet cells and lack of inflammatory cell infiltrates could be confirmed in all specimens after 2 weeks of diquafosol treatment (Figure 7B). The mean goblet cell counts in conjunctival specimens of the *Sod1*$^{-/-}$ and WT mice were 61±5 cells/mm^2 and 181±10 cells/mm^2, respectively, before diquafosol instillation. The mean goblet cell counts increased to 1090±50 cells/mm^2 and 545±70 cells/mm^2, respectively, after diquafosol treatment. These increases in goblet cell densities were significant ($p<0.01$).





Sod1-/-

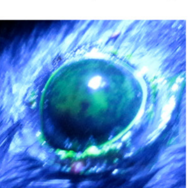
WT

Wilcoxon matched pairs test *p<0.01

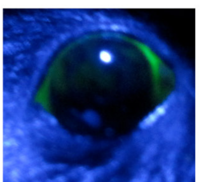
Figure 1. Tear film breakup time changes with 3% topical diquafosol application in the *Sod1*-/- mice and wild type (WT) mice. Note the significant improvement in tear stability with 2 weeks of diquafosol sodium treatment. * represents $p < 0.01$.

Changes of corneal fluorescein staining with 3% diquafosol application

Sod1-/- Before eye drops

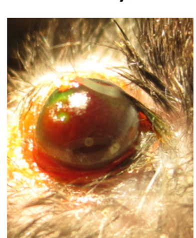


Sod1-/- After eye drops



Changes of corneal Rose Bengal staining with 3% diquafosol application

Sod1-/- Before eye drops



Sod1-/- After eye drops

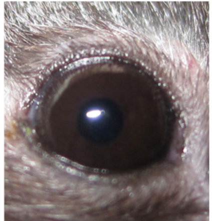


Figure 2. Anterior segment photographs showing changes in vital staining with 3% topical diquafosol application in the *Sod1*-/- mice. Upper inserts: Note the improvement in fluorescein staining after diquafosol sodium application. Lower inserts: Note the improvement in Rose Bengal staining after diquafosol sodium application.

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Changes of corneal fluorescein staining with 3% diquafosol application

WT Before eye drops

WT After eye drops

Changes of corneal Rose Bengal staining with 3% diquafosol application

WT Before eye drops

WT After eye drops

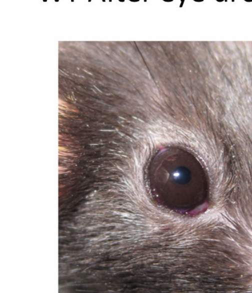


Figure 3. Anterior segment photographs showing changes in vital stainings with 3% topical diquafosol application in the wild-type (WT) mice. Upper inserts: Note the improvement in fluorescein staining after diquafosol sodium application. Lower inserts: Note the improvement in Rose Bengal staining after diquafosol sodium application.

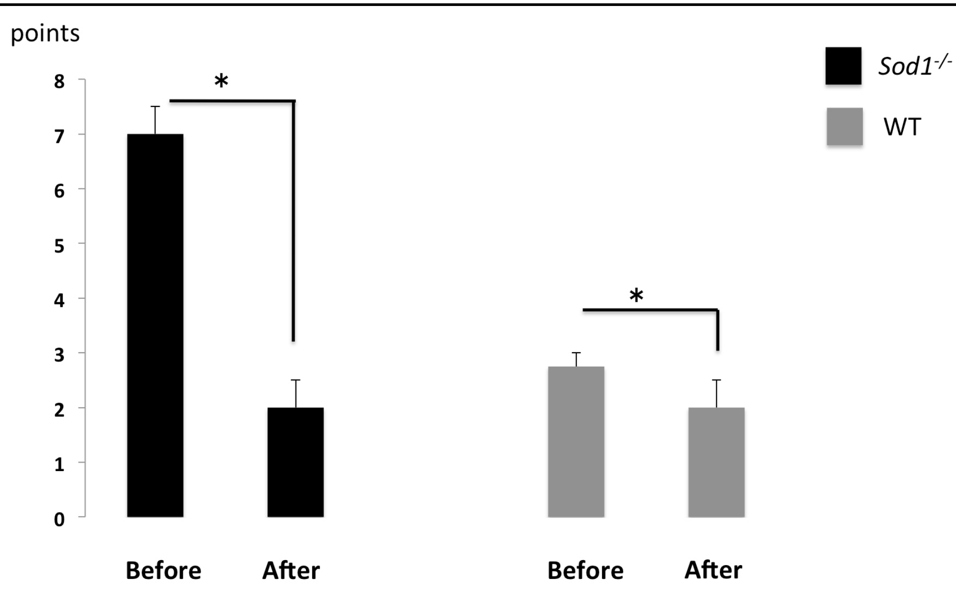
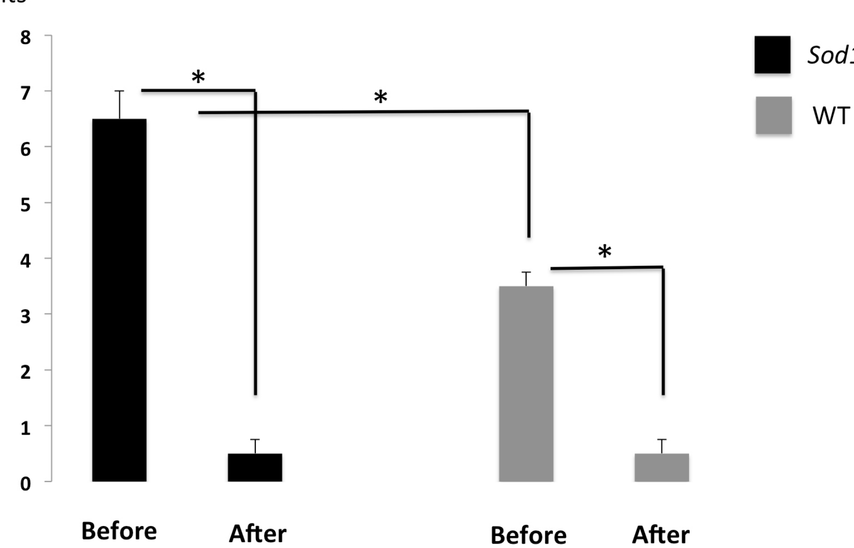


Figure 4. Changes of fluorescein staining scores with 3% topical diquafosol application in the *Sod1*$^{-/-}$ mice and wild type (WT) mice. Note the significant improvement in the fluorescein staining score in the *Sod1*$^{-/-}$ mice with 2 weeks of diquafosol sodium treatment. * represents $p < 0.01$.



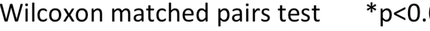
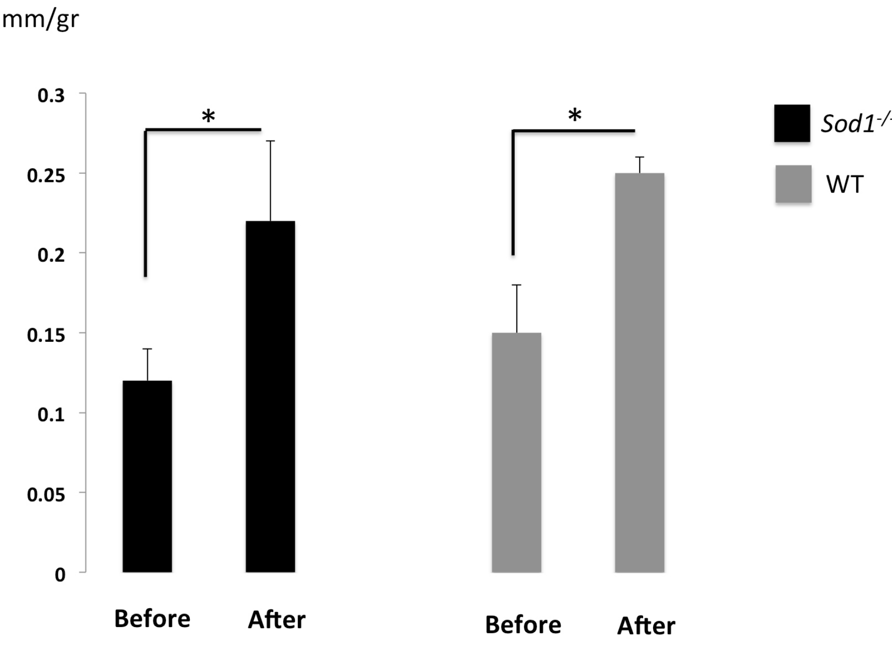


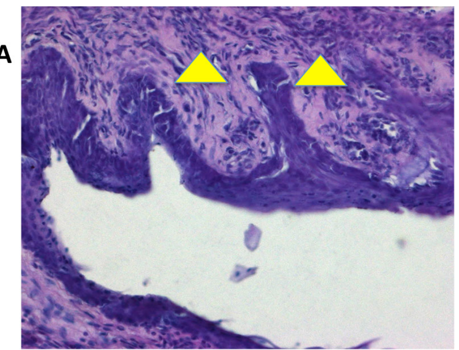
Figure 5. Changes in Rose Bengal staining scores with 3% topical diquafosol application in the *Sod1*$^{-/-}$ mice and wild type (WT) mice. Note the significant improvement in the Rose Bengal staining score in the *Sod1*$^{-/-}$ mice with 2 weeks of diquafosol sodium treatment. * represents $p < 0.01$.



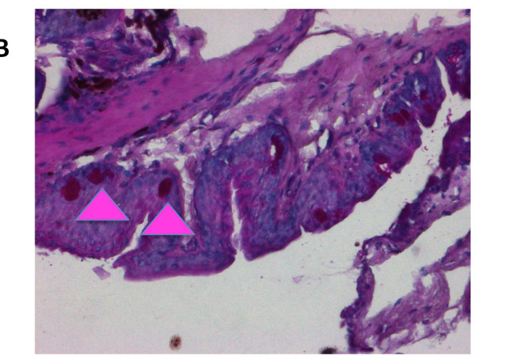
Wilcoxon matched pairs test *p<0.01

Figure 6. Weight-adjusted tear quantity changes with two weeks of 3% topical diquafosol application in the *Sod1*$^{-/-}$ mice and wild type (WT) mice. Note the significant improvement in tear quantity in both the *Sod1*$^{-/-}$ and WT mice with 2 weeks of diquafosol sodium treatment. * represents $p < 0.01$.

Conjunctival PAS staining before 3% DQS application 40 week *Sod1$^{-/-}$* mouse



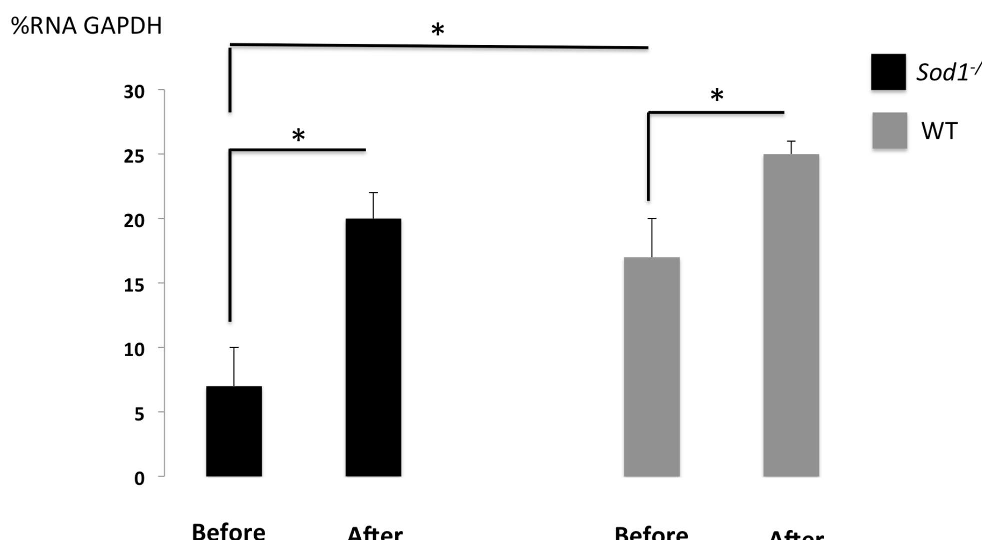
Conjunctival PAS staining after 3% DQS application 42 week *Sod1$^{-/-}$* mouse



Goblet cells

Inflammatory cells

Figure 7. Periodic acid Schiff stainings of conjunctival specimens showing changes in the *Sod1$^{-/-}$* mice before and after 3% topical diquafosol (DQS) application. **A**: Note the extensive inflammatory cell infiltration and lack of goblet cells in the conjunctival epithelium. **B**: Note the marked decrease in inflammatory cells and the presence of numerous goblet cells.



Wilcoxon matched pairs test *p<0.01

Figure 8. Real time reverse transcriptase polymerase chain reaction mucin 5AC glycoprotein messenger RNA expression changes with diquafosol application in the *Sod1$^{-/-}$* mice and wild type (WT) mice. Note the significant increase in muc 5Ac mRNA expression in both WT and the *Sod1$^{-/-}$* mice with 2 weeks of diquafosol sodium treatment. * represents $p < 0.01$.

Conjunctival muc5AC messenger RNA expression alterations: The expression of conjunctival muc5AC mRNA was significantly lower in the *Sod1*$^{-/-}$ mice than the WT mice before eye drop instillations (p<0.01), as shown in Figure 8. The expression of conjunctival muc5AC mRNA significantly increased both in the *Sod1*$^{-/-}$ and the WT mice after diquafosol instillations (p<0.01; Figure 8).

DISCUSSION

Oxygen free radicals and antioxidant systems have been demonstrated to be potentially important in the pathogenesis of ocular diseases such as cataracts, uveitis, retinopathy of prematurity, age-related macular degeneration, keratitis, keratoconus, and bullous keratopathy [18]. The role of oxidative stress in the pathogenesis of dry eye disease and its relationship with this disease have not previously been investigated in detail in an aging animal model or in humans. Because the amount and activity of Sod1 are the highest among the three isozymes in humans, it seemed reasonable to hypothesize that the lack of Sod1 would accelerate oxidative stress and age-related pathological changes in the lacrimal glands of the *Sod1*$^{-/-}$ mice [18]. We previously demonstrated that the lack of Sod1 led to increased oxidative lipid and DNA damage, increased $CD4^+$ T-cell inflammation, and epithelial-mesenchymal transition in the lacrimal glands of the current mouse model. This interfered with glandular secretory functions, resulting in dry eyes; moreover, this translated into an ocular surface disease [18]. In addition, we also reported that the *Sod1*$^{-/-}$ mouse model had signs of increasing oxidative stress and conjunctival inflammatory cell infiltrates from 10 to 40 weeks (unpublished data, presented at 2010 Gordon Conference, March 7–12, Ventura).

In this study, we investigated the effect of 3% diquafosol ophthalmic solution treatment for 2 weeks on the tear functions and conjunctival epithelial status in the *Sod1*$^{-/-}$. First, we noted that the 40-week-old *Sod1*$^{-/-}$ mice had tear instability and ocular surface epithelial damage, as evidenced by increased fluorescein and Rose Bengal staining scores, as well as a loss of goblet cells in the conjunctiva. These observations were consistent with our previous findings on age- and sex-matched *Sod1*$^{-/-}$ mice, in which we revealed certain pathological alterations in the conjunctiva, including increased apoptosis of the conjunctival epithelium, decrease in goblet cells, and increase of subconjunctival inflammatory cell infiltration (unpublished data, reported at 2010 Gordon Conference March 7–12, Ventura, CA). To further investigate whether these changes translated into disturbances of the most abundant ocular surface mucin expression (the investigation of which might have explained the tear instability, since muc5AC is a well-known ocular surface mucin contributing to tear stability [23,24]), we performed real-time RT-PCR for muc5AC mRNA expression. These efforts revealed that the expression of conjunctival muc5AC mRNA was significantly lower in the *Sod1*$^{-/-}$ mice than in the WT mice before eye drop instillation. The decrease in goblet cells and muc5AC expression and increased inflammatory cell density in the conjunctiva of the *Sod1*$^{-/-}$ mice may all be held responsible for the tear instability and ocular surface disease. To clarify whether the diseased ocular surface could be salvaged by a tear and mucin secretagogue, we used 3% diquafosol sodium eye drops in both the *Sod1*$^{-/-}$ and the WT mice.

Recently, diquafosol has been reported to be an agonist of the purinergic P_2Y_2 receptor, which is expressed in several ocular tissues, including the conjunctival epithelium and goblet cells. At the cellular level, the P_2Y_2 receptor is known to contribute to water transfer and mucin secretion [20-22]. In animal studies involving rabbits, diquafosol has been shown to stimulate both water secretion from conjunctival epithelial cells and mucin secretion from conjunctival goblet cells via the P_2Y_2 receptors [25]. Diquafosol has also been shown to prevent corneal epithelial damage in a rabbit dry eye model [26,27]. In a rat model of dry eye disease, diquafosol was demonstrated to improve tear secretion and restore the corneal epithelial barrier function [27].

Interestingly, we observed that tear stability, Rose Bengal staining (which is also known to indicate mucin-secreting cells and assess disorders of mucin secretion [28]) scores, and muc5 mRNA expression improved significantly with diquafosol treatment in both the WT and our KO mice, which was consistent with the observations in other animal models. We presume that the improvements in Rose Bengal scores were due to an increase of mucin expressions on the ocular surface. Fluorescein staining and aqueous tear quantity significantly improved in the *Sod1*$^{-/-}$ mice with diquafosol treatment. The increase in tear quantity may have occurred due to stimulation of tear secretion from the lacrimal glands or increased water secretion from the conjunctiva, the mechanisms of which need to be clarified in future studies. We also suggest that the improvement in tear stability may have resulted from an increase in goblet cell density or increased mucin expression.

Future investigation into the relationships between the changes in mucin expression, goblet cell differentiation, role and type of inflammatory pathway involvement, and P_2Y_2 receptor stimulation should provide very interesting information. Studies looking into P_2Y_2 receptor changes in this age-related dry eye disease mouse model and the possibility of upregulation with diquafosol should also provide

invaluable knowledge for the literature. In conclusion, this study revealed that 3% diquafosol ophthalmic solution was effective in the treatment of ocular surface disease in this KO mouse model; the mechanisms of action need to be clarified in future studies.

ACKNOWLEDGMENTS

This work was presented at the 2012 Japan Cornea Conference, February 23–25,2012, Tokyo, Japan. The authors have no proprietary interests in any of the products written in the paper. Santen Pharmaceuticals made a grant donation to Keio University School of Medicine, Department of Ophthalmology in the amount of one million JY (12.000 USD) for the conduct of the animal study and for the purchase of antibodies and solutions. Santen also provided the 3% diquafosol ophthalmic solutions for the experiments. The mice were kept, and cared for at the Tokyo Dental College, Ichikawa Hospital where the study was initiated and completed. Neither Santen was involved with the scientific content of the research projects run by the Ophthalmology departments of Keio University School of Medicine or Tokyo Dental College Ichikawa Hospital nor the authors were paid employees or received any kind of fees from Santen Pharmaceuticals. Drs Kojima and Dogru contributed equally to the work and request acknowledgment as double first authors.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 28 June 2014. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.