Effects of Topical Mucolytic Agents on the Tears and Ocular Surface: A Plausible Animal Model of Mucin-Deficient Dry Eye

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PURPOSE. A topical mucolytic agent, N-acetylcysteine (NAC), has been used to create an animal model without the intestinal mucus layer. In this study, we investigated the effects of topical NAC on the tears and ocular surface.

METHODS. NAC-treated models were established by topically administering 10% NAC four times daily for 5 days in male Sprague-Dawley rats. Clinical parameters and the expression of mucin proteins and genes were evaluated. Alterations in the conjunctival epithelium and goblet cells were monitored.

RESULTS. The NAC group showed significant decreases in tear secretion, corneal wetting ability, tear MUC5AC concentration, and conjunctival goblet cell numbers as compared with the control group (all P < 0.01). In addition, significant increases in corneal fluorescein score and rose bengal scores were observed in the NAC group versus in the control group (P < 0.05 and P < 0.01, respectively). Hematoxylin and eosin (H&E) staining and scanning electron microscopy clearly showed damage in the epithelial cell layer and microvilli of the NAC group. Although there was no significant difference in MUC16 gene expression, the MUC16 concentration of the tear film and ocular surface tissue was significantly increased in the NAC group versus in the control group (P < 0.01 and P < 0.05, respectively). Five-day treatment with 3% diquafosol had minimal therapeutic effect in NAC-treated rat eyes.

CONCLUSIONS. Topical administration of 10% NAC induced ocular surface damage and tear film instability by prompting MUC16 disruption and release from the ocular surface. This animal model could be used to study dry eye disease, especially the mucin-deficiency subtype.

Keywords: N-acetylcysteine, ocular surface damage, goblet cell, MUC5AC, MUC1, MUC16, dry eye

The precorneal tear film is vital for the maintenance of a healthy, wet ocular surface. The tear film consists of three layers: an outermost lipid layer; a middle aqueous layer, and an inner mucus layer.1 Recently, particular attention has been paid to the function of ocular mucins in dry eye disease.2–5 Mucins on the ocular surface and other wet-surfaced epithelia can be classified into two main categories: secreted or membrane-associated mucins (MAMs).4 Secreted mucins including MUC2 classified into two main categories: secreted or membrane-associated mucins (MAMs).4 Secreted mucins including MUC2 and MUC5AC have no transmembrane-spanning domains and are produced by goblet cells;5,5; they can trap allergens, cell debris, and pathogens to facilitate their clearance from mucosal surfaces.6 MAMs such as MUC1, MUC4, and MUC16, which are concentrated on the tips of apical cell microvilli, form a dense glyocalyx at the epithelial-tear film interface,7 but MAMs’ extracellular domains can also be released from the epithelial cell surface as soluble forms and are found in the tear film (due to shedding).8,9 The functions of the MAMs include antiadhesive, lubrication, water retention, pathogen barrier, and anti-inflammatory actions.7,10–12 Thus, a clear understanding of mucin-associated tear film dynamics is essential in order to successfully treat diseases with accompanying mucin deficiency.

Animal models provide ideal tools to understand the mechanisms underlying the function of the tear film on the ocular surface and of dry eye disease. For the sake of simulating different etiologies, several strategies have emerged for the creation of animal models of dry eye, such as placement in a desiccating environment,13,14 application of a muscarinic receptor antagonist,15 surgical extirpation of the lacrimal gland or testicles,16,17 and topical administration of benzalkonium chloride or atropine.18,19 However, none of these models seem to directly control mucin expression and/or precisely mimic the mucin-deficient form of ocular surface disease.

N-acetylcysteine (NAC) is a mucolytic and a reducing agent that is generally recognized as being able to cause a shift to low-molecular-weight mucin molecules via breakage of mucoprotein disulfide bonds.20 Mucolytic activity of NAC has been evaluated in a variety of respiratory and gastrointestinal tract diseases associated with mucus hypersecretion.21,22 Recently, Sharpe et al.23 and Qin et al.24 have constructed a gut injury model involving the loss of the intestinal mucus layer by gut segment exposure to 10% NAC solution in normal rats and demonstrated that the treatment of the gut with NAC causes a dose-dependent decrease in mucosal hydrophobicity and an increase in microbial invasion.23,24
increase in intestinal permeability. A previous study also reports that multiple or long-time topical instillations with 0.1 M or 10% NAC (dissolved in saline) for 1 day induce the loss of the conjunctival mucus layer or mucin-like substances in rabbit eyes.\textsuperscript{25,26} However, the effects of NAC topical administration on the ocular surface, such as the type of mucin gene and protein changes that occur in the tears and ocular surface, remain unclear, and clinical parameters have not yet been established in any model system.

Thus, in this study, we attempted to evaluate the effects of the topical administration of 10% NAC on mucin expression of the tears and ocular surface and to develop a plausible animal model of mucin-deficient dry eye.

**Materials and Methods**

Forty-two inbred male Sprague-Dawley rats, each weighing 200 to 300 g (age, 6–8 weeks old), were used in this study. The rats were kept in standard environment throughout the study as follows: room temperature 22°C ± 2°C, relative humidity 60% ± 10%, and alternating 12-hour light-dark cycles (8 AM–8 PM). All procedures adhered to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research (ARVO Animal Policy). Institutional Review Board approval was obtained from Korea University Animal Research Center.

**Experiment 1: The Effects of 10% NAC Topical Administration on the Tear Film and Ocular Surface**

**NAC-Treated Model.** To establish the NAC-treated model, 20 μL of 10% (wt/vol) NAC (Muteran; Han Wha Pharma Co., Ltd., Seoul, South Korea) solution was topically administered four times (10:00 AM, 12:00 PM, 2:00 PM, 4:00 PM) a day for 5 days into the right eye of each rat as the NAC group (n = 32). The left eye of the each rat was treated with normal saline as the control group (n = 32).

**Experimental Procedure.** Clinical parameters, including tear volume, tear film stability, corneal fluorescein staining scores, and ocular surface rose bengal staining scores, were measured in five NAC-treated and control eyes (n = 5 for each group). The other 15 NAC-treated and control eyes (n = 15 for each group) were used for the measurement of MUC5AC, MUC1, and MUC16 concentration from tear samples and MUC1 and MUC16 concentration from ocular surface tissue samples, using an enzyme-linked immunosorbent assay (ELISA). In addition, the remaining NAC-treated and control eyes (n = 12 for each group) were removed and the tissue samples were harvested to perform histologic examination, real-time polymerase chain reaction (PCR), and scanning electron microscopy (SEM).

General anesthesia was induced via an intramuscular injection of xylazine hydrochloride (Rompun 2%, 1 mg/100 g body weight; Bayer, Leverkusen, Germany) and zoletil (8 mg/100 g body weight; Virbac laboratories, Carros, France) before tear sample collection, tear film stability detection, ocular surface staining, and euthanasia.\textsuperscript{27}

**Tear Volume Measurements.** Tear production was measured in five eyes from each group by using phenol red-impregnated cotton threads (Zone-Quick; Showa Yakuhin Kako Co., Ltd., Tokyo, Japan). Neither topical nor general anesthesia was administered before the test. Smooth forceps were used to insert the 2-mm folded portion of the thread into the lateral canthus of the lower eyelid for 30 seconds. After 30 seconds, the thread was removed and the tear secretion was measured by evaluating the length of the wet portion (i.e., the red portion).\textsuperscript{26,27}

**Tear Film Stability.** The tear film stability of five eyes from each group was evaluated according to the previous method used to measure corneal wetting ability 15 minutes after instillation of eye drops.\textsuperscript{30,31} The corneal wetting ability was defined as the duration (in seconds) between the time immediately after blinking and the time at which the light beam image began to blur. At the starting point immediately after blinking, the stereomicroscope ring-shaped light beam was clearly visible on the corneal surface; however, at the endpoint, the ring-shaped light beam began to blur. A stereomicroscope (Olympus SZ6; Olympus Corporation, Tokyo, Japan) was used to measure corneal wetting ability, with the distance from microscope to the cornea being approximately 10 cm. The duration was directly measured twice by an independent observer (BK) in a blinded fashion and the mean value was used to compare corneal wetting ability.

**Ocular Surface Staining.** Fluorescein and rose bengal staining of the ocular surface were graded with slit lamp examination under general anesthesia by an independent observer (BK) in a blinded fashion after the last administration of 10% NAC solution or normal saline in five eyes of each group. Fluorescein staining was performed by using a fluorescein sodium-impregnated paper strip (Haag-Streit, Bern, Switzerland) and was graded by using the National Eye Institute (NEI) staining protocol, in which a score of between 0 and 3 (0 being normal and 3 being severe) was assigned to each of the five corneal regions (nasal, central, temporal, inferior, and superior), for a maximum total score of 15.\textsuperscript{32} The rose bengal staining score (0–3) was assigned by using a rose bengal strip (Akriti, Hyderabad, India) and evaluated according to the Shimmmura method for the superior, inferior, and mid-cornea and the temporal and nasal bulbar conjunctiva.\textsuperscript{33}

**Sample Collection of Tear and Ocular Surface Tissue.** Tear samples were obtained from 15 eyes in each group in order to measure the concentration of MUC5AC, MUC1, and MUC16 in tear (n = 5 for each group) by using the flush tear collection method, as previously described.\textsuperscript{34} To obtain at least 100 μL of eye-flush tears from each eye, 60 μL sterile normal saline was introduced into the space between the eye and the lateral canthus, and fluid was aspirated from the inferior meniscus by using a micropipette tip. In each collection of eye-flush tears, approximately 50 to 60 μL tear volume was collected. Sterile normal saline was instilled twice and the tear sampling was performed within 1 minute. After tear samples were obtained, the rats were humanely euthanized in a CO\textsubscript{2} chamber. Ocular surface tissue samples were surgically harvested from 15 eyes of each group (n = 5 for each group) and homogenized together in a Tper tissue protein extraction reagent containing a protease inhibitor mixture (Thermo Scientific, Rockford, IL, USA). The supernatant from the homogenates was then collected following centrifugation for 15 minutes at 1500g. All samples were stored at −20°C until ELISA analysis.\textsuperscript{27}

**Enzyme-Linked Immunosorbent Assay**

The level of secreted mucin in the tear of each group (n = 5 for each group) was measured with an ELISA kit for mucin-5 subtype AC (catalog No. MSB729889; Mybiosource, San Diego, CA, USA),\textsuperscript{26,31} and the levels of MUC1 and MUC16 in the tear and ocular surface tissue (all n = 5 for each group) were measured with the MUC1 ELISA kit (catalog No. MSB204481; Mybiosource) and the MUC16 ELISA kit (catalog No. MSB7228775; Mybiosource), respectively.\textsuperscript{35} All measurements were conducted according to the manufacturer\textquoteleft s protocol by using a
microplate spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA).

**Total RNA Isolation and Real-Time Polymerase Chain Reaction.** For the extraction of RNA, ocular surface tissue samples from three eyes of each group were collected and homogenized together in TRIzol solvent (Takara Bio, Inc., Shiga, Japan) and total RNA was isolated from the homogenized samples according to the product protocol. Complementary DNA was generated by using 2 μg RNA from the ocular surface tissue by a one-cycle reverse transcriptase reaction with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Quantitative real-time PCR was performed by using SYBR Premix Ex Taq II (Tli RNase H Plus) on Quant Studio 6 Flex system (Applied Biosystems, Life Technologies; Caramarillo, CA, USA). Primer sequences were as follows: GAPDH (5'-CATACCAAGAAATGAGCTCCTCAAA-3' and 5'-TCCACCACCGTGGTGCTGTA-3'); MUC1 (5'-TTGTTGGGCTTTGATGTCTG-3' and 5'-CATTGCCCAGACCAATGCTC-3'); and MUC16 (5'-TGGTACCCAGCTGTAAGACCTTG-3' and 5'-TAGGCGGTGTACGTTGAAGTTG-3'). To quantify the relative expression of each gene, the threshold cycle (CT) values were normalized for endogenous reference (ACT = CT target - CT GAPDH) and a calibrator using the ΔΔCT method (ΔΔCT = ΔCT sample - ΔCT calibrator).

**Histology.** After the last administration of 10% NAC solution or normal saline, the entire eyeball (including the eyelids and conjunctiva) was surgically enucleated from six eyes of each group. Enucleated eyeballs were fixed in PBS-buffered 4% formalin at 4°C overnight and embedded in paraffin. Tissue blocks were cut into 4-μm-thick sections and stained with hematoxylin-eosin and PAS dye. The morphology of the conjunctival epithelium was assessed under a microscope and images were recorded with a digital camera. Goblet cells were counted in nine representative slices of homologous positions of tissues from each group. The respective average number of goblet cells was compared between the two groups. In addition, the conjunctival epithelium was categorized into smooth surface and defective surface categories in nine representative slices (n = 9 for each group), and the percentage was compared between NAC-treated and control eyes.

**Scanning Electron Microscopy**

After the last administration of 10% NAC solution or normal saline, the conjunctival tissues from three eyes in each group were gently excised at the superior bulbar conjunctiva and prefixed in 2% glutaraldehyde in 0.1 M phosphate buffer. Samples were then postfixed for 2 hours in 1% osmic acid dissolved in PBS for SEM. Then, conjunctival tissues were treated in a graded series of ethanol and t-butyl alcohol, dried in a freeze dryer (ES-2030; Hitachi, Tokyo, Japan), and coated with platinum by using an ion coater (IB-5; Eiko, Ibaraki, Japan). The appearance of conjunctival epithelial cells was observed via FE-SEM (S-4700; Hitachi). The microvilli area per conjunctival unit area was defined as the ratio of the conjunctival microvilli area to the total area in each representative digital image from SEM. First, the unit of measurement of the still pictures was changed from distance in pixels to millimeters, based on the graduated ruler on the bottom right of the digital images, as previously described.

Next, the nine different areas (82.5 μm²) of each digital image were selected and measured by using the "Image Adjust Brightness/Contrast," "Edit Invert," and "Image Adjust Threshold" of ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) (Fig. 1).

**Experiment 2: The Effect of Topical Diquafosol Tetrasodium on Tear MUC5AC Concentration in a NAC-Induced Mucin-Deficient Rat Model**

Diquafosol tetrasodium is a novel mucin secretagogue that has been used to treat dry eye disease in Japan and South Korea. In our previous study, diquafosol ophthalmic solution effectively stimulated MUC5AC secretion in both normal rat and a dry eye animal model induced by subcutaneous scopolamine injection and exposure to environmental desiccating stress. Therefore, we sought to evaluate the effect of diquafosol on MUC5AC secretion in this animal model of mucin-deficient dry eye.

First, 20 μL of 10% NAC solution was topically administered four times a day for 5 days into the right eyes of each rat as described above, and the left eyes of each rat were untreated. After establishing the mucin-deficient model, 10 NAC-treated rats (n = 10) were randomly divided into two groups. Next, 20 μL of 3% diquafosol solution (Diquas Ophthalmic Solution 3%; Santen, Osaka, Japan) was topically instilled six times a day (9:00 AM, 10:30 AM, 12:00 PM, 13:30 PM, 15:00 PM, 16:30 PM) for 5 days into the right eyes of five rats as the diquafosol group (n = 5), while the right eyes of the remaining five rats were treated with normal saline as the saline (control) group (n = 5). Clinical parameters including tear volume, tear film corneal wetting ability, corneal fluorescein staining scores, ocular surface rose bengal staining scores, and tear MUC5AC concentration were compared between the diquafosol and saline groups (n = 5 for each group). All of the methods described above were also used in experiment 2.

**Statistical Analyses**

Statistical analyses were performed by using the Mann-Whitney U test in SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). Values were expressed as the median and interquartile range (IQR). Results were considered statistically significant at a P value less than 0.05.

**Results**

**Experiment 1: The Effect of 10% NAC Topical Administration on the Tears and Ocular Surface**

**Effect of NAC on Clinical Evaluation.** Topical treatment with a mucolytic agent induced significant decreases in tear secretion and corneal wetting ability. The median (IQR) tear secretion in the NAC group (5.0 mm [5.0–5.0]; Fig. 2A; Table 1) was significantly decreased as compared with that of the control group (9.5 mm [9.0–10.0]; P = 0.008; Fig. 2A; Table 1). The median (IQR) corneal wetting ability in the NAC group (69.0 seconds [66.0–72.0]; Fig. 2B; Table 1) was significantly shorter than that of the control group (119.0 seconds [102.0–120.0]; P = 0.008; Fig. 2B; Table 1). In addition, ocular surface damage was found in the NAC group compared with the control group. The median (IQR) corneal fluorescein staining score in the NAC group (7.0 [6.0–9.0]; Figs. 2C, 2F; Table 1) was significantly higher than that of the control group (1.0 [1.0–2.0]; P = 0.016; Figs. 2C, 2E; Table 1). The median (IQR) ocular surface rose bengal staining score in the NAC group (6.0 [6.0–7.0]; Figs. 2D, 2H, 2J; Table 1) was significantly higher than that of the control group (1.0 [0.0–2.0]; P = 0.008; Figs. 2D, 2G, 2I; Table 1).

**Effects of NAC on the Conjunctival Epithelia and Goblet Cells.** After topical treatment with 10% NAC, reductions in goblet cell density and ocular surface damage were observed. The median (IQR) PAS-positive conjunctival goblet cell numbers in the NAC group (41.0 [37.0–48.0]; Fig. 3;
Table 1) were significantly decreased versus those of the control group (90.0 [76.0–100.0]; \( P = 0.000 \); Fig. 3; Table 1). The percentage of defective surface category in the NAC group (88.8%) was significantly higher than that of the control group (11.1%).

Evidence of conjunctival epithelial damage was visible on SEM in the NAC group but not in the control group. NAC-treated eyes clearly showed desquamation in conjunctival epithelial cells but not in the control group (Figs. 4A, 4B). The median (IQR) conjunctival microvilli area in the NAC group (5.49% [5.29%–5.58%]; Figs. 4D, 4F; Table 1) was significantly decreased as compared with that of the control group (58.86% [58.72%–67.98%]; \( P = 0.000 \); Figs. 4C, 4E) (Fig. 4G).

**Effects of NAC on Mucin Subtypes.** After topical treatment with 10% NAC, the tear MUC5AC concentration was significantly decreased in NAC-treated eyes. The median (IQR) tear MUC5AC concentration in the NAC group (11.04 ng/mL [10.05–11.06]; Fig. 5; Table 1) was significantly decreased as compared with the control group (16.65 ng/mL [16.38–16.81]; \( P = 0.008 \); Fig. 5; Table 1).

In the evaluation of the effects of NAC on the protein and gene expression of MUC1 and MUC16, there were no significant differences in the MUC1 concentration of tears and ocular surface tissue. However, the median (IQR) MUC16 concentrations of tears and ocular surface tissue in the NAC group (80.43 pg/mL [69.97–111.56] and 45.37 pg/mL [33.33–54.23], respectively) were significantly increased versus those of the control group (15.34 pg/mL [13.11–18.20] and 26.25 pg/mL [17.17–27.28], respectively; \( P = 0.008 \) and \( P = 0.032 \), respectively) (Fig. 6; Table 1). The gene expression of MUC1 and MUC16 mRNA from ocular surface tissue was not significantly different between the NAC and control groups (Fig. 7; Table 1).

**Experiment 2: The Effect of Topical Diquafosol Sodium on Tear MUC5AC Concentration in a NAC-Induced Mucin-Deficient Model**

Although 3% diquafosol solution, a mucin secretagogue, was topically instilled six times a day for 5 days, there were no significant differences in tear secretion, corneal wetting ability, corneal fluorescein staining score, and rose bengal staining score between the diquafosol and saline groups (Table 2). In addition, the tear MUC5AC concentration in the diquafosol group did not vary from that of the saline group (Table 2).
DISCUSSION

Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability, with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. Tear film instability results from a relative loss of tear volume or from imbalanced tear composition; tear film instability may induce chronic ocular surface dryness. Hormones, mucins, and lipids are all associated with tear film stability. The disease mechanism includes long-standing local inflammation in the conjunctiva and cornea, dysfunction of the lacrimal glands with the disturbance of tear secretion, meibomian gland dysfunction, reduction of mucin expression, and others, all of which potentially result in damage to the ocular surface epithelia. Thus, a good animal model that simulates various clinical subtypes of dry eye problems would be a useful tool, not only for investigation of the pathophysiology and pathogenesis of dry eye but also for...

FIGURE 2. Clinical ocular surface evaluations of the control and NAC groups including tear volume (A), tear film corneal wetting ability (B), fluorescein staining (C), and rose bengal staining (D) after treatment with normal saline or 10% NAC for 5 days and representative images of corneal fluorescein in the control group (E) and NAC group (F). The representative front and side images of rose bengal staining in the control group (G and I, respectively) and NAC group (H and J, respectively) can be seen. The asterisks indicate a P value < 0.05 as determined by the Mann-Whitney U test.

TABLE 1. A Comparison of Clinical Parameters and Mucin Expression in Normal Rat Eyes Between the Control and NAC Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Group</th>
<th>NAC Group</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tear secretion, mm</td>
<td>9.5 (9.0–10.0)</td>
<td>5.0 (5.0–5.0)</td>
<td>0.008</td>
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<tr>
<td>Corneal wetting ability, s</td>
<td>119.0 (102.0–120.0)</td>
<td>69.0 (66.0–72.0)</td>
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<td>Corneal fluorescein staining score</td>
<td>1.0 (1.0–2.0)</td>
<td>7.0 (6.0–9.0)</td>
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<tr>
<td>Ocular surface rose bengal staining score</td>
<td>1.0 (0.0–2.0)</td>
<td>6.0 (6.0–7.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>Conjunctival goblet cell number</td>
<td>90.0 (76.0–100.0)</td>
<td>41.0 (37.0–48.0)</td>
<td>0.000</td>
</tr>
<tr>
<td>Conjunctival microvilli area per unit area, %</td>
<td>58.86 (58.72–67.98)</td>
<td>5.49 (5.29–5.58)</td>
<td>0.000</td>
</tr>
<tr>
<td>Tear MUC5AC, ng/mL</td>
<td>16.63 (16.38–16.81)</td>
<td>11.04 (10.05–11.06)</td>
<td>0.008</td>
</tr>
<tr>
<td>Tear MUC1, ng/mL</td>
<td>0.30 (0.27–0.32)</td>
<td>0.25 (0.21–0.25)</td>
<td>0.222</td>
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<tr>
<td>Tear MUC16, pg/mL</td>
<td>15.34 (13.11–18.20)</td>
<td>80.43 (69.97–111.56)</td>
<td>0.008</td>
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<td>Ocular surface tissue MUC1, ng/mL</td>
<td>0.71 (0.69–0.86)</td>
<td>0.66 (0.66–0.73)</td>
<td>0.421</td>
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<td>Ocular surface tissue MUC16, pg/mL</td>
<td>26.25 (17.17–27.28)</td>
<td>45.37 (33.53–54.23)</td>
<td>0.032</td>
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<td>Ocular surface tissue MUC1/GAPDH mRNA</td>
<td>1.33 (1.32–1.33)</td>
<td>1.29 (1.28–1.30)</td>
<td>0.100</td>
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<tr>
<td>Ocular surface tissue MUC16/GAPDH mRNA</td>
<td>1.37 (1.35–1.47)</td>
<td>1.34 (1.32–1.34)</td>
<td>0.400</td>
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</table>

Data are expressed as the median (interquartile range) of each eye from each group. The experimental groups are the same as those in Figure 2. * Mann-Whitney U test.
**Figure 3.** A comparison of the number of goblet cells in the conjunctiva as observed by PAS staining (magnification: ×400) and the alterations of conjunctival epithelium integrity observed by H&E staining (magnification: ×400) in the control and NAC groups after treatment with normal saline or 10% NAC for 5 days. The experimental groups are the same as those in Figure 2. (A) PAS staining of the conjunctival fornix in the control group. (B) PAS staining of the conjunctival fornix in the NAC group. (C) H&E staining of the conjunctival fornix in the control group. (D) H&E staining of the conjunctival fornix in the NAC group. (E) Median (IQR) PAS-positive cell numbers in each group. The asterisk indicates a *P* value < 0.05 as determined by the Mann-Whitney *U* test.

**Figure 4.** A comparison of the alterations in the conjunctival epithelium as observed by SEM in the control and NAC groups after treatment with normal saline or 10% NAC for 5 days. The experimental groups are the same as those in Figure 2. (A) Conjunctival epithelial cells in the control group (magnification: ×1000). (B) Conjunctival epithelial cells in the NAC group (magnification: ×1000). (C) Microvilli of conjunctival epithelial cells in the control group (magnification: ×4000). (D) Microvilli of conjunctival epithelial cells in the NAC group (magnification: ×4000). (E) Digital image prepared in (C) by ImageJ for analysis of microvilli area. (F) Digital image prepared in (D) by ImageJ for analysis of microvilli area. (G) Comparison of conjunctival microvilli area between the control and NAC groups. The asterisk indicates a *P* value < 0.05 as determined by the Mann-Whitney *U* test.
the development of new therapeutic treatments. Based on the mucolytic effect of NAC, this study attempted to control mucin expression and mimic the mucin-deficient subtype of ocular surface disease. Our findings indicated that the rat mucin-deficient model induced by topical medication of 10% NAC demonstrated characteristics consistent with human dry eye syndrome, including secreted mucin deficiency and ocular surface damage, as well as those seen with tear film instability and tear deficiency.

Tear break-up time (BUT) is a parameter commonly measured to assess tear film stability in dry eye syndrome. However, it is difficult to measure BUT in animal models, especially in small animals. Corneal wetting ability, introduced by Miyake et al. and applied in our previous study, is an alternative method used to evaluate tear film stability. In our previous study, 15 minutes after the instillation of 3% diquafoxol solution into both eyes of normal rats, the mean corneal wetting time is significantly increased. On the other hand, Anderton and Tragoulias report that, after the instillation of 20% NAC into the eyes of rats, the thickness of the tear fluid layer is reduced to 19 µm compared with 32 µm after the administration of only physiologic saline solution. In our study, the mean corneal wetting time was decreased significantly in the NAC group. Fluorescein staining and rose bengal staining are effective methods for ocular surface evaluation. Fluorescein staining is the result of uptake caused by the disruption of corneal epithelial cell–cell junctions or damaged corneal epithelial cells. Rose bengal has been demonstrated to stain corneal and conjunctival epithelial cells that are not adequately protected by the preocular tear film. According to Feenstra and Tseng, rose bengal can stain live and dead cells if they are not protected by an intact mucin layer. On the other hand, Argueso et al. have suggested that MUC1 and MUC16 form a protective barrier on the cell surface, thereby reducing the internalization of rose bengal and cell surface binding of cationized ferritin. As shown in our results, the fluorescein staining and rose bengal staining scores

![Figure 5](image1.png)

**Figure 5.** A comparison of MUC5AC levels in rat tear samples among the control and NAC groups after treatment with normal saline or 10% NAC for 5 days. The experimental groups are the same as those in Figure 2. The *asterisk* indicates a *P* value < 0.05 as determined by the Mann-Whitney *U* test.

![Figure 6](image2.png)

**Figure 6.** A comparison of MUC1 and MUC16 levels in rat tear and ocular surface tissue samples among the control and NAC groups after treatment with normal saline or 10% NAC for 5 days. The experimental groups are the same as those in Figure 2. (A) A comparison of MUC1 levels in tears between the control and NAC groups. (B) A comparison of MUC16 levels in tears between the control and NAC groups. (C) A comparison of MUC1 levels in ocular surface tissues between the control and NAC groups. (D) A comparison of MUC16 levels in ocular surface tissues between the control and NAC groups. The *asterisks* indicate a *P* value < 0.05 as determined by the Mann-Whitney *U* test.
significantly increased following topical instillation of a mucolytic agent.

Goblet cells are highly specialized epithelial cells located on the apical surface of the conjunctiva. The main functions of goblet cells are the synthesis, storage, and secretion of MUC5AC in the tear film. As shown in our results, the concentration of tear MUC5AC was significantly decreased in the NAC group, which is supported by the significant loss of goblet cell number in the NAC group. MAMs on the ocular surface projected from microplicae on the anterior surface of corneal and conjunctival epithelial cells are important components of the mucous layer of the tear film. Their functions include antiadhesive, lubrication, water retention, pathogen barrier, and anti-inflammatory effects. The most prevalent mucins expressed on the ocular surface are MUC1, MUC4, and MUC16. Urashima et al. report that on the 14th day after administration of six topical doses of 10% NAC at approximately 2-hour intervals during a single day into the eyes of rabbits, the amounts of conjunctival and corneal mucin-like substances are significantly decreased. In their study, mucin-like substances having high-molecular glycoproteins on the corneal and conjunctival surfaces are measured by using the Alcian-blue binding method. However, this method cannot be used to verify the mucin subtype. To the best of our knowledge, this is the first study to quantitatively evaluate the concentration of several mucin subtypes after topical NAC treatment. The concentration of tear MUC5AC was significantly decreased, whereas the MUC16 concentrations in tear and ocular tissue increased unexpectedly. At present, the different responses between MUC1 and MUC16 cannot be properly explained. However, MUC1 is the smallest and MUC16 is the largest membrane-associated mucin. MUC16 is more than twice as long as MUC1. Previous studies have demonstrated that neutrophil elastase, MMP-7, MMP-9, and bacterial metalloprotease induce the release of the extracellular domain of MUC16. Therefore, MUC16 can be more easily released from the cell surface after topical NAC treatment and can be present at higher concentrations in tear and ocular tissues. Further studies are needed to determine the mechanism by which NAC acts differently on mucin subtypes.

In our animal model of mucin-deficient dry eye, tear secretion was significantly decreased as compared with that of the control group (median tear secretion: 5.0 mm versus 9.5 mm). However, a previous study using Spdef knockout mice to block goblet cell differentiation has produced contrasting results. Although Spdef knockout mice, which lack conjunctival goblet cells and exhibit down-regulation of goblet cell-specific genes, showed a mild dry eye phenotype, the tear volumes of Spdef knockout mice were significantly higher than those of age-matched Spdef wild-type mice. The authors suggest that the observed increase in tear volume may be due to a compensatory mechanism for the loss of the secretory mucins. However, other dry eye animal models induced by topical administration of benzalkonium chloride or air pollution significantly.
tion particulate matter show a significant decrease in tear secretion even when scopolamine is not used to inhibit aqueous tear production. As compared with Sfpdef knockout mice, ocular surface damage was more severe in our model. For this reason, the compensatory mechanism may not work. In addition, severe conjunctival damage may affect tear secretion from accessory lacrimal glands within the conjunctiva.

SEM was used to study the normal and pathologic ultrastructure of ocular surfaces to evaluate the local tolerance of ophthalmic preparations. Thermes et al. report that, following the application of 0.1 M NAC 12 times at approximately 5-minute intervals for 1 hour into the eyes of rabbits, the conjunctival epithelial cells are not covered with a mucus layer and the microvilli are clearly visible on SEM. In their study, the authors note that a 0.1 M solution of NAC is adequate to dissolve the mucus deposited on the conjunctival and corneal surfaces by blinking. On the other hand, they also show that the topical application of NAC solution into rabbit eyes induces epithelial cell degeneration and desquamation and microvilli loss in a dose-dependent manner. In our study, SEM evaluation confirmed that topical treatment of 10% NAC solution administered for 5 days caused severe damage to the conjunctival epithelium. NAC-treated rats showed desquamation of conjunctival epithelial cells and a significant reduction in microvilli area, which was supported by the higher percentage of defective conjunctival surface in the NAC group and consistent with the findings of a previous study of NAC-treated rabbits.

Diquafosol tetrasodium (P2Y2 purinogenic receptor agonist), rebamipide, and vitamin A (VA, retinoic acid), which promote tear and mucin secretion, have been reported to be effective in improving the clinical symptoms of various dry eye diseases. In our previous study, diquafosol ophthalmic solution effectively stimulated MUC5AC secretion in both normal and dry eye animal model, which was induced by subcutaneous scopolamine injection and exposure to environmental desiccating stress. Urashima et al. report that treatment with 1% rebamipide increases the amount of mucin-like substances covering the cornea and conjunctiva of 10% NAC-treated rabbit eyes. However, the findings of our study revealed minimal therapeutic effects of 3% diquafosol in mucin-deficient rat models after 5 days of treatment. We think that a 5-day treatment period was insufficient to show the possible therapeutic effects. Longer-term treatment with 3% diquafosol is needed to explore the therapeutic effects in an animal model of ocular surface mucin deficiency.

There were some limitations to this study, which aimed to create a mucin-deficient dry eye animal model using a topical mucolytic agent. First, the animal sample size was relatively small. In addition, a reduction in all mucin subtypes was expected in this animal model, but different results were obtained depending on the mucin subtype. After topical application of 10% NAC solution, the fluorescein and rose bengal staining of the ocular surface increased dramatically and the microvilli of conjunctival epithelial cells were significantly decreased. In addition, the tear concentration of MUC5AC secreted from goblet cells decreased. However, the tear concentration of MUC1 did not change, whereas that of MUC16 increased. To establish a more ideal mucin-deficient subtype model, we need to use NAC solutions at various concentrations and increase the duration of NAC topical application. Ten percent NAC solution seems to be a relatively high concentration, so we cannot exclude the possibility of toxicity. Additional research is needed in this regard.

In conclusion, the topical administration of 10% NAC induced ocular surface damage and tear film instability by affecting MUC16 to the point of disruption and release from the ocular surface. This animal model could be used to study dry eye disease, especially the mucin-deficient subtype.

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