Title of Thesis

Development of clinical and non clinical method for dry eye

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**List of Abbreviation**

ARVO, Association for Research in Vision and Ophthalmology

AzaSite®, Azithromycin ophthalmic solution

CFA, complete Freund’s adjuvant

DED, dry eye disease

DEWS, Dry eye workshop

HE, hematoxylin-eosin

MGD, meibomian gland dysfunction

MRKC, meibomitis-related keratoconjunctivitis

ND, normal diet

OCT, optical coherence tomography

SLE, systemic lupus erythematosus

TFOT, tear film oriented therapy

TBUT, tear film break-up time

Tob/Dex, Tobramycin/dexamethasone ophthalmic suspension
Chapter 1  General introduction

What is dry eye?

Dry eye is one of the most common ophthalmic diseases. Studies on the overall prevalence of dry eye have shown that dry eye incidence increases worldwide with age, longer life spans, aging populations, and contact lens use. Based on the 2007 Dry Eye Workshop (DEWS) report, the prevalence of dry eye ranges from 5% to 30% and occurs in 25% of patients over 65 years of age.\(^1\) Furthermore, lifestyle changes are becoming an increasing contributor to dry eye as more people spend substantial time working on computers and staring at the electronic screens of televisions, tablets, and smart phones. Recently, the Asia Dry Eye Society\(^2\) has provided the following definition of dry eye: “Dry eye is a multifactorial disease characterized by an unstable tear film causing a variety of symptoms and/or visual impairment, potentially accompanied by ocular surface damage.” This definition suggests that an unstable tear film is the pivotal mechanism underlying dry eye-induced symptoms and/or visual impairment.

According to the definition, a new concept of treatment strategy was developed by the Japanese and the Asian Dry Eye Societies.\(^2\) Based on the new definition, an unstable tear film is the major factor involved in the diagnosis of dry eye, suggesting a crucial role of the condition of the tear film in maintaining a healthy ocular surface. The tear film comprises three layers: lipid, aqueous, and mucin. When one layer becomes abnormal, the tear film layer becomes unstable, possibly leading to the development of dry eye. This has led to the development
of a new strategy called tear film-oriented therapy (TFOT). The TFOT concept suggests that if an abnormal layer is present, then that layer should be targeted for treatment. For example, if the lipid layer is affected, such as that in meibomian gland dysfunction (MGD), then it is appropriate to treat the meibomian glands.

**Challenges of Dry Eye**

Clarifying the causative factors of dry eye will allow the administration of appropriate drugs to the patients. Measuring tear film stability and the quantity and quality of tears is essential. Recent diagnostic technologies have greatly improved the process and ability to better characterize the various types of dry eye disorders; however, measuring tear volume has continued to remain a challenge. Although the Schirmer test, which measures tear volume, has been available for over a century, clinical studies have shown that it does not accurately detect the efficacy of drugs in patients with dry eye and that variability is a potential cause. The methodology of this test has changed over the years because of its variability. Detecting the precise tear volume allows the clinician to not only diagnose dry eye but also more efficiently develop drugs for it.

The oily layer plays an important role in maintaining the stability of the tear film by preventing tears from evaporating, reducing the surface tension of tears, and reducing the friction of blinking; MGD, in which the meibomian glands do not secrete sufficient oil into the tears, impairs the quality of life and vision. No approved drugs for MGD exist. The causes and progression of MGD should be further investigated to develop better pharmacologic treatments. Appropriate
animal models mimicking MGD with a pathogenesis similar to that in humans are strongly desired for understanding the pathophysiology of this disease and developing potential drugs.
Chapter 2 Tear volume estimation using a modified Schirmer test: a randomized, multicenter, double-blind trial comparing 3% diquafosol ophthalmic solution and artificial tears in dry eye patients.

2.1 Introduction

One of the characteristic signs of dry eye patients is a low tear production. Lower tear volume leads to tear film instability, superficial punctual keratitis and dry eye symptoms, such as dryness, foreign body sensation and ocular discomfort. Drugs that enable an increase in tear volume are used for the treatment of dry eye patients. Detecting the precise tear volume allow the clinician to not only diagnose dry eye but also develop dry eye drugs more efficiently.

More than a century ago, Schirmer reported a method to detect tear volume. The device is made of strip filter paper and is able to be performed at any medical facility due to its low cost and lack of special equipment. The Schirmer test is one of the most important tests to diagnose dry eye disease and clinical endpoints. A Schirmer score of greater than 10 mm in 5 minutes is widely accepted as the normal value, whereas a score less than 5 mm is indicative of tear deficiency.

Although the Schirmer test has been available for over a century, clinical studies have shown that it does not properly detect the efficacy of drugs in patients with dry eye and that variability is one of the potential causes. The methodology of the test has changed over the years because of its variability. Some reports have investigated various factors, such as whether the test should
be performed with eyes open or eyes closed\textsuperscript{10,11}, eye position\textsuperscript{12}, measurement time\textsuperscript{11,13}, and with or without anesthesia.\textsuperscript{14}

Some new tests are now emerging, such as the tear meniscus height by the optical coherence tomography (OCT) test\textsuperscript{15,16}, the radius curvatures by the meniscometer test\textsuperscript{17,18} and the tear volume by the strip meniscometry test.\textsuperscript{19} The OCT and meniscometer tests can precisely measure the tear meniscus height and radius curvatures. However, the equipment would not be appropriate for the subset of dry eye patients who do not form a normal tear meniscus, eg, patients with an irregular lid margin, conjunctivochalasis and severely low tear production.

Diquafosol ophthalmic solution 3\% (diquafosol 3\%) has been launched as a drug for dry eyes in some Asian countries. Diquafosol stimulates the P2Y2 receptors on the ocular surface. A previous report concluded that diquafosol 3\% increases the tear meniscus height by OCT in dry eye patients after a long treatment\textsuperscript{20} and the tear meniscus radius curvature by meniscometry in healthy subjects\textsuperscript{21} and Sjögren's syndrome patients.\textsuperscript{22} Diquafosol 3\% also stimulates the secretion of sialic acid in tears, which is a mucin-like substance, in healthy subjects after a single dosing.\textsuperscript{23} Diquafosol 3\% improved dryness after 4 weeks of treatment in a phase 2 clinical trial.\textsuperscript{24} To our knowledge, there are no reports using the Schirmer test to detect a significant increase in tear volume and an improvement in symptoms after a single dosing of diquafosol 3\% in dry eye patients.
The question remains as to whether the Schirmer test is a useful way to evaluate the efficacy of drugs that produce tears in dry eye patients. The purpose of this study was to use a modified Schirmer test to investigate the increase in tear volume after a single administration of diquafosol 3% in dry eye patients.

2.2 Materials & Methods

2.2.1 Subjects

One hundred and two eligible eyes from 51 Japanese patients with dry eye (≥ 20 years) were enrolled in this clinical study. The inclusion criteria were as follows: (1) a definitive diagnosis of dry eye based on the 2006 dry eye diagnostic criteria of the Japanese Dry Eye Society⁹ (subjective symptoms, abnormal tear state and superficial punctate keratitis) in both eyes, and (2) a result (average of the longest part and the shortest part) of the Schirmer test without anesthesia for 5 minutes of less than 10 mm in both eyes. Patients who had an intraocular operation within 90 days before the screening or treatment to close the punctal within 30 days before the screening were excluded. Patients with ocular disease requiring treatment other than for dry eye, allergic conjunctivitis that worsened during the study period, contact lens use or ophthalmic solution use on the study drug dosing day were also excluded.

2.2.2 Study Materials

Diquafosol 3% (Diquas® ophthalmic solution 3%, Santen Pharmaceutical Co, Ltd, Osaka, Japan) and artificial tears (Soft Santear®, Santen Pharmaceutical Co, Ltd,
Osaka, Japan: as the control) were used as study drugs.

2.2.3 Randomization

The subjects were randomized corresponding to allocation codes generated for group A (right eye: diquafosol 3%, left eye: artificial tears) and group B (right eye: artificial tear, left eye: diquafosol 3%) by a randomization manager using the permuted block method.

2.2.4 Study design and treatment

This study was a randomized, multicenter, double-blind, right-eye and left-eye comparison clinical study from November 2014 to February 2015. The study protocol comprised screening and treatment periods, both of which occurred on the same day (Fig. 2-1). All patients who agreed to participate in this study provided written informed consent. After informed consent, subjects received the 1st Schirmer test without anesthesia (9:30 ± 1 hour). Eligible subjects were randomized into two groups and administered one drop of each study drug in each eye at 3 hours 20 minutes after the 1st Schirmer test. Ten minutes after administration, the 2nd Schirmer test was implemented because diquafosol 3% significantly increases tear volume within 15 minutes.\(^\text{21}\) Finally, the 3rd Schirmer test was performed 3 hours 40 minutes after administration. The Schirmer test was performed at intervals of more than 3 hours to confirm the disappearance of efficacy of diquafosol 3%.\(^\text{25}\) The subjective symptoms associated with dry eye were interviewed prior to each Schirmer test.

The primary endpoint was the amount of change in tear secretion from baseline
at the time of the second Schirmer test. The secondary endpoints were as follows: (1) the change in tear secretion from baseline at the time of the third Schirmer test and (2) subjective symptoms. The presence of ocular symptoms was evaluated by conducting interviews; 12 items evaluated subjective symptoms. The severity of each ocular symptom was assessed on a 4-point scale ranging from 0 to 3 as follows: 0=no symptoms, 1=mild, 2=moderate, and 3=severe. This study was performed in 4 sites in Japan, conducted in compliance with the Declaration of Helsinki, and was approved by the Ethical Review Board of Yokohama Minoru Clinic. This study was also registered at www.clinicaltrials.jp under the identifier JapicCTI-142713 (website registration date: November 19, 2014). In addition, we received an external audit by the Intellim Corporation (Osaka, Japan) regarding the data collection, data management and statistical analysis.

Figure 2-1. Study design
The study protocol was a randomized, multicenter, double-blind, right-eye and left-eye comparison clinical study. The screening and treatment periods occurred on the same day.

2.2.5. Schirmer test procedure

Schirmer test strips (Shirumeru test paper, Showa Yakuhin Kako Co, Ltd, Tokyo, Japan) were used in this study. The examiner held the tip of the Schirmer test strip in a hook-like conformation to the lower eyelid of the outside 1/3 of the conjunctival sac of each subject without anesthesia. Subjects closed their eyelids during the test. After 5 minutes, the investigators removed the test strip and traced the tear edge with a pen, measuring the shortest and longest parts with an electronic caliper. The mean value between the longest and shortest parts was used for the statistical analysis. The Schirmer test, administered three times a day, was implemented by the same investigator.

2.2.6 Evaluation of ocular symptoms

Prior to each Schirmer test, the effects of a single dose of diquafosol 3% and artificial tears on subjective ocular symptoms was evaluated by conducting interviews regarding symptoms, such as foreign body sensation, photophobia, itching, eye pain, dryness, heavy eye feeling, blurred vision, asthenopia, eye discomfort, eye discharge and tearing. The severity of ocular symptoms was assessed by on a four-point scale from 0-3 (0=no symptoms, 1=mild, 2=moderate, 3=severe).
2.2.7 Statistical Analyses

Data are shown as the mean ± standard error. The change in tear secretion from baseline was compared between the study drug groups by paired t-tests. SAS software version 9.4 (SAS Inc., Cary, NC, USA) was used for statistical analyses.

2.3 Results

2.3.1 Patients

Informed consent was obtained from 66 patients. Fifteen subjects who did not meet the criteria of the Schirmer test value were removed during the screening period. Finally, 51 subjects were randomized. The randomized 51 subjects all completed the schedule without discontinuation. One subject was excluded from the efficacy analysis set because a protocol deviation affecting the efficacy evaluation occurred. Table2-1 shows that there were no differences between groups A and B with regard to the demographic data of the efficacy analysis set. At baseline (1st test), the Schirmer scores of the diquafosol 3% group and artificial tear group were 2.886 ± 0.399 mm and 3.010 ± 0.387 mm, respectively (Table 2-2). There was no significant difference between the study drug groups.
Table 2-1. Demographic data.

<table>
<thead>
<tr>
<th></th>
<th>Group A (N=25)</th>
<th>Group B (N=25)</th>
<th>Total (N=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>3 (12.0)</td>
<td>4 (16.0)</td>
<td>7 (14.0)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>22 (88.0)</td>
<td>21 (84.0)</td>
<td>43 (86.0)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>25</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>59.4 (16.7)</td>
<td>58.1 (18.4)</td>
<td>58.7 (17.4)</td>
</tr>
<tr>
<td>Min, Max</td>
<td>26, 83</td>
<td>26, 82</td>
<td>26, 83</td>
</tr>
<tr>
<td><strong>Age Group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 65 years, n (%)</td>
<td>14 (56.0)</td>
<td>12 (48.0)</td>
<td>26 (52.0)</td>
</tr>
<tr>
<td>65 years or more, n (%)</td>
<td>11 (44.0)</td>
<td>13 (52.0)</td>
<td>24 (48.0)</td>
</tr>
<tr>
<td><strong>Sjogren Disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No, n (%)</td>
<td>24 (96.0)</td>
<td>22 (88.0)</td>
<td>46 (92.0)</td>
</tr>
<tr>
<td>Yes, n (%)</td>
<td>1 (4.0)</td>
<td>3 (12.0)</td>
<td>4 (8.0)</td>
</tr>
</tbody>
</table>

2.3.2 Primary endpoint

The change in the amount of tear secretion from baseline is shown in Fig. 2-2. The measured value and the change in tear secretion from baseline are shown in Tables 2 and 3, respectively. In the second Schirmer test, the Diquafosol 3% group showed a statistically significant increase in tear volume compared to baseline (1.131 ± 0.470 mm, P = 0.020), whereas the artificial tear group showed the same tear fluid level as baseline (0.083 ± 0.442 mm, P = 0.852). In addition, the difference in the change between the study drug groups (Diquafosol 3% group - artificial tear group) was 1.048 ± 0.449 mm. This difference was
statistically significant \((P = 0.024)\). In terms of the change ratio in tear volume, the diquafosol 3\% group showed an increase in tear volume of 39.2\%. The artificial tear group showed an increase of 2.8\%.

**Table 2-2.** Actual measured value of the Schirmer test score at each point.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Schirmer Test (m)</th>
<th>3% diquafosol ophthalmic solution (N=50)</th>
<th>Artificial tear (N=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st test</td>
<td>n</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mean (SE)</td>
<td>2.886 (0.399)</td>
<td>3.010 (0.387)</td>
</tr>
<tr>
<td></td>
<td>Min, Max</td>
<td>0.00, 9.80</td>
<td>0.00, 9.30</td>
</tr>
<tr>
<td>2nd test</td>
<td>n</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mean (SE)</td>
<td>4.017 (0.499)</td>
<td>3.093 (0.477)</td>
</tr>
<tr>
<td></td>
<td>Min, Max</td>
<td>0.00, 15.85</td>
<td>0.00, 16.32</td>
</tr>
<tr>
<td>3rd test</td>
<td>n</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mean (SE)</td>
<td>2.641 (0.398)</td>
<td>3.250 (0.521)</td>
</tr>
<tr>
<td></td>
<td>Min, Max</td>
<td>0.00, 10.33</td>
<td>0.00, 15.73</td>
</tr>
</tbody>
</table>

**2.3.3 Secondary endpoint**

As shown in Table 2-3, in the third Schirmer test, the Diquafosol 3\% group showed the same tear fluid level as baseline \((0.055 \pm 0.448 \text{ mm}, P = 0.902)\) as did the artificial tear group \((0.240 \pm 0.477 \text{ mm}, P = 0.617)\). In addition, the difference in the change between the study drug groups (diquafosol 3\% group – artificial tear group) was \(-0.185 \pm 0.436 \text{ mm}\). This difference was also not statistically significant \((P = 0.673)\). The diquafosol 3\% and artificial tear groups showed a significant improvement in the total dry eye symptom scores.
compared to the baseline score. However, there was no significant difference between the diquafoisol 3% group and the artificial tear group in the total symptom scores from baseline.

**Table 2-3.** Change in tear secretion from baseline.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Change from Baseline (mm)</th>
<th>3% diquafoisol ophthalmic solution (N=50)</th>
<th>Artificial tear solution (N=50)</th>
<th>3% diquafoisol ophthalmic solution</th>
<th>Artificial tear</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd test</td>
<td>n</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mean (SE)</td>
<td>1.131 (0.470)</td>
<td>0.083 (0.442)</td>
<td>1.048 (0.449)</td>
<td>-9.28, 7.39</td>
</tr>
<tr>
<td></td>
<td>Min, Max</td>
<td>-5.90, 10.11</td>
<td>-7.60, 8.87</td>
<td>-9.28, 7.39</td>
<td>-7.68, 6.99</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.020</td>
<td>0.852</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>3rd test</td>
<td>n</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mean (SE)</td>
<td>0.055 (0.448)</td>
<td>0.240 (0.477)</td>
<td>-0.185 (0.436)</td>
<td>-7.68, 6.99</td>
</tr>
<tr>
<td></td>
<td>Min, Max</td>
<td>-7.80, 6.90</td>
<td>-6.70, 8.72</td>
<td>-7.68, 6.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.902</td>
<td>0.617</td>
<td>0.673</td>
<td></td>
</tr>
</tbody>
</table>

P-value: Comparison with baseline by Paired t-test for each group. Comparison between groups by Paired t-test for difference.

**2.4. Discussion**

The Schirmer test is one of the most important tests used to evaluate the potential for tear production and to diagnosis dry eye disease.\(^8,9\) The test can be performed at any medical facility because of its simplicity and lack of special equipment. Although the Schirmer test has been available for over a century\(^6\), in clinical studies, the Schirmer test does not properly evaluate the efficacy of
drugs in patients with dry eye due to its variability. Improvements in the method and study design allow more reproducible and reliable tear volumes to be measured.\textsuperscript{10,12,14} In this study, we investigated whether a modified Schirmer test and study design were useful for evaluating tear secretion drugs, such as diquafosol 3%.

Fifty-one dry eye patients were enrolled, and 86\% were female. The average age was 58.7 years old. This population coincides with the characteristics of actual dry eye patients, who tend to be post-menopausal women.

![Graph showing change in tear secretion from baseline.](image)

**Figure 2-2.** Change in tear secretion from baseline.

Notes: *P=0.024 for diquafosol 3\% vs artificial tear, paired t-test. Each point shows the mean ± S.E.
The original Schirmer test was described to be performed with the patient seated and with eyes open, blinking normally. Some authors have reported clinical studies using the test with eyes closed. The test performed with eyes closed could result in less variation in humidity, evaporation, reflex tearing and illumination. The 2007 Report of the International Dry Eye Workshop (DEWS) suggested to perform the Schirmer test with closed eyes.

Schirmer strips are printed with a scale ranging from 0 to 35 mm. However, the scale is not sufficiently fine for a precise measurement. In this study, the investigators measured the distance from the shortest wet part to the longest part using an electronic caliper and calculated the average. Moreover, the same investigator performed the Schirmer test three times a day to diminish variability among individuals.

Tear production volume is diurnal and is also influenced by environmental factors. Execution times were defined in our protocol to eliminate the influence of diurnal changes and daily variation. Moreover, tear production can be influenced by intrinsic causes. Examples of intrinsic causes are aging, sex hormone balance and systemic disease, eg, Sjögren's syndrome, diabetes and rheumatoid arthritis. To avoid the effects of intrinsic causes, one eye was treated with diquafosol 3% while the other eye was treated with artificial tears.

Soft Santear was selected for comparison because it is widely used as an artificial tear in Japan. Diquafosol 3% includes preservatives, whereas Soft Santear does not have preservatives. In Yokoi et al., a topical single application of diquafosol 3% significantly increased tear volume compared with Soft Santear,
as measured by using reflective meniscometry. The tear meniscus measurements were significantly higher after the single application of diquafosol 3% than after the application of saline by using optical coherence tomography.

Our results are consistent with these previous observations. In Shichijo et al., diquafosol resulted in a significant dose-dependent increase in tear volume according to the Schirmer strip test (compared with vehicle) in normal rabbits. Therefore, preservatives are unlikely impact on the efficacy of diquafosol 3% on tear volume.

Our results show that diquafosol 3% significantly increases increased tear volume (by 1.048 mm) 10 minutes after a single administration compared to artificial tears, and the effect disappeared after 3 hours and 40 minutes. At its peak, diquafosol 3% resulted in an approximately 30% increase in tear volume compared to the baseline score. Thus, diquafosol 3% improves ocular surface conditions by increasing tear volume in dry eye patients. To our knowledge, this is the first study to use the Schirmer test in dry eye patients to detect a significant increase in tear volume after a single dose of diquafosol 3%. Yokoi et al. also reported that diquafosol 3% shows a peak in tear volume 10 minutes after administration, which disappeared by 60 minutes according to meniscometry.

Our results correspond with this previous report.

This study was performed at 4 sites in Japan. The results from all sites show in the increase and disappearance of the effect in tear volume. These results suggest that our study design could be acceptable in multicenter clinical trials. Regarding symptoms, diquafosol 3% improved dryness after 4 weeks of treatment in a phase 2 clinical trial. Our results showed that both diquafosol 3%
and artificial tears significantly improve symptoms compared to baseline. There was no significant difference between the groups regarding the degree of improvement in symptoms. Further studies should be conducted to evaluate the effects on symptoms by single dosing.

One of the limitations of this study is that we did not account for the drainage and evaporation of tears. Tear volume in the conjunctival sac is determined by the balance between tear production and tear elimination. The influence of tear efflux should be studied further.

2.5 Conclusion

To evaluate the feasibility of using a modified Schirmer test to determine the increase in tear volume after administration of 3% diquafosol ophthalmic solution (diquafosol 3%) in dry eye patients.

A randomized, multicenter, prospective, double-blind clinical study recruited 50 qualified subjects. They received diquafosol 3% in one eye and artificial tears in the other eye. The study protocol comprised a screening and treatment period in one day. The Schirmer test was performed with closed eyes three times a day. The primary efficacy endpoints were the 2\textsuperscript{nd} Schirmer test scores 10 minutes after the single dose. Secondary endpoints were 3\textsuperscript{rd} Schirmer test scores 3 hours and 40 minutes after the single dose and symptom scores prior to 2\textsuperscript{nd} and 3\textsuperscript{rd} Schirmer tests.

According to the Schirmer test, 10 minutes after their administration, diquafosol 3% significantly increased tear volume compared to artificial tears.
Diquafosol 3% and artificial tears both showed a significant improvement in the symptom scores compared to baseline. However, there was no significant difference in the symptoms score between diquafosol 3% and artificial tears.

In conclusion, the modified Schirmer test can detect a minute change in tear volume in dry eye patients. The present study documents the ability to precisely measure tear volume in a various types of dry eye patients, within any area or clinical setting, in a single day and at low cost. Therefore, we believe that this method will be useful for the diagnosis of dry eye, the assessment of treatment benefits in daily clinical practice and the development of possible tear-secreting compounds for the treatment of dry eye.
Chapter 3 Meibomian gland dysfunction model in hairless mice fed a special diet with limited lipid content.

3.1 Introduction

Meibomian glands, a type of sebaceous glands, are arranged vertically within the upper and lower tarsal plates. Meibomian glands secrete lipids, which form a superficial oily layer on the tear film. Meibomian gland dysfunction (MGD) is defined as a chronic and diffuse abnormality of the meibomian glands, and it is commonly observed with terminal duct obstruction and/or qualitative or quantitative changes in glandular secretion. MGD results in alteration of the tear film, eye irritation, clinically apparent inflammation, and ocular surface disease. Some ophthalmic examinations have been developed to diagnose MGD. The most conventional and significant mean of diagnosing MGD is to use a slit lamp to thoroughly examine the lid margin, particularly that surrounding the orifice. In a patient with characteristic signs of obstructive MGD, slit-lamp microscopy reveals meibomian gland orifices that are closed with plugs comprised of thickened, opaque secretions containing keratinized material with telangiectasia around the orifice and lid margin rounding. MGD impairs the quality of life and the quality of vision because the oily layer plays an important role in maintaining the stability of the tear film by preventing tears from evaporation, reducing the surface tension of tears and lessening the friction of blinking. The causes and progression of MGD should be further investigated to develop better pharmacologic treatments. Thus, appropriate animal models mimicking MGD with a pathogenesis similar to that observed in humans are strongly desirable to understand the pathophysiology of the disease and to develop potential
pharmacologic interventions. There are several spontaneous genetic animal models and chemically induced models of MGD, including the rhino mouse model\textsuperscript{34}, ACAT-1 null mice\textsuperscript{35}, an adrenaline-induced rabbit model\textsuperscript{36,37}, a polychlorinated biphenyl-induced monkey model\textsuperscript{38}, an isotretinoin-induced blepharitis model\textsuperscript{39} and TRAF6-deficient mice.\textsuperscript{40} Although these models provide insight into the potential causes and key molecular events of disease, the etiology and pathophysiology of MGD remains unclear. Evaluating treatments for abnormal meibomian glands is difficult using these models because the clinical presentation is late-onset and/or irreversible. The lack of an appropriate model substantially contributes to the current lack of pharmacologic treatments for MGD.

Azithromycin ophthalmic solution (AzaSite\textsuperscript{®}) has been approved for the treatment of bacterial conjunctivitis. In addition to anti-bacterial effects, azithromycin suppresses the mRNA expression of inflammatory mediators toward normal levels in MGD patients.\textsuperscript{41} Azithromycin can also act directly to promote the lipid production in human meibomian gland epithelial cells.\textsuperscript{42} It has been reported that azithromycin significantly improves meibomian gland plugging, meibomian gland secretion, and eyelid redness and restores meibomian gland lipid secretion.\textsuperscript{43,44}

Tobramycin/dexamethasone ophthalmic suspension (Tob/Dex) is indicated for acute anterior blepharitis, although it is often used for posterior blepharitis.\textsuperscript{45} However, no reports have demonstrated that Tob/Dex can improve meibomian gland plugging.
It has been proposed that MGD is significantly associated with higher blood levels of total cholesterol and increased blood levels of low-density lipoprotein when compared with similarly aged controls without MGD.\textsuperscript{46,47} Oral linoleic and gamma-linolenic acid supplementation has been shown to be beneficial in the treatment of MGD.\textsuperscript{48} These data suggest that lipid composition in the diet may be implicated in the development of MGD.

Recent investigations have demonstrated that HR-1 hairless mice fed an HR-AD diet with limited lipid content, but not those fed a normal diet, developed atopic dermatitis-like symptoms, which are characterized by severely dry skin and inflammatory cellular infiltration into the skin.\textsuperscript{49} Because abnormal sebaceous glands are one of the causes of severely dry skin, HR-1 hairless mice with severely dry skin are likely to show not only abnormal sebaceous glands but also meibomian gland dysfunction. However, macroscopic and histopathologic observations of meibomian glands in HR-1 hairless mice fed an HR-AD diet have not yet been reported.

In this study, we investigated the pathology of MGD induced in HR-1 hairless mice fed HR-AD, including the occurrence of and temporal changes in morphology, to establish a novel MGD model to evaluate the treatment efficacy of eye drops.

### 3.2 Materials & Methods

#### 3.2.1 Animals

Four-week-old male HR-1 hairless mice were obtained from Hoshino
Experimental Animal Center (Yashio, Saitama, Japan). The mice were housed under 12-h light-dark cycles (lights on at 07:00 a.m.) at room temperature (23 ± 1°C) with a humidity of 55 ± 10%. Food and water were provided ad libitum. The mice were fed a standard laboratory diet (F-2, Funabashi Farm, Chiba, Japan) or a special diet with limited lipid content (HR-AD, Nosan Corp., Yokohama, Japan), which was prepared for the HR-1 hairless mice, for up to 16 weeks. After a set period of time, the HR-AD diet was changed to a normal diet to assess the recovery of the plugged orifices. The experimental procedures were performed in accordance with the statement by the Association for Research in Vision and Ophthalmology (ARVO) concerning the use of animals in ophthalmic and vision research. All the experimental procedures were approved by the Committee on Animal Research at Santen Pharmaceutical Co., Ltd. (Osaka, Japan).

3.2.2 Slit lamp examination

The meibomian gland orifices of mice under isoflurane anesthesia were assessed under a slit lamp (SL-D7; Topcon, Tokyo, Japan) and were imaged using a digital camera (D100; Nikon, Tokyo, Japan). All images were obtained using the same camera with the same settings. The number of plugged orifices among the eight meibomian gland orifices in the center of the upper eyelid was quantified. In this study, plugged orifices were defined as opaque and swollen meibomian gland orifices.

3.2.3 Histologic analysis

The eyelid tissues, which included the meibomian gland orifices, were dissected at 4, 6, 8, 12, and 16 weeks. The tissues were fixed with 10% neutral buffered
formalin solution, embedded in paraffin, and vertically cut into 2-μm-thick sections from the temporal, central and nasal portions of the eyelid. Next, the sections were stained with hematoxylin-eosin (HE) and rabbit polyclonal anti-cytokeratin 6 (CK 6; Abcam, Cambridge, UK) using the Simple Stain Mouse MAX-PO® kit (Nichirei Bioscience, Tokyo, Japan). The CK 6 signal was visualized with the chromogen diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin.

3.2.4 MGD treatment

Saline or AzaSite® (1% azithromycin ophthalmic solution; Merck & Co., Inc., Whitehouse Station, NJ, USA) was instilled into the right eye for 4 weeks (2 μL/eye; twice per day on days 1 and 2 of instillation, and once per day on day 3 and thereafter) after the plugged orifice had developed as a result of feeding with HR-AD for 4 weeks. Saline or TobraDex®ST ophthalmic suspension (2 μL/eye; 0.3% tobramycin and 0.05% dexamethasone, Alcon Laboratories, Inc., Fort Worth, TX, USA) was instilled q.i.d. into the right eye for 4 weeks after the plugged orifice had developed as a result of feeding with HR-AD for 2 weeks. The meibomian gland orifices were examined under a slit lamp 2 and 4 weeks after the treatment. The number of plugged orifices was quantified in the same manner as described above.

3.2.5 Statistical analysis

Data were expressed as the mean ± S.E. The statistical significance of differences was assessed using the Aspin-Welch test or the Student’s t-test. P-values less than 0.05 were considered statistically significant.
3.3 Results

3.3.1 MGD assessed by slit lamp examination

Fig. 3-1 shows an eyelid after 11 weeks of feeding with an HR-AD diet. A marked difference was observed at the lid margins between the groups fed the HR-AD diet and those fed the normal diet. Rounding of the posterior lid margin was observed in the mice fed the HR-AD diet (Fig. 3-1C), and many plugged orifices and instances of telangiectasia were observed around the orifices in the eyelid margin (Fig. 3-1D). These changes became more distinct when the HR-AD feeding period was extended. After 16 weeks, thickened secretions and a toothpaste-like meibum appeared (Fig. 3-1E). No changes in the eyelid or lid margin were observed in the mice fed a normal diet (Fig. 3-1A and B).

![Normal diet](image1)

![HR-AD diet](image2)

**Figure 3-1.** The eyelids of mice fed a normal diet (A, B) and the HR-AD diet (C, D, E) for 11 weeks from 5 weeks to 16 weeks of age. Rounding (C), plugging and telangiectasia (arrowheads in D), and toothpaste-like meibum (arrowheads in E).
3.3.2 Histopathologic test for MGD

We evaluated histologic changes in the eyelid after 4, 8, 12 and 16 weeks of feeding with HR-AD (Fig. 3-2). In the mice fed the HR-AD diet, the meibomian glands exhibited dramatic changes at all time points. At 4 weeks, the mice fed the HR-AD diet exhibited a thickening and hyperkeratinization of the ductal epithelium in the meibomian glands (Fig.3-2C). Keratin accumulation was detected in the ducts, and the orifices were plugged with a keratinized substance. In addition, a marked loss or atrophy of the acinar component was also observed in the glands. The thickness increase of the ductal epithelium and the volume loss of the acinar were enhanced as the HR-AD feeding period continued (Fig. 3-2C, D and E). Furthermore, the thickening of the epidermis was followed by hyperkeratosis in the HR-AD-fed mice (Fig.3-2D and E). These histological changes in the meibomian glands were observed in the whole eyelid. However, in control mice fed the normal diet, the meibomian glands did not show any changes over the experimental period (Fig. 3-2A and B). A few inflammatory cells infiltrated the tarsal plate in both the HR-AD-fed and normal diet-fed mice.
3.3.3 Amelioration of MGD signs after diet alteration

Next, we examined whether a normal diet could reduce the MGD signs induced by the HR-AD diet in mice. Five-week-old HR-1 hairless mice were fed an HR-AD diet for 14 days to develop plugged orifices. Once the mice had developed MGD signs, they were randomly divided into two groups. The mice in one group continued receiving an HR-AD diet (HR-AD group), and the mice in
the other group were fed the normal diet (ND group) for an additional 4 weeks. In

the HR-AD group, the number of plugged orifices continued to increase for 6
weeks (Fig. 3-3). In contrast, the mice in the ND group had a decreasing number
of plugged orifices. Significant improvements in MGD were observed by

reducing the number of plugged orifices 4 weeks after the diet conversion.

Moreover, immunohistological testing showed that the mice in the ND group

exhibited a histologic reversal of cytokeratin 6 staining in the meibomian ductal

epithelium compared with the mice in the HR-AD group (Fig. 3-4B, C). The

acinar atrophy was also recovered by diet conversion (data not shown).

![Graph showing effect of diet conversion on MGD model](image)

**Figure 3-3.** Effect of diet conversion on the number of plugged orifices in the MGD model

induced in HR-1 hairless mice fed the HR-AD diet. The number of plugged orifices was

recovered by switching the HR-AD diet to a normal diet at 2 weeks. Each bar represents the

mean ± S.E. of 6 eyes. *; p<0.05 vs. normal diet mice (Student’s t-test). ###; p<0.01 vs.

HR-AD diet mice (Student’s t-test).
Figure 3-4. Immunostaining of the meibomian gland with keratin 6. HR-1 hairless mice fed a normal diet (A, normal), MGD model fed the HR-AD diet (B), HR-1 hairless mice fed a normal diet for 4 weeks after the HR-AD-induced MGD (C). Overexpressed cytokeratin 6 in the meibomian ductal epithelium was recovered to normal levels (compare Fig. 3-4C to 4A). Bar: 200 μm.
3.3.4 Effects of drug treatment on the plugged meibomian gland orifices

Azithromycin was therapeutically administered to HR-1 hairless mice with plugged orifices induced by the HR-AD diet. Azithromycin significantly reduced the number of plugged orifices during treatment for 4 weeks (Fig. 3-5). Azithromycin also reduced the thickened and hyperkeratinized meibomian ductal epithelium and acinar atrophy (Fig. 3-6). However, tobramycin/dexamethasone, which is a mixture of steroids and antibiotics, did not show any efficacy on the plugged orifices under the experimental conditions (Fig. 3-7).

Figure 3-5. Effect of azithromycin on the number of plugged meibomian orifices in the HR-AD-induced MGD model in HR-1 hairless mice. Mice were treated with azithromycin for 4 weeks after the development of plugged orifices after feeding with the HR-AD diet for 4 weeks. Each bar represents the mean ± S.E. of 6 eyes. ###; p<0.01 vs. normal (Aspin-Welch t-test). **; p<0.01 vs. normal (Student’s t-test). $$; p<0.01$ vs. saline (Student’s t-test).
Figure 3-6. Histology of the meibomian gland in the MGD model induced by the HR-AD diet. The MGD model was maintained with HR-AD and treated with saline (A) or azithromycin (B) and was maintained with the normal diet (C) for 4 weeks. Azithromycin decreased the thickness and keratinization of the meibomian ductal epithelium. The acinar size also improved to a size similar to that observed in C (compare Fig. 3-6B to 6C). HE staining. Bar: 200 μm.
Figure 3-7. Effect of tobramycin/dexamethasone on the number of plugged orifices in the MGD model induced with the HR-AD diet. Mice were treated with tobramycin/dexamethasone for 4 weeks after the development of plugged orifices by the HR-AD diet for 2 weeks. Each bar represents the mean ± S.E. of 6 eyes. **; p<0.01 vs. normal (Student's t-test).

3.4 Discussion

HR-AD-fed HR-1 hairless mice showed plugged orifices, telangiectasia surrounding the orifices, posterior lid margin rounding, and toothpaste-like meibum, under slit lamp examination. These pathophysiologic characteristics are similar to those observed in MGD patients. The mice fed HR-AD diets for 4 weeks exhibited acinar atrophy, which was diagnosed using a histologic test. The onset of these MGD signs was also observed earlier than in previous reports. These data suggested that daily diet could be the cause of MGD,
and our model should soon be available to evaluate drug candidates and to elucidate the pathophysiology of MGD.

Because MGD is defined as a ‘diffuse abnormality’ of the meibomian glands, a wide range of meibomian gland conditions could be evaluated using this animal model. To the best of our knowledge, a mouse has approximately 10 or more meibomian glands in the upper eyelid. In the present study, we evaluated plugged orifices and selected 8 meibomian gland orifices that were close to the center of the upper eyelid due to the difficulty in evaluating the orifice that was proximately close to the lid aperture of the slit lamp. The orifices in this animal model closely resembled those of MGD patients under microscopy. Because microscopic observation with a slit lamp is important for diagnosing MGD in clinical practice, the animal model described in this study should be useful for studying the clinical state of the orifices of multiple meibomian glands by slit lamp.

Thickening and hyperkeratinization of the ductal epithelium and atrophy of acinar cells in the meibomian gland were observed by histological analysis in this model. Jester et al. reported the keratinized epithelium of the epidermis and markedly thickened and hyperkeratinized meibomian glands in rhino mice. Adrenaline treatment induced the accumulation of cell debris in the ducts of rabbits. In rhesus monkeys, PCB induced abnormal hyperkeratosis in the ductal epithelium, which was observed by histopathological analysis. Taken together, these previous observations in MGD animal models are consistent with the data from our model.
Although the mechanisms underlying the induction of MGD symptoms by HR-AD remain unclear, these symptoms resemble the characteristic changes observed in aging humans. HR-1 hairless mice fed the HR-AD diet have also been reported to develop skin barrier dysfunction characterized by increased transepidermal water loss and thickening of the epidermis. These observations suggest that breakdown of the meibomian ductal epithelium barrier may cause epithelial cell thickening and hyperkeratinization. Our data revealed that cytokeratin 6 was significantly overexpressed, and keratinized substances accumulated and plugged the meibomian orifices, resulting in an apparent extreme loss of mature acini followed by atrophy of the gland. The expression of the keratin 6 gene is also significantly increased in the meibomian glands of MGD patients.

Meibomian gland obstruction is reportedly influenced by endogenous factors, such as age, sex, and hormonal disturbances, as well as by exogenous factors, such as topical medications. However, the relevance of inflammation in the pathophysiology of MGD still remains controversial. In previous histopathologic studies on humans, the infiltration of inflammatory cells was not observed in specimens of either cystic dilatation or acinar atrophy of the meibomian gland. Few inflammatory cells were detected in the meibum of elderly patients, whereas keratinized material was observed in nearly all cases. However, inflammatory cytokines were recently detected in tear samples obtained from MGD patients. The proinflammatory state might have pathophysiologically occurred in these cases of MGD. Although minimal infiltration of inflammatory cells into the meibomian gland was observed under
the experimental conditions in our model, the instillation of steroid eye drops did not improve the obstructed meibomian gland orifices. The infiltration of inflammatory cells did not appear to be essential in the development of plugging. Taking these results into consideration, our model should pathophysiologically mimic human MGD.

More importantly, in this study, we found that a normal diet ameliorated the plugged orifices and acinar atrophy induced by special diet feeding. This is the first study to demonstrate improvements in the obstructed orifice and acinar atrophy using an animal model. Although HR-AD is known as a diet deficient in lipids and some types of minerals, few detailed analyses have been reported on the components of this diet. According to an investigation by Fujii et al., deficiency of n-6 polyunsaturated fatty acids, particularly linoleic acid, is mainly responsible for abnormal skin conditions because supplemental linoleic acid in HR-AD improved the dry skin symptoms in HR-1 hairless mice. Furthermore, the authors could not improve the skin condition by the supplementation of minerals, including magnesium. Meibum consists of substances such as wax esters, cholesteryl esters, triacylglycerols and free fatty acids. Arita et al. reported that the free fatty acid composition of human meibum correlated with meibum color, as determined using a slit lamp microscope. Oral fatty acids, particularly linoleic acid and gamma-linolenic acid, significantly reduced the secretion of turbid substances and meibomian gland obstruction in MGD patients. In this context, free fatty acids, particularly linoleic acid, should play an important role in the development of MGD signs in this model, and the mechanism should be similar to that of MGD in humans.
Azithromycin ophthalmic solution has been proposed as a novel treatment for posterior blepharitis, including MGD. Foulks et al. reported that topical therapy with azithromycin relieved the signs and symptoms of MGD and restored the lipid properties of meibomian gland secretion to a normal state. In our model, azithromycin improved plugged meibomian orifices. This is the first report to show an improvement in obstructed meibomian orifices using therapeutic treatment with any drugs in animal models. This finding indicates that our model should be useful for evaluating drug candidates for MGD. Azithromycin exerts not only an antibacterial action but also anti-inflammatory effects. In addition, azithromycin treatment has been shown to result in lipid accumulation in immortalized human meibomian gland epithelial cells. These properties contribute to the efficacy of azithromycin in this model. However, further study is required to reveal the pharmacologic mechanisms of the effect of azithromycin on plugged meibomian gland orifices and acinar atrophy.

In this study, although MGD is considered to be a leading cause of evaporative-type dry eye in human patients, corneal fluorescein staining did not change with the HR-AD diet. It is assumed that mice already showed variable staining before the HR-AD feeding period. The abnormal changes were thought to result from alterations to the ocular surface caused by small substances, such as dusts and chips because these mice lacked eyelashes. Therefore, the effects of plugging on tear stability in this model remain unclear. Although our model showed toothpaste-like meibum as a characteristic feature of MGD, it appears difficult to elucidate the components of the meibum due to the limited meibum volume in mice.
3.5 Conclusion

A novel meibomian gland dysfunction (MGD) model was developed to facilitate the understanding of the pathophysiology of MGD and to evaluate treatment with azithromycin ophthalmic solution (azithromycin). MGD was induced in HR-1 hairless mice by feeding a special diet with limited lipid content (HR-AD).

Male HR-1 hairless mice were fed an HR-AD diet for 16 weeks. The development of MGD was assessed by histopathology at 4-week intervals. The lid margin was observed by slit lamp examination. After cessation of the HR-AD diet, the mice were fed a normal diet to restore normal eye conditions. Cytokeratin 6 expression was determined by immunostaining. We evaluated the effects of topically applied azithromycin on the plugged orifice in this model.

After feeding the HR-AD diet, histopathology analysis showed hyperkeratinization of the ductal epithelium in the meibomian gland. The ductal hyperkeratinization resulted in the loss of acini, followed by atrophy of the gland. Slit lamp examination revealed a markedly plugged orifice, telangiectasia and a toothpaste-like meibum compared with the normal eyelid. Cessation of feeding with HR-AD ameliorated both the MGD signs and cytokeratin 6 expression, restoring the tissue to a histologically normal state. Azithromycin treatment significantly decreased the number of plugged orifices and ameliorated atrophy as revealed by histopathologic analysis.

Here we report the successful development of a novel MGD model induced by HR-AD in HR-1 hairless mice, demonstrating early onset of the characteristic clinical signs and atrophy of acinar cells in meibomian glands. A
normal diet ameliorated the plugged orifices and restored the tissues to normal. Topically applied azithromycin was also shown to have therapeutic effects on the number of plugged meibomian gland orifices and atrophy of acinar cells. Taken together, the results indicate that this model could be useful not only for the elucidation of the pathogenesis of MGD but also for the evaluation of the efficacy of drug candidates for treating MGD.
Chapter 4 A novel model of meibomian gland dysfunction induced with complete Freund's adjuvant in rabbits.

4.1 Introduction

The meibomian glands are a type of sebaceous gland that is vertically arranged within the upper and lower tarsal plates. These glands secrete lipids that form a superficial oily layer on the tear film. Meibomian gland dysfunction (MGD) is defined as a chronic and diffuse abnormality of the meibomian glands and is commonly observed with terminal duct obstruction and/or qualitative or quantitative changes in glandular secretion. MGD results in alteration of the tear film, eye irritation, clinically apparent inflammation, and ocular surface disease. The pathogenesis of both MGD and dry eye disease (DED) has recently been described in terms of a ‘vicious cycle’, wherein the underlying pathophysiological mechanisms of DED and MGD interact, thus resulting in pronounced clinical effects.

In patients with characteristic signs of obstructive MGD, slit lamp microscopy has revealed that the meibomian gland orifices are closed by plugs of thickened and opaque secretions containing keratinized material with telangiectasia around the orifice and eyelid margin rounding. Furthermore, MGD patients experience impaired quality of both life and vision because the oily layer plays an important role in maintaining the stability of the tear film by preventing tear evaporation and reducing tear surface tension and the friction of
blinking. However it is controversial whether the oily layer reduces tear evaporation. Given the increasing recognition of the importance of MGD, considerable attention has been paid to therapies targeting this condition. Traditional MGD treatments consist of warm compresses and eyelid hygiene to remove the obstructed meibum. Some pharmacological therapies such as azithromycin, doxycycline and others show the efficacy through the improvement of meibum quality in MGD patients. However, no pharmacological therapy for MGD has been approved to date. Thus, the causes and progression of MGD require further investigation to develop better pharmacologic treatments.

Both systemic and local factors may contribute to the pathogenesis of MGD. We have previously developed a novel MGD model induced by feeding a special diet containing limited lipid content to HR-1 hairless mice to demonstrate the characteristic clinical signs and atrophy of acinar cells in meibomian glands. Moreover, the switch to a normal diet ameliorated the plugged orifices and restored the tissue to normal. Our data revealed that inflammation was not critical for the development of MGD in this model.

However, the relevance of inflammation in the pathophysiology of MGD remains controversial. Suzuki, T. et al. have proposed that MGD is divided into two main types: 1) inflamed/obvious and 2) non-inflamed/ non-obvious, and that meibomitis is inflamed obstructive MGD. Topical loteprednol etabonate and eyelid scrubs with warm compresses have been found to improve clinical outcomes, tear film break-up time (TBUT), corneal and conjunctival fluorescein
staining, and meibum quality, thus indicating that inflammation plays a considerable role in the onset of MGD.

Appropriate animal models mimicking the pathogenesis of MGD with inflammation observed in humans are strongly desirable to understand the pathophysiology of the disease and to aid in development of potential pharmacologic interventions. There are several spontaneous genetic animal models and chemically induced models of MGD. Although these models suggest potential underlying causes and key molecular events of the disease, the roles of bacterial components and inflammation in the development of MGD remain unknown. Indeed, the lack of an appropriate model is a major reason for the lack of pharmacologic treatments for MGD.

We demonstrate the development of novel MGD with meibomitis model by injecting complete Freund’s adjuvant (CFA) containing heat-killed *Mycobacterium tuberculosis* and paraffin oil in rabbits. Simultaneously, we describe the pathology of MGD together with the temporal morphological changes in inflamed meibomian gland and the efficacy of Tobramycin/dexamethasone ophthalmic suspension (Tob/Dex) in the treatment using this model.

### 4.2 Materials & Methods

#### 4.2.1 Animals
Japanese white rabbits weighing 1.50 to 1.99 kg were obtained from Kitayama Labes Co., Ltd (Ina, Nagano, Japan). The rabbits were housed under a 12-h light-dark cycle (light on at 7:00 am) at room temperature (23 ± 1°C) and a humidity of 55 ± 10% and were given ad libitum access to tap water and a gamma-ray sterilized pellet diet (LRC4; Oriental Yeast Co., Ltd., Tokyo, Japan) of approximately 130 g/day.

4.2.2 Induction of the MGD model

The MGD model was induced with CFA containing killed Mycobacterium tuberculosis, strain H37Ra (Difco Lab, Detroit, MI, USA). Under topical anesthesia (Benoxil ophthalmic solution 0.4%, Santen, Osaka, Japan), CFA (each 10 μL) was injected into the nasal, central and temporal upper eyelid margin of the right eye. Saline was injected in the left eye as a control. The experimental procedure was performed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) concerning the use of animals in ophthalmic and vision research. All of the experimental procedures were approved by the Committee on Animal Research at Santen Pharmaceutical Co., Ltd (Osaka, Japan).

4.2.3 Slit lamp examination

The development of MGD was evaluated on the basis of the presence of plugged orifices and telangiectasia. The meibomian gland orifices of rabbits were assessed under a slit lamp (SL-D7; Topcon, Tokyo, Japan) and were imaged using a digital camera (D100; Nikon, Tokyo, Japan). All images were
obtained using the same camera with the same settings. The numbers of plugged orifices were separately scored in the nasal, central and temporal regions of the upper eyelid as follows: 0, none; 1, less than 4 plugged orifices; 2, greater than 4 and less than 7 plugged orifices; and 3, greater than 7 plugged orifices. Nine was the maximal total score for each region. In this study, plugged orifices were defined as opaque and swollen meibomian gland orifices.

The telangiectasia intensity around the orifice was separately scored in the nasal, central and temporal regions of the upper eyelid as follows: 0, none; 1, mild; 2, moderate; and 3, severe. Nine was the maximal total score for each region.

4.2.4 Histologic analysis

Eyelid tissue from the nasal, central and temporal portions of the eye, which included the meibomian gland orifices, was dissected at day 29. The tissue was fixed with 10% neutral buffered formalin solution, embedded in paraffin, and vertically cut into 2-μm-thick sections. The sections were stained with HE for light microscopic examination.

4.2.5 Treatment of MGD

Rabbits were randomized into two groups 4 days after the CFA injection. Saline or tobramycin (0.3%) and dexamethasone (0.05%) ophthalmic suspension (TobraDex®ST, ;Alcon Laboratories, Inc., Fort Worth, TX, USA) was instilled by 50 μL/eye four times daily into the right eye from day 5 to day 15. The plugged orifices and telangiectasia were scored in the same manner as described above
Statistical analysis

Data are expressed as the means ± S.E. The statistical significance of the differences was assessed using Aspin-Welch tests or Student’s t-tests. P-values less than 0.05 were considered to be statistically significant.

Results

MGD with meibomitis assessed through slit lamp examination

Fig. 4-1 shows photographs of the eyelid margin under a slit lamp. A marked difference was observed at the eyelid margins between normal control (Fig. 4-1A) and CFA-injected eyes (Fig. 4-1B-D). Telangiectasia around the orifices, palpebral conjunctiva hyperemia (Fig. 4-1B), and many plugged orifices (Fig. 4-1C, arrows) were observed in the eyelid margins. Slit lamp examination 4 days after the injection indicated that these changes surrounding the orifices progressed as the period after injection was extended. Thickened secretions were discharged, and a toothpaste-like meibum appeared at day 21 (Fig. 4-1D). No changes in the eyelid or eyelid margin were observed in rabbits injected with saline (Fig. 4-1A).

Histopathologic analysis of MGD with meibomitis

We evaluated histologic changes in the nasal, central and temporal portions of the eyelid after CFA injection. Fig. 4-2 presents the Hematoxylin-enosine (HE).
staining results for the central portion in the eyelid. CFA injection induced granulomatous inflammation with destruction of the meibomian glands (Fig. 4-2C, E). The eyelids also exhibited thickening and hyperkeratinization of the ductal epithelium and dilation of the duct in the meibomian glands (Fig. 4-2C, D arrowheads). Proteinaceous substances accumulated in the ducts and plugged the orifices (Fig. 4-2D arrows). In addition, acinar cells became hypertrophic and/or hyperplastic (Fig. 4-2D asterisks). These changes were identical for each portion of the eyelid. However, the meibomian glands in the rabbits injected with saline did not exhibit any changes throughout the experimental period (Fig. 4-2A, B).

Figure 4-1. Eyelids of rabbit MGD model. Slit lamp photographs of the eyelid margins injected with saline (A) or CFA (B-D). Rounding, plugging (arrows) and telangiectasia at day 15 (B, C) and a toothpaste-like meibum at day 21(D) in CFA-injected rabbits.
**Figure 4-2.** HE staining of the sections of meibomian gland. Twenty-nine days after saline injection (A, B) and CFA injection (C, D, E). B and D show higher-power views within the rectangular enclosure in A and C, respectively. E shows damaged glands by granulomatous inflammation (g). Thickening and hyperkeratinization of the ductal epithelium (arrowheads in D). Plugging with a proteinaceous substance (arrows in D). Hypertrophy and/or hyperplasia in the acinar cells (asterisks in D). Bar = 100 µm (B, D and E), 200 µm (A and C).
4.3.3 Treatment with Tob/Dex

At each site with CFA injection, the telangiectasia score and plugged orifice score increased up to 11 days after randomization (Table 4-1). Topically applied Tob/Dex significantly suppressed the increase in the telangiectasia score for the temporal, central and whole eyelid sections on days 7 and 11. The plugged orifice score was also significantly low for the temporal, central and whole eyelid sections on day 11 (Table 4-1; Fig. 4-3A, B). Typical clinical photograph provided evidence that plugging and telangiectasia was reduced by the Tob/Dex(Fig.4-4B) compared with that of saline (Fig.4-4A) for 11 days.

![Figure 4-3. Effect of Tob/Dex on the telangiectasia (A) and plugged orifice (B). CFA-induced MGD in rabbits was treated with the drug for 11 days. The scores were monitored in the presence or absence of the drug. Tob/Dex eye drops significantly suppressed the increase in both scores in the telangiectasia and plugged orifice. Each bar represents the mean ± S.E. of 7 eyes. *; p<0.05, **; p<0.01 vs. saline (Student’s t-test)
Table 4-1: Change of telangiectasia score and plugged orifice score with treatment of tobramycin/dexamethasone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after randomization</th>
<th>Telangiectasia score (Mean ± SE)</th>
<th>Plugged orifice score (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Temporal</td>
<td>Central</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0.3 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Tobramycin/Dexamethasone</td>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.3 ± 0.2*</td>
<td>0.3 ± 0.2**</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.2*</td>
</tr>
</tbody>
</table>

Note: n = 7, *; p<0.05, **; p<0.01 compared with Saline groups at each time point (Student’s t-test).
Figure 4-4. Slit lamp photographs of the eyelid margins injected with CFA. CFA-induced MGD in rabbits was treated with saline (A) and Tob/Dex (B) for 11 days.

4.4 Discussion

CFA containing heat-killed *Mycobacterium tuberculosis* and paraffin oil will form a viscous water-in-oil emulsion when mixed with aqueous solutions. Injection of the emulsion induces chronic inflammation, such as the infiltration of inflammatory cells and cell-mediated immunity. However, macroscopic and histopathologic observations of meibomian glands in CFA-injected rabbits have not been reported to date. In this study, we successfully developed a novel MGD with meibomitis model induced by CFA injection in rabbits. CFA injection exhibited the early onset of characteristic clinical signs of MGD with meibomitis and cystic dilation of ducts and granulation tissue in the meibomian glands. Topically applied Tob/Dex eye drops demonstrated efficacy in avoiding the plugged meibomian gland orifices and telangiectasia.

Gram-positive bacteria, such as *Coagulase-negative Staphylococcus*, *Propionibacterium acnes* and *Coryneform bacteria*, are the most common
bacteria isolated from the eyelids of MGD patients as well as of healthy humans.\textsuperscript{36,38,39,75} However, the role of commensal bacteria in the development of MGD remains unclear. It has been reported that some species of gram-positive bacteria in eyelids of MGD are significantly present more often than those in healthy eyelids.\textsuperscript{76} It is possible that innate immunity without tolerance is essential for the pathogenesis of MGD. CFA can be used to generate an MGD animal model because it contains heat-killed gram-positive bacteria.

MGD is a chronic and diffuse abnormality of the meibomian glands. Suzuki et al. have proposed a new disease subset termed meibomitis-related keratoconjunctivitis (MRKC).\textsuperscript{42} Meibomitis is thought to be an inflammatory form of MGD, and the primary clinical feature of MRKC is the occurrence of meibomitis, which is defined as stagnation of meibum and swelling of the eyelid margin and palpebral conjunctiva hyperemia, and particularly telangiectasia around the meibomian gland orifices. This study has revealed that plugged orifices, palpebral conjunctiva hyperemia, telangiectasia surrounding the orifices, and a toothpaste-like meibum were observed under slit lamp examination in rabbits. Because the vast majority of MGD associated with dry eye is rather characterized by MG dropout due to plugging and acinar atrophy without severe inflammation, our novel model may mirror the pathophysiology of a meibomitis as a subtype of human MGD.\textsuperscript{69}

Histological examination of the eyelids revealed that CFA induced inflammatory cell infiltration, ductal epithelial hyperkeratinization, granulation, meibomian hypertrophy, hyperplasia and the plugged orifices. Granulation tissue is an indicator of a chronic inflammatory response. Obata has reported the
histopathology of cystic dilatation of acini and/or ducts and granulation tissue in the meibomian gland in aging subjects.\textsuperscript{51} Samples of abnormal human meibum collected from MGD patients exhibited elevated level of total protein\textsuperscript{77}, and increase of keratins/cytokeratins.\textsuperscript{78,79} Viscous ordered lipids blocked the ducts sometimes followed by the proteinaceous conjunction.\textsuperscript{80} Regarding histological tests, our present model resembles the observations reported in human subjects.

However, histological data in our current model did not reveal atrophy of acinar cells, whereas acinar atrophy was observed in the specimens of human meibomian gland. The meibum component, blink rate and TBUT in rabbits are so different from those of humans that these facts should be noted when biochemistry and biophysics of the meibomian lipid are studied\textsuperscript{81,82,83} to clarify the relationship between MGD and evaporative-type dry eye in humans.

In the model of experimental systemic lupus erythematosus (SLE) reported by Chan et al., the histology of the eyelids shows marked infiltration of polymorphonuclear neutrophils, macrophages, and lymphocytes.\textsuperscript{84} In addition, the meibomian glands in this model exhibit varying stages of hypertrophy and mild hyperplasia with inflammation. Our findings also suggest the possibility of the development of hypertrophy and hyperplasia as a compensatory response to the loss of acinar cells due to granulomatous inflammation or as a regenerative process for tissues injured by chronic inflammation. In future studies, we will investigate the mechanism of hypertrophy and hyperplasia in detail and the subtype of lymphocytes involved.

Tob/Dex is used as a combination steroid and antibiotic eye drop indicated for acute anterior blepharitis. This formulation is often used for posterior blepharitis.\textsuperscript{45} However, the functional role of topical corticosteroids in
the effect on MGD is controversial because inflammation is not clearly identified in MGD.\textsuperscript{45} In this study, Tob/Dex suppressed not only telangiectasia but also plugging of the orifice. Steroid eye drops could be one of the options to treat MGD, but the chronic use should be refrained to avoid the side effects.

\section*{4.5 Conclusion}

A novel meibomian gland dysfunction (MGD) model induced by the injection of complete Freund’s adjuvant (CFA) in rabbits was developed to facilitate the understanding of the pathophysiology of MGD with meibomitis. In addition, we sought to evaluate treatment with steroid eye drops in this model. Male Japanese white rabbits were subcutaneously injected with CFA into the upper eyelid margin. The eyelid margins of the rabbits were chronologically observed through slit lamp examination. The development of meibomitis was assessed through histopathology. We evaluated the effects of topically applied tobramycin/dexamethasone (Tob/Dex) eye drops on the plugged orifices and telangiectasia. After the injection of CFA, slit lamp examination revealed markedly plugged orifices, telangiectasia around the orifices and a toothpaste-like meibum, as compared with the normal eyelids. Histopathology revealed granulation tissue with infiltration of inflammatory cells, hyperkeratinization of the ductal epithelium, and cystic dilatation of ducts in the meibomian gland. The orifices were plugged with a proteinaceous substance. Tob/Dex eye drops significantly suppressed the plugging and telangiectasia around the orifices.

To our knowledge, this is the first report to demonstrate the peripheral
inflammation induced in the eyelid by CFA containing bacterial components leads to meibomitis as a subtype of obstructive MGD. In addition, steroid/antibiotics eye drops exhibit significant efficacy in alleviating the symptoms. This model should help the development of new treatments elucidating the pathogenesis of MGD with meibomitis on a molecular basis.
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