• Basic Research •

Meibomian gland dysfunction model induced with complete Freund's adjuvant in C57BL/6 mice

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Abstract

● **AIM:** To establish a new inflammatory animal model of meibomian gland dysfunction (MGD) in C57BL/6 mice.

● **METHODS:** C57BL/6 mice were randomly divided into complete Freund's adjuvant (CFA) group (14 animals, 14 eyes), naphthazolin hydrochloride (NH) group (14 animals, 14 eyes) and control group (14 animals, 14 eyes). In CFA group, CFA was used in eyelid conjunctiva injection; in NH group, NH eye drops were used twice a day; control group was injected with equal dose of saline at the same time point and same site with animals in CFA group. The meibomian gland orifices score (MGOS) was evaluated on a scale of 0 to 3 in the middle five meibomian gland orifices of the upper and lower eyelid using slit lamp. After the successful induction of each animal model, intense pulsed light (IPL) was introduced on each mouse in CFA and NH group. Oil red O (ORO), hematoxylin and eosin (H&E) staining were performed before and after successful induction of CFA, NH and control group.

● **RESULTS:** At 12wk after CFA injection, inflammatory cell infiltration and fiber necrosis was observed, with acinar density and duct dilatation significantly lower compared with control group. In NH group, the meibomian gland acini were relatively smaller and deformed compared with control group, the number of meibomian gland acini was also slightly lower. No inflammatory cell or fiber necrosis was observed in NH group. After three times of IPL treatment (5/10 mice in each group, and the other 5 mice served as non-IPL control), MGOS was significantly lower in IPL-treated mice in NH group (P<0.01). After three times of IPL treatment, the MGOS of NH group was significantly lower than that in the CFA group (P<0.01).

● **CONCLUSION:** We develop a novel animal model that studies the role of inflammation in the development of MGD and IPL treatment. This model indicates that persistent inflammatory state may be the cause of MGD and weaken the therapeutic effect of IPL.

● **KEYWORDS:** meibomian gland dysfunction; complete Freund's adjuvant; inflammation; acinar atrophy; intense pulsed light

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INTRODUCTION

Meibomian gland dysfunction (MGD) is a complication of posterior blepharitis, which is considered to be the main cause of evaporative dry eye[1]. The meibum released by meibomian glands in patients with MGD is insufficient or of poor quality, which results in thinning of lipid layer and excessive evaporation of tear. Although MGD is a common disease worldwide, it is still overlooked clinically and considered to be less prevalent than dry eye disease[2].

In order to better understand the pathogenesis of MGD, a large amount of research has been undertaken. In recent years, due to the frequent occurrence of various infectious diseases in dry eye patients, such as anterior blepharitis and keratitis, more and more researches focus on the relationship between inflammation and dry eye. The pathogenesis of MGD has been described in terms of a "vicious cycle"[3]. Anterior blepharitis, clinically observed as crust or flakes on eyelashes and eyelid margin, can cause toxic environment that damages tear film and promotes ocular surface inflammation. A wide range of bacteria, such as *Staphylococcus aureus*[4] and *Klebsiella*[5], have been identified in patients with ocular surface infections mentioned above.

According to the research of ophthalmic bacteria in recent years, antibiotics have been used to treat MGD[6-8]; however, the relationship between inflammation and pathophysiology of MGD remains controversial. Therefore, it is very difficult to develop a more specific and feasible treatment. Suzuki *et al*[9] divided MGD into two main types: inflammatory/obvious

and non-inflammatory/non-obvious, with meibomianitis as inflammatory obstructive MGD. At present, local azithromycin alone or combined oral has been reported to alleviate the symptoms and signs of dry eye[8], and intense pulsed light (IPL), which also has been proved to have anti-inflammatory effect[10], has been widely used in the treatment of MGD. These evidences suggest that inflammation may play an important role in the pathogenesis of MGD.

A large number of MGD animal models have been established, including closure of meibomian gland orifices[11-12], diet or drug induction[13-15], and gene induction[16]. However, few of them were induced by inflammation. In this research, we established a novel MGD model by inflammation, which is injecting complete Freund's adjuvant (CFA) in C57/BL6 mice. CFA is a solution of antigen (heat-killed and dried *Mycobacterium tuberculosis*) emulsified in paraffin oil and used as an immunopotentiator. Previously, CFA has been widely used in inflammatory animal models, such as arthritis[17-19], prostatitis[20] and dermatitis[21-22]. Epinephrine has long been used in MGD model[15]. In this research, naphthazolin hydrochloride (NH), an alpha adrenoceptor agonist, was also used to induce MGD for comparing the difference between CFA model and traditional MGD model. To the best of our knowledge, this is the first inflammatory MGD animal model induced in C57/BL6 mice.

MATERIALS AND METHODS

Ethical Approval The use of animals in this study was in accordance with the Association for Research in Visual Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of Meibomian Gland Dysfunction in C57 Mice A total of 42 healthy 6-week-old, female C57BL/6 mice purchased from the Medical Animal Center of Ninth People's Hospital Affiliated to Shanghai Jiao Tong University were used in our study. The mice were housed under 12-hour light/dark cycles (lights on at 07:00 a.m.) at room temperature (22°C±1°C) with humidity of 55%±10%. Food and water were provided *ad libitum*. Mice were randomly assigned to CFA group (14 animals, 14 eyes), NH group (14 animals, 14 eyes) and control group (14 animals, 14 eyes).

Complete Freund's adjuvant group CFA (Sigma, USA) was injected into palpebral conjunctiva of the upper and lower eyelid of both eyes (50 μL in each site, day 1). Additional injections (50 μL) into the same position were given on day 2, 6, 10, 14 and 21. Each mL of CFA contains 1 mg of heat-killed and dried *Mycobacterium tuberculosis* (strain H37Ra, ATCC 25177), 0.85 mL paraffin oil and 0.15 mL of mannide monooleate. After successful modeling, the eye with higher meibomian gland orifices score (MGOS) was recruited.

Naphthazolin hydrochloride group Adrenergic agonists were given according to literature[15]. In brief, both eyes of each animal were given one drop of NH (0.2 mg/10 mL), twice a day (7 a.m., 5 p.m.), 6d a week for 6mo. After successful modeling, the eye with higher MGOS was recruited.

Control Right eye of each animal were injected with equal dose of saline at the same time point and same site with animals in CFA group to serve as control group. In brief, 50 μL saline was injected into palpebral conjunctiva of the upper and lower eyelid of right eye on day 1, 2, 6, 10, 14 and 21. Right eye of each animal was recruited.

Criteria for successful blepharitis induction According to the Preferred Practice Patter (PPP) published by American Academy of Ophthalmology (AAO) in September 2018[23], one of the following eyelid abnormalities and accompanying signs is considered as blepharitis. Eyelid abnormalities: including abnormal position, palpebral margin hyperemia, sparse or disordered eyelashes, palpebral margin ulcer and rounding of eyelid margin; Conjunctival signs: foam-like exudates on lid margin, conjunctival congestion, follicular keratoconjunctivitis, filamentous keratitis, corneal epithelial punctate staining, corneal ulcers, *etc*.

Criteria for successful meibomian gland dysfunction induction At least three of the five meibomian gland orifices in the middle of the upper and lower eyelids were blocked (MGOS≥2).

Intense Pulsed Light Treatment Ten animals randomly selected from CFA group and NH group were examined by slit lamp microscope and treated with IPL. The other four mice were killed at different times for further study. All mice were killed by cervical dislocation under ketamine induced anesthesia. Every effort has been made to minimize the suffering of animals and to reduce the number of animals used.

Complete Freund's adjuvant and naphthazolin hydrochloride group Five mice chosen randomly were performed with IPL (MED-230, YourGa) at 7th, 14th and 21st day after the successful MGD induction. The rest were considered as non-IPL control.

Control group No IPL therapy was performed.

Intense pulsed light parameters Energy density was 10 J/cm^2, with pulse width of 5ms, pulse interval of 15ms, filter of 590 nm, 3 pulses per shot. One shot each at the both cheeks and the area between upper eyelids (Figure 1A, red areas were the treatment site of IPL). After hair removal, medical ultrasonic couplants were coated on the treatment site and IPL was performed under ketamine induced anesthesia (Figure 1B).

Meibomian Gland Function Assessment MGOS were assessed under microscope on a scale of 0 to 3 in 5 orifices in the middle part of the upper and lower lid: 0, no orifice plugged; 1, 1 or 2 orifices plugged; 2, 3 or 4 orifices plugged; and 3, all orifices plugged. Final score was the sum of the upper and lower eyelid scores.

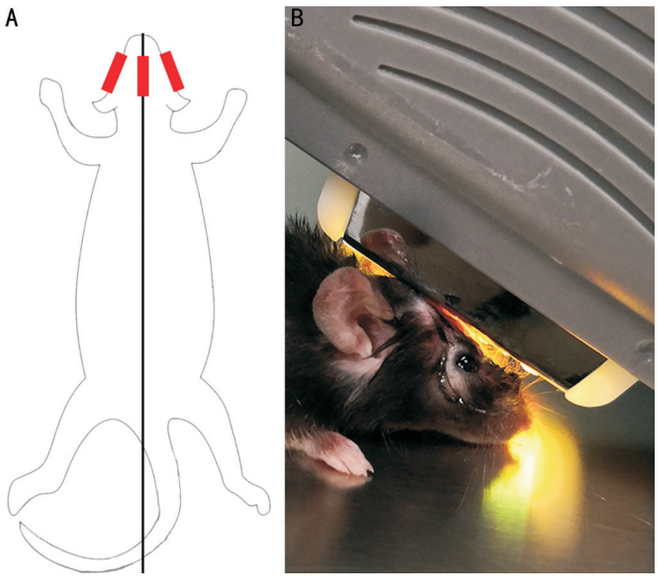

Figure 1 Treatment site of IPL A: Red zone represents the treatment site of IPL, which was the middle of the face (between the two upper eyelids) and the both cheeks (under the lower eyelid of each eye); B: Photographs of IPL treatment, IPL after skin preparation and coating of medical ultrasonic couplants.

Meibomian Gland Histopathological Assessment After successful induction of MGD in CFA group and NH group, 4 mice in each group were randomly selected and killed. Eyelid tissues, which included the meibomian gland orifices, were dissected. The tissues were immerged in 4% paraffin and vertically cut into 4 μm-thick paraffin sections using a microtome by standard technique and processed for hematoxylin and eosin (H&E) staining according to conventional histological techniques.

Lipid Staining Assessment We used an oil red O (ORO) staining method to detect the morphology of neutral lipids and lipid droplets (LD). We studied the ORO staining of every group at the same time point with H&E assessment. We dissected meibomian glands after intraperitoneal anesthesia from animals. Specimens were then fixed with 4% paraformaldehyde and embedded in the compound at the optimum cutting temperature (OCT). Then the meibomian gland was frozen rapidly (-20°C) with liquid nitrogen (N_2). We collected tissues in sections and 4 μm-thick slides with different depths from meibomian gland to the same slide in order to provide a good overview of the tissue. The frozen sections were incubated in 60% 2-propanol for 5min, then stained with ORO (Merck KGaA, Darmstadt, Germany) solution for 20min. Finally, phosphate buffered saline (PBS) was used to clean the sections strictly, and hematoxylin was used for counterstaining.

Statistical Analysis Data were expressed as mean±SD. The statistical significance of differences was assessed using the Mann-Whitney *U* test or the Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Table 1 MGOS between CFA, NH and control groups after successful modeling

Group	MGOS	*P* (compared with control group)
CFA	4.60±1.26	<0.01
NH	4.40±1.07	<0.01
Control	0.80±0.42	/

MGOS: Meibomian gland orifices score; CFA: Complete Freund's adjuvant; NH: Naphthazolin hydrochloride.

Table 2 MGOS of CFA group and NH group before and after IPL treatment

Time	CFA (*n*=5)	NH (*n*=5)	*P*
Baseline	4.80±0.84	4.60±1.14	0.760
3wk	4.20±0.84	2.00±1.00	0.005

MGOS: Meibomian gland orifices score; CFA: Complete Freund's adjuvant; NH: Naphthazolin hydrochloride; IPL: Intense pulsed light.

RESULTS

Meibomian Gland Function Assessment

Morphology of lid margin The eyelid margin photos were taken after successful induction of MGD. It took 12wk in CFA group and 24wk in NH group. Figure 2 shows the morphology of eyelid margin in control group at 12wk, CFA group and NH group when the model was successfully induced. Significant differences were observed among the three groups. In CFA group, obvious blockage and telangiectasia were observed around meibomian gland orifices. Depigmentation, atrophic meibomian gland, swollen and rounded meibomian margin were also observed. However, in NH group, only one or two occluded orifices were observed at 12wk, which was not enough to diagnose MGD. It was not until 24wk that half or more of the orifices were plugged. The meibomian gland morphology of NH group was slightly smaller than that of control group at 24wk, but still better than CFA group. During this period (0-12wk), the eyelid or meibomian gland of the control group did not change.

Meibomian gland orifices score As shown in Table 1, after 12wk in CFA group and 24wk in NH group, MGOS of CFA and NH groups were significantly higher than control group (P<0.01), indicating both groups were successful in modeling. After 3 times of IPL treatment (5/10 mice in each group, the other 5 mice served as non-IPL control group), there was no significant difference in MGOS level between IPL and non-IPL intervention groups in CFA group; after three IPL interventions in NH group, MGOS in IPL intervention group was significantly lower than that in non-IPL intervention group (P<0.01; Figure 3). After three IPL treatments, the MGOS of IPL intervention group in NH group was significantly lower than that of IPL intervention group in CFA group (P<0.01; Table 2).

Meibomian Gland Histopathology Test H&E sections (Figure 4) showed the acini of the control group (Figure 4A-4C)

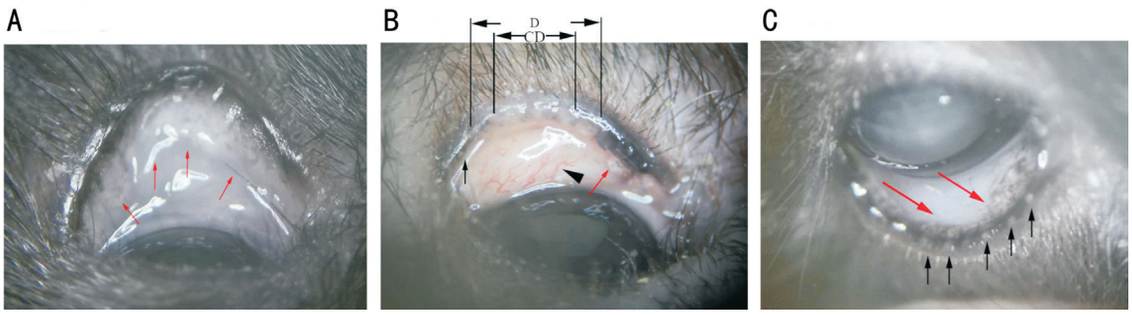

Figure 2 Morphological changes of lid margin A: Lid margin at week 12 in control group was intact. White and clear meibomian glands (red thin arrows) were seen without atrophy. B: The eyelid of mice injected with CFA for 12wk (before IPL). Rounding and depigmented lid margin was seen with plugging orifices (black thin arrows). Depigmentation (D) and complete depigmentation (CD) were observed. Conjunctival hyperemia (black triangle) and fewer meibomian glands could be seen under microscope compared with control group. C: The eyelid of mice in NH group at 24-weeks' time showed an increasing number of plugged orifices (black thin arrows) and slightly atrophic meibomian glands (red thin arrows).

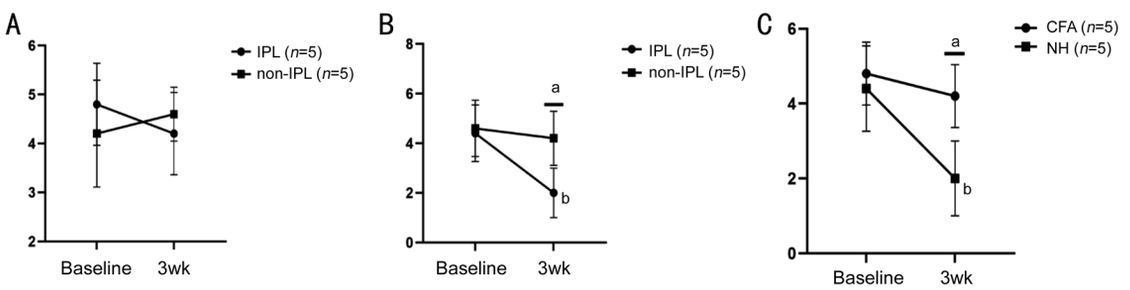

Figure 3 MGOS before and after three IPL treatments A: No significant difference was observed in the CFA group before and after three IPL treatments, and there was no statistical difference between the IPL and the non-IPL intervention in CFA group. B: In NH group, MGOS of mice treated with IPL showed a significant lower score than non-IPL mice in NH group. C: IPL-treated mice in CFA group. [a]P<0.05 inter group differences; [b]P<0.01 intra group differences.

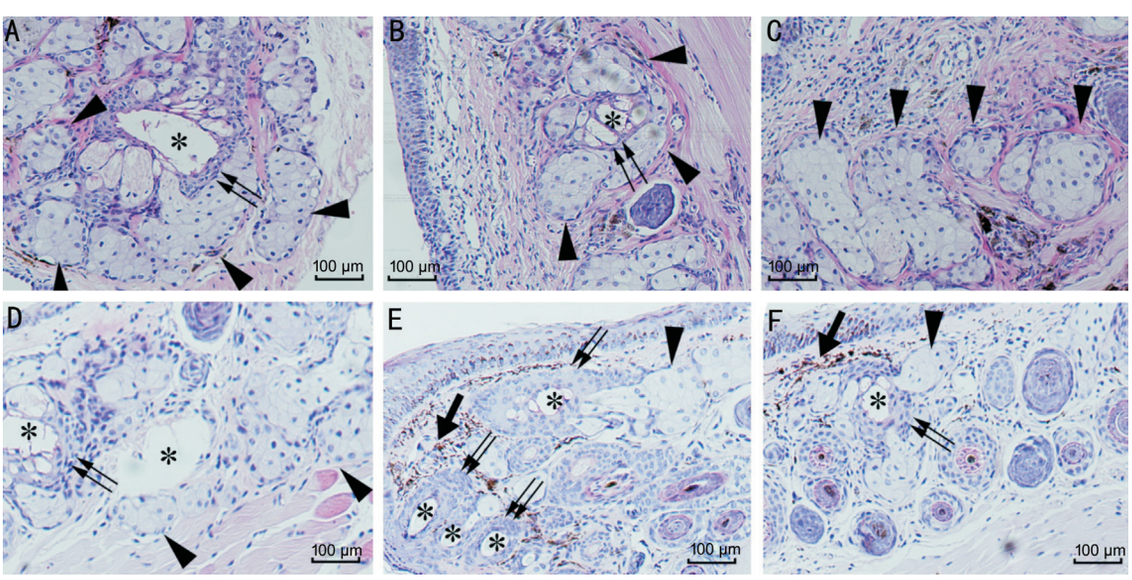

Figure 4 H&E sections of CFA and control group A-C: H&E staining of control group showed the central duct was composed of stratified squamous epithelium and keratinized in some parts of the duct (double thin arrow). Acinus were plump and normal in shape (single triangle). D-F: Number of acini was reduced compared to control group (single triangle, D, E) even after IPL treatment (single triangle, F). Perinuclear infiltration (thick arrows) also increased in CFA group (E, F). Central glandular tube (asterisk) was mostly intact in week 8 (D) but significantly smaller in week 12 and 15 (E, F). The epithelium of the ductus was thicker (double thin arrow) in week 12 (E) but slightly recovered after IPL treatment (F).

were significantly fuller and more regular than that of the CFA group (Figure 4D-4F).

Control group Each acini consists of a flat layer of basal cells and a round mass of cellular foam cytoplasm. At various stages

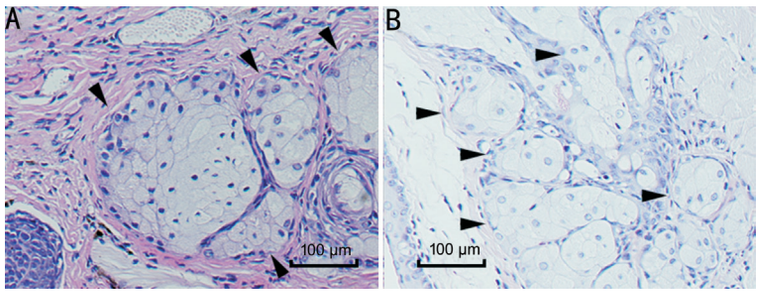

Figure 5 H&E section of NH and control group A: H&E section of control group at 24-week showed full and normal acini (black triangle) around ductus; B: Acini (black triangle) was deformed in NH group at 24-week' time.

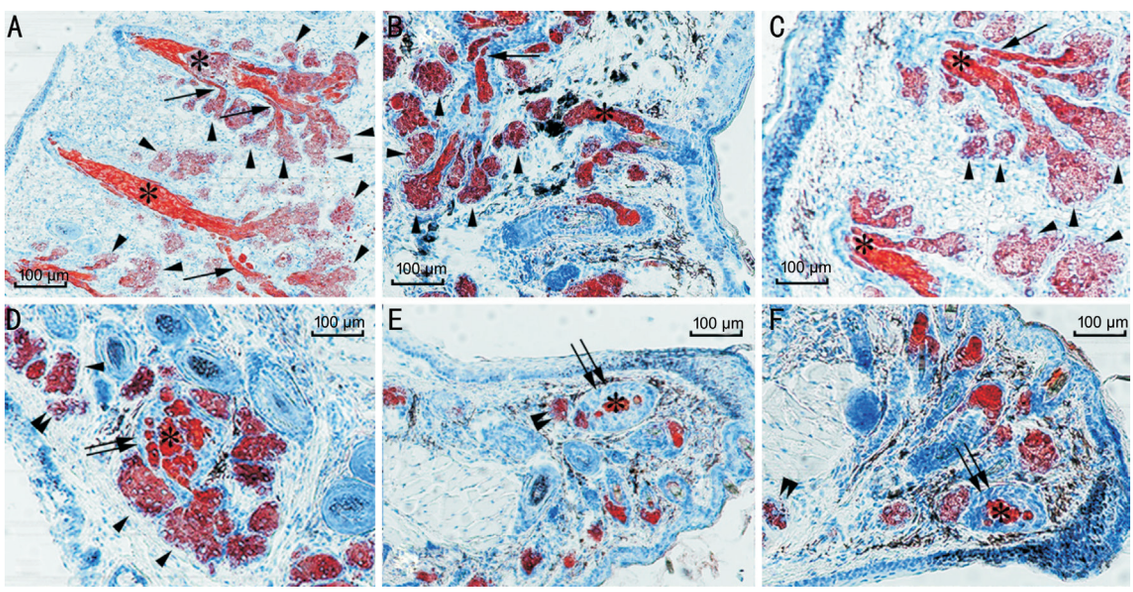

Figure 6 ORO staining of CFA and control group A-C: ORO staining of control group showed the typical histological structure of the meibomian gland which was a simple branched gland composed of many branched acini (single triangle), opening to short ducts (thin arrows) and end into a long central glandular tube (asterisk). Acinus was plump and normal in shape. D-F: Lipid drops in meibomian gland decreased significantly after CFA injection. Thickening of the epithelium of the ductus was observed (double thin arrow). Normal (single triangle) and atrophic (double triangles) acini both existed in transverse plane of ORO staining at week 8. The radius of central glandular tube (asterisk) was similar to that of control group in this period (D). However, only atrophic (double triangles) acini, smaller central glandular tube (asterisk) and thicker epithelium of the ductus (double thin arrow) were observed in mice at week 12 (E, before IPL) and IPL-treated mice at week 15 (F).

of differentiation, acinar cells can be identified and release their contents into the duct. The central tube was lined with a layer of squamous epithelium, and only part of the tube was keratinized.

Complete Freund's adjuvant group In the mice injected with CFA, however, the meibomian glands exhibited dramatic changes at all time points (Figure 4D-4F). The H&E staining sections of CFA group showed that the tissue of meibomian acini was relatively smaller, with the number of acini significantly reduced. Thickening and hyperkeratinization of ductal epithelium in the meibomian glands were also observed. It also demonstrated increased inflammatory cell infiltration and fibrous necrosis, with reduced acinar density and ductal expansion in 8 and 12-weeks' time after CFA injection (Figure 4E, 4F).

Naphthazolin hydrochloride group The H&E staining sections of NH group at 6-month time (Figure 5B) showed that meibomian acini was relatively smaller and deformed, with the number of acini slightly reduced compared to control group (Figure 5A).

Lipid Staining Changes Assessment ORO staining section in both control groups showed the characteristic structure of the meibomian gland, which demonstrated central duct containing branched acini (Figure 6A-6C, Figure 7A). Acini were connected to a long central duct through short ductules. ORO staining in the control group did not change over time. In CFA group, however, there was a significant decrease in ORO staining over time, even after IPL treatment, no significant increase in ORO staining was observed (Figure 6D-6F). ORO staining in NH group at 6-month time (Figure 7B) was fewer compared to control group (Figure 7A).

DISCUSSION

MGD is a common cause of evaporative dry eye. Awareness

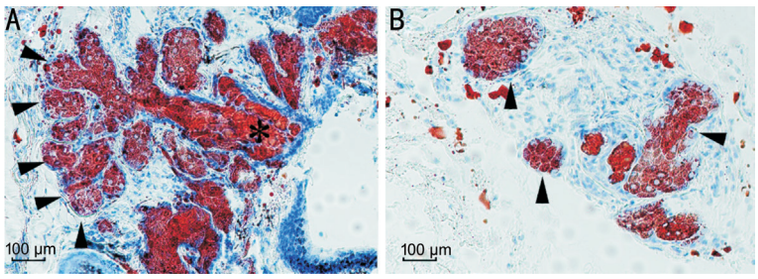

Figure 7 ORO staining of NH and control group A: Acinus (black triangle) were plump and normal, opening to a long central glandular tube (asterisk) in control group at 6-months' time; B: Lipid drops in meibomian gland decreased significantly after NH intervention for 6mo. So was the number of acini (black triangle).

of the disease is improving both clinically and in research[24]. Although the development of MGD treatment has received much attention, not many options are available, such as warm compress, eyelid hygiene, IPL and antibiotics, *etc*. Therefore, the management of MGD is still a challenging problem.

According to slit lamp examination, C57BL/6 mice treated with CFA showed plugged meibomian gland orifice, conjunctival telangiectasia, and rounding of lid margin. These pathophysiological characteristics were similar to those of MGD patients. Histological examination confirmed that acinar atrophy occurred at most 12wk after CFA injection. The presence of these signs of MGD has been previously reported[15,25]. Since meibomianitis is considered to be an inflammatory form of MGD according to literature[9], our research might suggest that inflammation might be one of the direct causes of MGD. Our model should soon be available for evaluating treatment candidates and elucidating the pathophysiology of MGD. A wide range of meibomian gland conditions could be evaluated using this animal model.

To the best of our knowledge, about ten meibomian gland orifices exist in the lid margin of mice. In this study, the central 5 orifices of the upper and lower eyelids, which may have the greatest influence on cornea, were selected for scoring. Modeling in CFA group was verified to be successful in 12wk and NH group in 24wk. The morphology of meibomian gland orifices obstruction in these two groups was similar to that in patients with MGD. CFA group and NH group started IPL treatment from the verification of successful modeling. However, compared with the other half without IPL treatment, the average MGOS of mice treated with IPL in CFA group was lower, but there without statistical difference. For these mice, H&E confirmed irreversible meibomian atrophy, which may be one of the reasons for the nonresponse of IPL. If the CFA group received IPL treatment before most of the glands atrophied, such as 8wk, the results might be different. The small sample size may also be the reason for no statistical difference. In NH group, the MGOS of IPL mice was statistically lower, which confirmed the therapeutic effect of IPL on NH induced MGD model, indicating that NH model was partially reversible. It was also confirmed that blepharitis and meibomian gland atrophy did weaken the therapeutic effect of IPL, but IPL played a therapeutic role in NH group without meibomian gland atrophy.

Ductal epithelial thickening and acinar cell atrophy with inflammatory cell infiltration were observed in CFA group. A previous model of using CFA to induce rabbit MGD did not induce acinar atrophy[26], which may due to the fact that only one additional injection of CFA was given in previous study and the anti-inflammatory treatment was soon performed. In this study, in order to ensure the complete development of MGD, it was necessary to ensure the persistence of blepharitis in mice, thus a total of 6 CFA injections (5 additional injections) were given. The long course of inflammation may be the cause of acinar atrophy and MGD. Studies have shown that continuous or repeated exposure to inflammation may potentially deplete meibocyte stem cells, leading to early aging changes and glandular atrophy[27-28]. The meibomian glands in this model exhibit varying stages of hypertrophy and mild hyperplasia with inflammation. Overall, persistent inflammation has a greater effect on meibomian gland function, which is consistent with our animal model. According to literature, meibomian gland obstruction is affected by endogenous factors including age, gender and hormone disorders, as well as exogenous factors, such as local medication[27]. However, the relationship between inflammation and pathophysiology of MGD remains controversial. In the previous study of human histopathology, no infiltration of inflammatory cells was observed in the specimens of meibomian gland cystic expansion or acinar atrophy[29-30]. Previous studies have suggested that infiltration of inflammatory cells does not seem to be important in the development of MGD[13,26], however, these studies have failed to control the duration of inflammation. Our study suggests that persistent inflammatory states may cause pathophysiological changes in meibomian gland.

In this study, corneal fluorescein staining was not measured in synchronization with the course of the disease. In view of the

influence of inflammation itself on corneal fluorescein staining, it could be hard to explain whether the increase in fluorescein staining score was caused by MGD or inflammation itself. Even allergic inflammation can damage corneal epithelium[31]. Therefore, the effects of plugging on tear stability in this model remain unclear. Considering compress was needed to evaluate the meibum quality, which might improve the function of meibomian gland and have an unexpected therapeutic effect on meibomian, the examination of meibum quality was not conducted. Although our model showed toothpaste-like meibum at the orifices of plugged meibomian gland and it appeared to be a characteristic feature of MGD, it was still difficult to elucidate the components of the meibum due to the limited meibum volume. Furthermore, our study failed to confirm that the perinuclear infiltrate was due to inflammatory cells. The evidence for inflammatory infiltrates was relatively weak, even with the presence of blepharitis. Inflammatory cytokines test will be needed in future studies on this model.

In this research, we report the successful development of a novel MGD model induced by CFA in C57BL/6 mice. The model showed characteristic clinical symptoms, atrophy of meibomian gland acinar cells, involvement of inflammatory cells. Persistent inflammatory state may be the cause of MGD. This model can be available for elucidating the pathophysiology of inflammation related MGD and faster than traditional NH model. It may have the potential to evaluate the treatment of the disease.

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REFERENCES

1 Schaumberg DA, Nichols JJ, Papas EB, Tong L, Uchino M, Nichols KK. The international workshop on meibomian gland dysfunction: report of the subcommittee on the epidemiology of, and associated risk factors for, MGD. *Investig Ophthalmol Vis Sci* 2011;52(4):1994-2005.

2 Arita R, Mizoguchi T, Kawashima M, Fukuoka S, Koh S, Shirakawa R, Suzuki T, Morishige N. Meibomian gland dysfunction and dry eye are similar but different based on a population-based study: the hirado-takushima study in Japan. *Am J Ophthalmol* 2019;207:410-418.

3 Baudouin C, Messmer EM, Aragona P, Geerling G, Akova YA, Benítez-Del-castillo J, Boboridis KG, Merayo-Lloves J, Rolando M, Labetoulle M. Revisiting the vicious circle of dry eye disease: a focus on the pathophysiology of meibomian gland dysfunction. *Br J Ophthalmol* 2016;100(3):300-306.

4 Watters GA, Turnbull PR, Swift S, Petty A, Craig JP. Ocular surface microbiome in meibomian gland dysfunction. *Clin Exp Ophthalmol* 2017;45(2):105-111.

5 Graham JE, Moore JE, Xu JR, Moore JE, Goodall EA, Dooley JSG, Hayes VEA, Dartt DA, Downes CS, Moore TCB. Ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. *Invest Ophthalmol Vis Sci* 2007;48(12):5616.

6 Sabeti S, Kheirkhah A, Yin J, Dana R. Management of meibomian gland dysfunction: a review. *Surv Ophthalmol* 2020;65(2):205-217.

7 Wladis EJ, Bradley EA, Bilyk JR, Yen MT, Mawn LA. Oral antibiotics for meibomian gland-related ocular surface disease: a report by the American academy of ophthalmology. *Ophthalmology* 2016;123(3):492-496.

8 Kashkouli MB, Fazel AJ, Kiavash V, Nojomi M, Ghiasian L. Oral azithromycin versus doxycycline in meibomian gland dysfunction: a randomised double-masked open-label clinical trial. *Br J Ophthalmol* 2015;99(2):199-204.

9 Suzuki T, Teramukai S, Kinoshita S. Meibomian glands and ocular surface inflammation. *Ocular Surf* 2015;13(2):133-149.

10 Albietz JM, Schmid KL. Intense pulsed light treatment and meibomian gland expression for moderate to advanced meibomian gland dysfunction. *Clin Exp Optom* 2018;101(1):23-33.

11 Eom Y, Han JY, Kang B, Hwang HS, Lee HK, Kim HM, Song JS. Meibomian glands and ocular surface changes after closure of meibomian gland orifices in rabbits. *Cornea* 2017;37(2):218-226.

12 Dong ZY, Ying M, Zheng J, Hu LJ, Xie JY, Ma Y. Evaluation of a rat meibomian gland dysfunction model induced by closure of meibomian gland orifices. *Int J Ophthalmol* 2018;11(7):1077-1083.

13 Miyake H, Oda T, Katsuta O, Seno M, Nakamura M. Meibomian gland dysfunction model in hairless mice fed a special diet with limited lipid content. *Investig Ophthalmol Vis Sci* 2016;57(7):3268-3275.

14 Osae EA, Steven P, Redfern R, Hanlon S, Smith CW, Rumbaut RE, Burns AR. Dyslipidemia and meibomian gland dysfunction: utility of lipidomics and experimental prospects with a diet-induced obesity mouse model. *Int J Mol Sci* 2019;20(14):3505.

15 Jester JV, Rife L, Nii D, Luttrull JK, Wilson L, Smith RE. *In vivo* biomicroscopy and photography of meibomian glands in a rabbit model of meibomian gland dysfunction. *Investig Ophthalmol Vis Sci* 1982;22(5):660-667.

16 Wang YC, Li SM, Chen XX, Ma BK, He H, Liu TT, Yu JW, Zhang LY, Chen YX, Liu ZG, Li W. Meibomian gland absence related dry eye in ectodysplasin A mutant mice. *Am J Pathol* 2016;186(1):32-42.

17 Hammell DC, Zhang LP, Ma F, Abshire SM, McIlwrath SL, Stinchcomb AL, Westlund KN. Transdermal cannabidiol reduces inflammation and pain-related behaviours in a rat model of arthritis. *Eur J Pain* 2016;20(6):936-948.

18 Silva Rodrigues J, Silva e Silva C, França Muniz T, *et al*. Sulforaphane modulates joint inflammation in a murine model of complete freund's adjuvant-induced mono-arthritis. *Molecules* 2018;23(5):988.

19 Bonezzi FT, Sasso O, Pontis S, Realini N, Romeo E, Ponzano S, Nuzzi A, Fiasella A, Bertozzi F, Piomelli D. An important role for N-acylethanolamine acid amidase in the complete freund's adjuvant rat model of arthritis. *J Pharmacol Exp Ther* 2016;356(3):656-663.

20 Lin L, Zhu BP, Cai L. Therapeutic effect of melittin on a rat model of chronic prostatitis induced by complete Freund's adjuvant. *Biomed Pharmacother* 2017;90:921-927.

21 Jing PB, Cao DL, Li SS, Zhu MX, Bai XQ, Wu XB, Gao YJ. Chemokine receptor CXCR3 in the spinal cord contributes to chronic itch in mice. *Neurosci Bull* 2018;34(1):54-63.

22 Ichikawa H, Armstrong RB, Harber LC. Photoallergic contact dermatitis in Guinea pigs: improved induction technique using Freund's complete adjuvant. *J Investig Dermatol* 1981;76(6):498-501.

23 Amescua G, Akpek EK, Farid M, Garcia-Ferrer FJ, Lin A, Rhee MK, Varu DM, Musch DC, Dunn SP, Mah FS. Blepharitis preferred practice pattern®. *Ophthalmology* 2019;126(1):P56-P93.

24 Messmer EM. The pathophysiology, diagnosis, and treatment of dry eye disease. *Deutsches Arzteblatt Int* 2015;112(5):71-81.

25 Jester JV, Rajagopalan S, Rodrigues M. Meibomian gland changes in the rhino (hrrhhrrh) mouse. *Investig Ophthalmol Vis Sci* 1988;29(7): 1190-1194.

26 Miyake H, Oda T, Katsuta O, Seno M, Nakamura M. A novel model of meibomian gland dysfunction induced with complete Freund's adjuvant in rabbits. *Vision* 2017;1(1):10.

27 Knop E, Knop N, Millar T, Obata H, Sullivan DA. The international workshop on meibomian gland dysfunction: report of the subcommittee on anatomy, physiology, and pathophysiology of the meibomian gland. *Investig Ophthalmol Vis Sci* 2011;52(4):1938-1978.

28 Olami Y, Zajicek G, Cogan M, Gnessin H, Peer J. Turnover and migration of meibomian gland cells in rats' eyelids. *Ophthalmic Res* 2001;33(3):170-175.

29 Obata H. Anatomy and histopathology of human meibomian gland. *Cornea* 2002;21(7 Suppl):S70-S74.

30 Reneker LW, Irlmeier RT, Shui YB, Liu Y, Huang AJW. Histopathology and selective biomarker expression in human meibomian glands. *Br J Ophthalmol* 2020;104(7):999-1004.

31 Leonardi A, Lazzarini D, la Gloria Valerio A, Scalora T, Fregona I. Corneal staining patterns in vernal keratoconjunctivitis: the new VKC-CLEK scoring scale. *Br J Ophthalmol* 2018;102(10): 1448-1453.