Isotretinoin Impairs the Secretory Function of Meibomian Gland Via the PPARγ Signaling Pathway

Peng Zhang, Lei Tian, Jiayu Bao, Shang Li, Ao Li, Ya Wen, Jingyi Wang, and Ying Jie

Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology and Visual Sciences Key Lab, Beijing, China

Correspondence: Ying Jie, Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology and Visual Sciences Key Lab, No. 1 Dong Jiao Min Xiang, Dong Cheng District, Beijing 100730, China; jie_yingcn@aliyun.com.

PZ and LT contributed equally to this study.

Received: November 4, 2021
Accepted: March 14, 2022
Published: March 30, 2022

Citation: Zhang P, Tian L, Bao J, et al. Isotretinoin impairs the secretory function of meibomian gland via the PPARγ signaling pathway. *Invest Ophthalmol Vis Sci.* 2022;63(3):29. https://doi.org/10.1167/iovs.63.3.29

Purpose. To investigate the effects of isotretinoin on the ocular surface and to explore the possible mechanisms.

Methods. Rats were treated with isotretinoin 20 mg/kg/d for five months and tested monthly for tear secretion, fluorescein staining, and infrared photography. After five months of treatment, tissues were harvested for routine staining to evaluate the morphological changes; and real-time polymerase chain reaction, Western blot, and immunohistochemistry to study the expression of associated genes and their products such as forkhead box protein O1 (*FoxO1*), forkhead box protein O3, peroxisome proliferator–activated receptor γ (*PPARγ*), adipose differentiation–related protein, elongation of very long chain fatty acids protein 4, fatty acid binding protein 4, matrix metalloproteinase-9, and interleukin-6.

Results. Systemically, isotretinoin-treated rats have a significantly lower body weight that controls and apparent skin damage. Locally, although there was no alteration in tear secretion, a significant corneal involvement indicated by increased fluorescein staining scores, and also the contrast of meibomian gland was significantly reduced but no significant atrophy of the acinus was found. In addition, isotretinoin causes a decrease in conjunctival goblet cells. Furthermore, isotretinoin treatment did not cause the upregulation of *FoxO1* and inflammation related genes but significantly suppressed the expression of *PPARγ* pathway.

Conclusions. Isotretinoin does not cause a significant atrophy of the acinus and a significant change of *FoxO1* expression in the meibomian gland. Isotretinoin causes meibomian gland dysfunction, affecting meibocyte differentiation and qualitative and quantitative changes in the meibum, through *PPARγ* pathway.

Keywords: isotretinoin, meibomian gland dysfunction, PPARγ, sebaceous gland, FoxO1

Acne vulgaris is a common disease affects the sebaceous gland that in young patients during their puberty. It is a serious clinical and social problem as epidemiologic studies reported that acne has a prevalence rate of 85% among people aged from 12 to 25.[1] For severe acne in which conventional treatment is ineffective, isotretinoin is the drug of choice for treatment.[2] Isotretinoin, also known as 13-cis retinoic acid, is a natural derivative of vitamin A in the body and belongs to the first generation of non-aromatic hydrocarbon retinoids.[3] Isotretinoin binds weakly to its receptor, but in sebaceous gland cells, it can be converted to all-trans retinoic acid. These active products bind to their cytoplasmic chaperons and then enter the nucleus, where they further bind to retinoic acid receptors and thus regulate gene expression and exert biological effects. Isotretinoin is used for the treatment of acne with a standard oral dosage of 0.5 mg to 2 mg/kg/d for four to six months. Isotretinoin has the most potent anti-lipid activity among all retinoids, reducing sebum production by 90% in only six weeks. European guidelines for acne treatment have listed it as the first-line agent for severe acne,[4] and American Academy of Dermatology guidelines have given low-dose oral isotretinoin a Class A recommendation for the treatment of severe acne.[5]

Although isotretinoin is the most effective drug available for the treatment of acne, its adverse effects are also obvious, and several studies have reported adverse involvement of mucosa, skin and other systemic impacts. Ocular symptoms caused by isotretinoin are a growing concern, as the meibomian gland (MG) is a large, specially differentiated sebaceous gland and oral administration of isotretinoin can lead to meibomian gland dysfunction (MGD) resulting in evaporative dry eye.[6,7] Studies have shown that 30% to 50% of patients received isotretinoin for acne treatment developed dry eye symptoms. It is generally accepted that the effects of isotretinoin on the MG are similar to its effects on the sebaceous glands of the skin.[8] In the studies of sebaceous gland, isotretinoin inhibits lipid synthesis in sebocyte, suppresses cell proliferation, and induces sebocyte apoptosis through upregulation of the transcription factor forkhead box protein O1 (*FoxO1*).[9,10]

However, no studies have shown the role of *FoxO1* in isotretinoin induced MGD, our study is to investigate the effect of isotretinoin treatment on the ocular surface, and

Copyright 2022 The Authors

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

the change of *FoxO1* expression in the MG and to explore the role of the peroxisome proliferator–activated receptor γ (*PPARγ*) pathway involved in it. We found that isotretinoin induced MGD is not FoXO1 mediated but through *PPARγ* pathway.

Materials and Methods

Animal Model and Treatment

Thirty-two 12-week-old female Wistar rats (purchased from Charles River Laboratory Animal Center, Beijing, China) were used in this study. Prior to initiation of treatment, the eyes of each rat were evaluated using a slit-lamp microscope (BX900; Haag-Streit AG, Koeniz, Switzerland). All animal eyes appeared normal. Subsequently, rats were randomly divided into two groups, the control group and the isotretinoin group. Isotretinoin was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (I129856 Aladdin). It was supplied as a yellow-orange to orange crystalline powder that was stored at −20°C in a sealed container away from light. Before its use, the isotretinoin was brought to room temperature and dissolved in glycerol. The isotretinoin group was given 20 mg/kg per day of isotretinoin for five months, and the control group received the vehicle only. The drug was administered by oral intubation, using glycerol as a vehicle.[11] All animal experiments were complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Review Board of Capital Medical University (Beijing, China; approval ID: AEEI-2020-152).

Measurement of Tear Production

Phenol red cotton thread (Tianjin Jingming New Technology Development Co., Ltd., Tianjin, China) was used to collect tear for measurement. Animals were anesthetized by intraperitoneal injection of pentobarbital. The thread was then placed on the lower conjunctival fornix near the lateral canthus at approximately one-third of the length of the lower lid. After one minute, the thread was removed, and the red wetted length was measured.

Corneal Fluorescein Staining

Three blinks after 2 µL of 0.1% sodium fluorescein solution was dropped into the conjunctival sac, and after 60 seconds, the corneal epithelial integrity was graded with a slit-lamp microscope under cobalt blue filter. Punctuate epithelial staining was recorded according to the standard National Eye Institute grading system of 0–3 to each of five areas of the cornea: central, superior, inferior, nasal, and temporal (total, 15 points).[12]

Infrared Imaging and Slit-Lamp Photography of the MG

In this study, MG dropout score and change of contrast were measured using the Oculus Keratograph 5M (Oculus GmbH, Wetzlar, Germany) one day before euthanization. MG morphology was evaluated via a slit-lamp microscope after the animal was euthanized.

RNA Isolation and Real-Time PCR

The upper and lower eyelids were completely excised, meibomian gland tissues were isolated by removing skin, subcutaneous tissue, muscle, and palpebral conjunctiva under a dissecting microscope.[13] Total RNA was extracted with TRIZOL reagent (Yeasen Biotech Co., Ltd., Shanghai, China) and quantified with a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was reverse transcribed of total RNA to with a cDNA Synthesis Super Mix. Gene expression was then measured by quantitative PCR with SYBR Green Master Mix (Yeasen Biotech Co., Ltd., Shanghai, China) using a Bio-Rad CFX96 cycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). *GAPDH* was used as a reference gene. Primer sequences are listed in Table 1.

Western Blot Analysis

Tissues were collected and lysed in RIPA buffer supplemented with protease inhibitor cocktail, and quantified with BCA protein analysis kit (Yeasen Biotech Co., Ltd., Shanghai, China). Then, an equal amount of protein (30 µg) was subjected to SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 5% non-fat milk for one hour, the membranes were incubated with the primary antibodies over night at 4°C. After the membranes were rinsed thoroughly with Tris-buffered saline solution with Tween 20, they were incubated with secondary antibodies for one hour at room temperature. Finally, Western Lightning Plus-ECL (PerkinElmer, Inc, Waltham, MA, USA) was added to magnify the HRP signals, which were detected using a Bio-Rad system (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). Image analysis was performed using the Image Lab software. Primary antibodies used for western blotting are listed below: *FoxO1* (ET1608-25, 1:1000) and fatty acid binding protein 4 (*FABP4*; ET1703-98, 1:1000) were from Huaan Biotechnology (Hangzhou, China). Forkhead box protein O3 (*FoxO3A*) (66428-1-Ig, 1:1000), elongation of very long chain fatty acids protein 4 (*ELOVL4*; 55023-1-AP, 1:500), adipose differentiation–related protein (*ADRP*; 15294-1-AP, 1:1000) and matrix metalloproteinase-9 (*MMP9*) (10375-2-AP, 1:600) were from Proteintech Group Inc (Rosemont, IL, USA). *PPARγ* (2435S, 1:1000) was from Cell Signaling Technology (Danvers, MA, USA).

Table 1. Rat Primer Sequences Used for Real-Time PCR

Genes	Forward Primer	Reverse Primer	PCR Product, bp
FoxO1	TGGGGCAACCTGTCGTA	GGGCACACTCTTCACCATC	108
FoxO3A	GGGTCACGACAAGTTCCC	GGCATCCATGAGTTCGCT	100
ELOVL4	GGACATACAATGAGCCCAA	GCTTCCCGTTCTTCTGG	129
ADRP	CGGCCTCTGCTCTGAAT	AAGCCACACAAGAACATGC	88
PPARγ	GGGAGTTCCTCAAAAGCC	TTCACGTTCAGCAAGCC	163
FABP4	TTCGCCACCAGGAAAGT	ACGCCCAGTTTGAAGGA	125
Caspase3	AGCTGGACTGCGGTATTG	CGGGTGCGGTAGAGTAAG	105
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452

Investigative Ophthalmology & Visual Science

IL6 (abs135607, 1:1000) was from Absin Bioscience Inc (Shanghai, China). *GAPDH* (30202, 1:2000) and secondary antibodies were from Yeasen Biotechnology (Shanghai, China).

Histological Stains

The eyes including the lids were excised and fixed in 10% buffered formalin and embedded in Paraffin. Sections were collected at a thickness of 4 µm from the same locations, each spaced approximately 200 µm apart to span the ocular surface in a lateral orientation.[14] Tissue sections were stained with hematoxylin & eosin (H&E) and periodic acid-Schiff (PAS) to visualize and evaluate meibomian gland and goblet cell morphology and number. Images were acquired using PANNORAMIC whole slide scanners (3DHISTECH Ltd, Budapest, Hungary). The acini area of meibomian gland and the thickness of the ductal epithelium were analyzed by CaseViewer software (3DHISTECH Ltd, Budapest, Hungary). Conjunctival goblet cell counts were assessed with reference to the methods documented in the literature.[14] Five sections from four samples in each group were stained with H&E and PAS stain to observe and evaluate the morphology of meibomian gland and number of GCs.

Oil Red O Staining

Frozen eyelids were sectioned in the vertical plane at a thickness of 10 µm, washed in phosphate-buffered saline solution (PBS) for five minutes and 60% isopropyl alcohol for 20 seconds, and stained with freshly prepared oil red O solution for 15 minutes, and rinsed with 60% isopropanol alcohol for one minute followed by water. The sections were counterstained with hematoxylin. Then the sections were mounted in glycerol and photographed using an upright microscope (Leica Microsystems GmbH, Wetzlar, Germany). The oil red O staining was analyzed with Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA), expressed in mean optical density (MOD). First, the software is required to detect the valid measurement area and then measure the integrated optical density of the targeting stain region within that area. The percent of integrated optic density of the area was MOD.[15]

Immunohistochemistry

Paraffin sections were rehydrated and blocked with 3% hydrogen peroxide for 10 minutes, followed by washing three times with PBS for five minutes each. Sections were subsequently treated with 0.2% Triton X-100 for 20 minutes. After washing three times each with PBS for five minutes, they were incubated with 5% BSA for 60 minutes, followed by incubation with *FoxO1* (ET1608-25, 1:200), *FoxO3A* (66428-1-Ig, 1:250), *PPARγ* (2435S, 1:400), *Elovl4* (55023-1-AP, 1:200), and *ADRP* (15294-1-AP, 1:200) antibodies over night at 4°C. The sections were further incubated with secondary antibodies for one hour. The reaction product was then developed with diaminobenzidine for one minute, mounted with mounting medium, and examined under a light microscope. The immunohistochemical staining was analyzed with Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA), expressed in MOD.

Statistical Analysis

Results in this present study were performed at least three independent experiments and presented as mean ± SD. Student's *t*-test was conducted for statistical comparison of body weight, tear secretion, fluorescein staining scores and relative mRNA and protein expression between two individual groups and the Mann-Whitney U test was used to mean optical density of Immunohistochemistry using SPSS 17.0 software (SPSS, Chicago, IL, USA). Statistical significances were determined as $P < 0.05$.

Results

Isotretinoin Induces Dry Eye-like Ocular Surface Damages

After 5 months of isotretinoin treatment, animals showed clinical signs of systemic toxicity, including significantly lower body weight than controls, dry skin, and abnormal secretions on the back. Animals in the control group showed no signs of toxicity (Figs. 1A, 1B). During isotretinoin treatment, no changes in tear secretion at all time points was observed, and there was no difference in tear secretion after 5 months of isotretinoin treatment compared with the

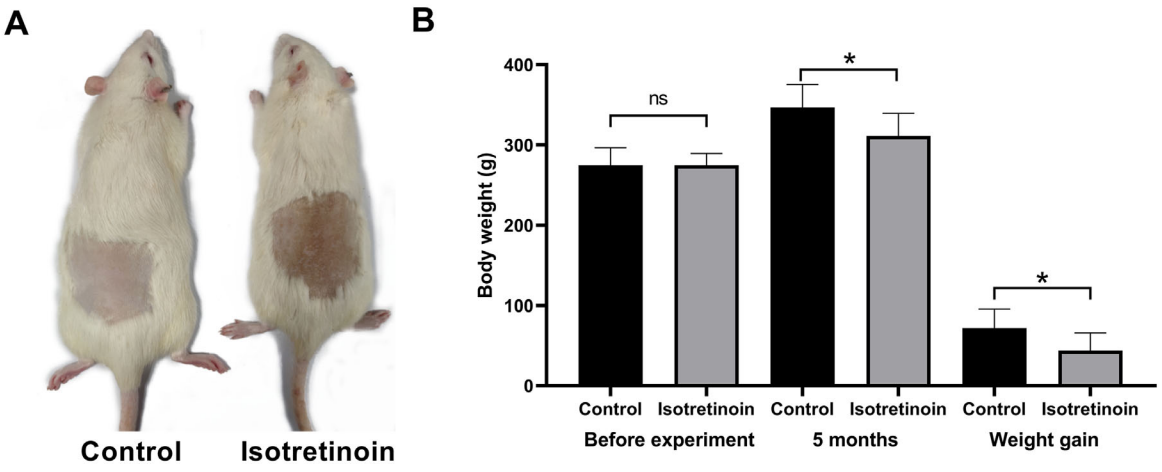

Figure 1. Body weight and skin changes of rats. Body size, skin changes (**A**), and body weight (**B**) decreased in the isotretinoin group compared with the control group. Data are presented as the mean ± SD. $^{*}P < 0.05$ versus control group.

Investigative Ophthalmology & Visual Science

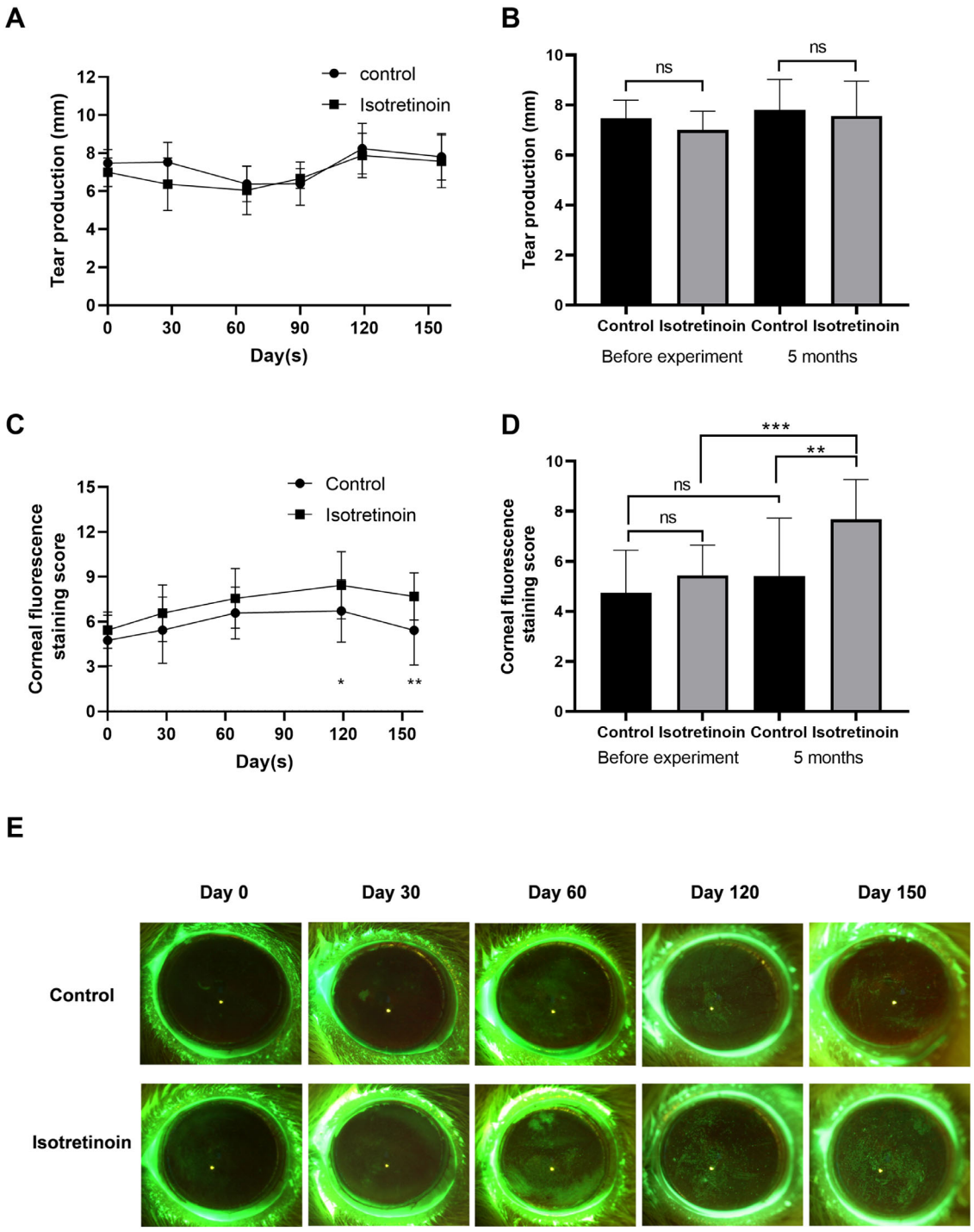

Figure 2. Clinical examination of animals treated with continuous isotretinoin. The tear production (**A**) and corneal fluorescein staining (**C**) were examined monthly. The tear production and staining scores before experiment and after the last treatment were compared (**B, D**). Representative pictures of corneal fluorescein staining were shown (**E**). Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group.

control group (Figs. 2A, 2B). After five months of isotretinoin treatment, it is observed an increase in corneal fluorescein staining scores. After five months of isotretinoin treatment, corneal fluorescein sodium staining was significantly higher in the isotretinoin group compared with the control group; and also significantly higher than the baseline (Figs. 2C, 2D). Representative pictures of corneal fluorescein staining were shown (Fig. 2E).

Isotretinoin Causes Thickening of the Epithelium in MG Ducts, a Decrease in Lipid Secretion and the Number of Conjunctival Goblet Cells

In the isotretinoin treated group, there was no significant difference in the morphology and size of the MGs from the control group by slit lamp examination (Fig. 3A). It is observed in the upper MG, the contrast of the gland was

Investigative Ophthalmology & Visual Science

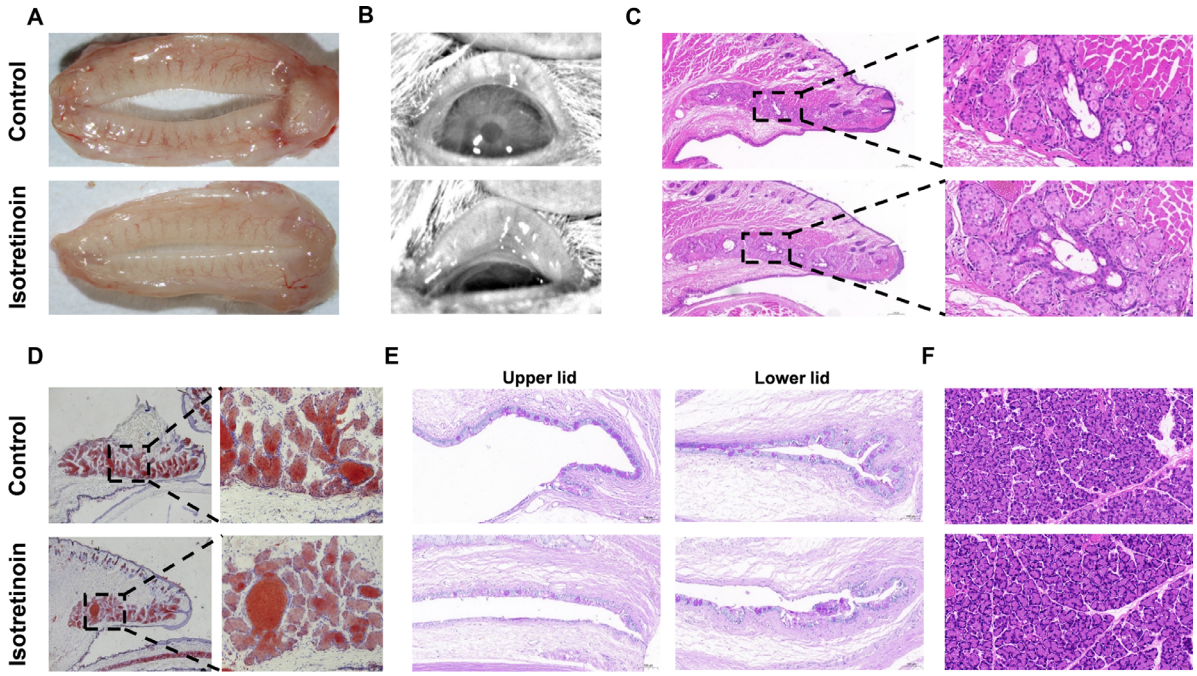

Figure 3. The slit-lamp photography of the eyelids (**A**). The infrared photography of the meibomian glands (**B**). H&E staining of duct and acini of meibomian gland (**C**) (n = 4). *Scale bars*: 50 µm. Oil red O staining of meibomian gland of the isotretinoin and control rats (**D**) (n = 4). *Scale bars*:25 µm. Representative images of the PAS staining. The number of PAS-positive cells was significantly decreased in the Isotretinoin group (**E**) (n = 4). *Scale bars*: 100 µm. H&E staining of lacrimal gland (**F**). *Scale bars*: 50 µm.

Table 2. Comparison of Characteristics Between Isotretinoin and Controls

	Control	Isotretinoin	*P* Value
Meibomian gland Acini size (upper lid), mm^2	0.443 ± 0.048	0.438 ± 0.051	0.809
Ductal epithelial thickness, µm	8.39 ± 1.83	15.91 ± 3.79	0.0001*
Conjunctival goblet cell, cell/mm	12.30 ± 2.10	8.28 ± 1.06	0.0412*
Oil red O mean optical density	77.81 ± 19.15	43.76 ± 15.26	0.0031*

* Statistically significant at $P \leq 0.05$.

significantly lower in the isotretinoin treated group than in the control group by infrared imaging (Fig. 3B). H&E staining of the MG showed that after isotretinoin treatment, the duct epithelium was significantly thickened without significant atrophy of the acinus compared to the control group (Fig. 3C). Oil red O staining showed a significant decrease in the isotretinoin group, indicating a decrease in lipid production (Fig. 3D). PAS staining was performed and most of the PAS-positive cells in the control group resided in the superficial epithelium of conjunctival fornix. The isotretinoin treatment showed a decreased number of mucin-filled goblet cells (Fig. 3E). H&E staining of the lacrimal gland did not show significant changes in the isotretinoin group compared with the control group, and there was no inflammatory infiltration (Fig. 3F). Statistical analysis of the acini area of meibomian gland, duct thickness, the number of conjunctival goblet cells and the mean optical density of oil red O staining were presented in Table 2.

Isotretinoin Did Not Stimulate Inflammatory Cytokine Production in the Meibomian Gland

Upregulation of *MMP9* is associated with ocular surface damage, which contributes to ocular surface inflammation and dry eye.[16] Its activity was significantly elevated in patients with MGD as well.[17] We found that it was not significantly changed in the isotretinoin group. At the same time, inflammatory cytokines *IL6* protein was significantly downregulated by western blot (Figs. 4A, 4B), indicating that isotretinoin did not cause any inflammatory change in the MG.

Isotretinoin Treatment Does Not Upregulate in *FoxO1* Expression

Literature suggests that the effect of isotretinoin on the MGs may be similar to its effects on the sebaceous glands of the skin in the treatment of acne.[8,10,18] *FoxO1* plays an important role in the isotretinoin treatment of sebaceous acne, and there are hypotheses that the side effects of isotretinoin are also caused by elevated *FoxO1* in the nucleus. To investigate whether isotretinoin affects the MG via *FoxO1*, *FoxO1* and *FoxO3A* expression in the MG was measured. It is found that *FoxO1* was not significantly changed. However, *FoxO3A* was significantly decreased (Figs. 5A–C). From the immunohistochemical staining, *FoxO1* expression in normal rat meibomian gland and sebaceous gland tissues is mainly in the nucleus, with weak staining in the cytoplasm.

Investigative Ophthalmology & Visual Science

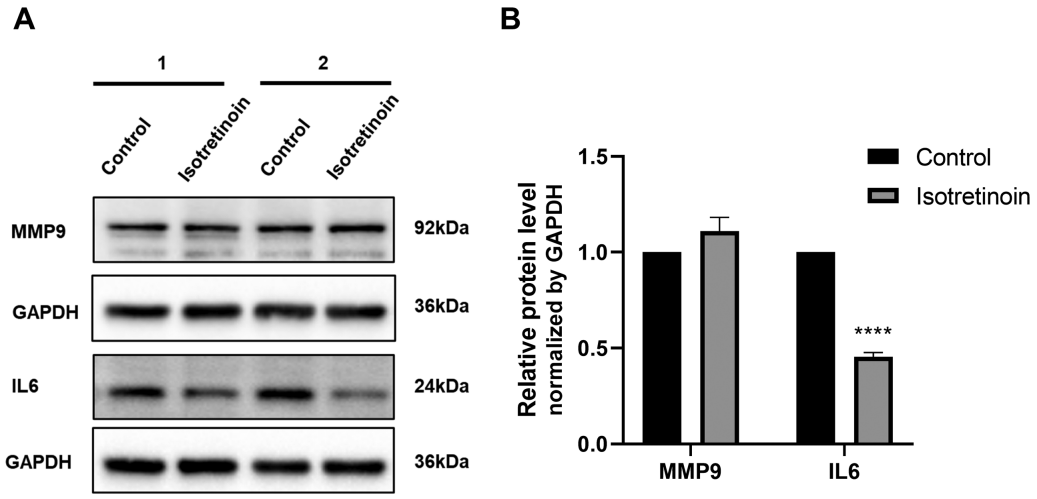

Figure 4. Western blot assay of indicated *MMP9* and *IL6* proteins in the meibomian gland homogenates (**A**) and densitometry analyses of the Western blotting results (**B**). Data are presented as the mean ± SD (n = 6). $^{****}P < 0.0001$ versus control group.

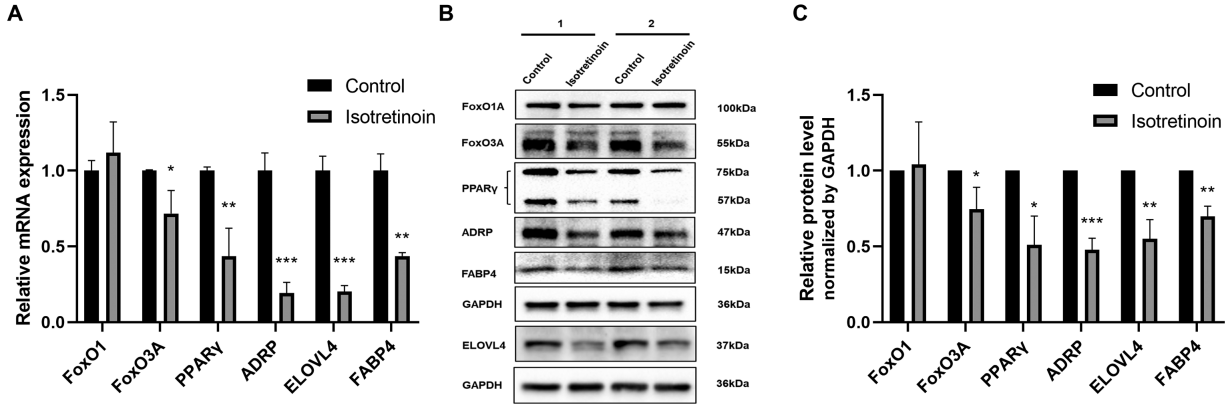

Figure 5. *FoxO1*, *FoxO3A*, *PPARγ*, *ADRP*, *ELOVL4* and *FABP4* mRNA levels in the meibomian gland were measured by qPCR (**A**) (n = 6). Western blot assay of indicated *FoxO1*, *FoxO3A*, *PPARγ*, *ADRP*, *ELOVL4*, and *FABP4* proteins in the meibomian gland homogenates (**B**) and densitometry analyses of the western blotting results (**C**) (n = 6). Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group.

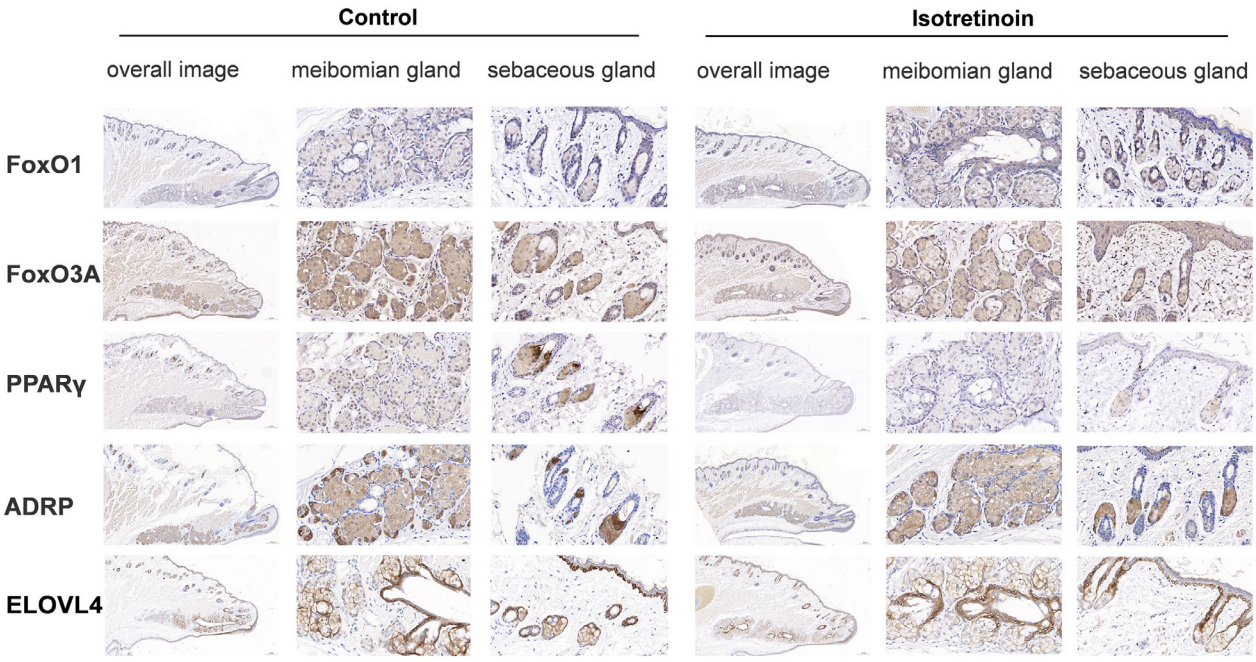

Figure 6. Immunohistochemical staining to detect meibomian gland and sebaceous gland *FoxO1*, *FoxO3A*, *PPARγ*, *ADRP*, and *ELOVL4* expression (n = 4). *Scale bars*: 20 µm.

Investigative Ophthalmology & Visual Science

Table 3. Comparison of Immunohistochemistry Between Control and Isotretinoin Group

	Control	Isotretinoin	*P* Value
Meibomian gland			
FoxO1 (nucleus)	27.53 ± 4.85	30.08 ± 1.35	0.2899
FoxO3A	172.87 ± 35.35	75.37 ± 51.78	0.0002*
PPARγ	30.36 ± 19.26	6.66 ± 2.48	0.0012*
ADRP	51.09 ± 14.98	19.72 ± 5.68	0.0286*
ELOVL4	291.60 ± 102.3	111.71 ± 60.52	0.0003*
Sebaceous gland			
FoxO1 (nucleus)	27.75 ± 3.55	27.20 ± 3.85	0.8968
FoxO3A	109.75 ± 25.19	47.47 ± 23.85	0.0005*
PPARγ	101.85 ± 12.41	11.61 ± 4.88	0.0022*
ADRP	142.45 ± 41.42	38.85 ± 9.27	0.0004*
ELOVL4	129.64 ± 12.17	61.62 ± 25.73	0.0002*
Lacrimal gland			
PPARγ	61.67 ± 8.25	25.62 ± 9.25	0.0022*
FoxO3A	107.18 ± 24.11	22.92 ± 1.10	0.0286*

* Statistically significant at $P \leq 0.05$.

Immunohistochemistry also did not reveal significant changes of the expression of FoxO1 in the nucleus in the MGs and sebaceous glands (Fig. 6, Table 3).

Isotretinoin Induces a Decreased Expression of Lipid Metabolism Associated Genes

In recent years, several studies have also been performed in MG cells, and it was found that PPARγ was significantly downregulated in aging MGs.[19–21] PPARγ is a lipid-sensitive transcription factor that plays an important role in governing morphology, lipid metabolism and cell differentiation of the MG.[22] To further determine the lipid-associated genes in the MG after isotretinoin treatment, we investigated several lipogenesis-related factors including *PPARγ*, and its downstream component *ADRP*, *ELOVL4* and *FABP4* using both real-time polymerase chain reaction and Western blot. The results showed that the expression of *PPARγ*, *ADRP*, *ELOVL4* and *FABP4* were significantly downregulated after isotretinoin treatment (Figs. 5A–C). The results by immunohistochemistry also showed that *PPARγ*, *ADRP* and *ELOVL4* were significantly decreased in the MGs and sebaceous glands (Fig. 6, Table 3).

In the lacrimal gland tissue, after isotretinoin treatment, no significant differences were seen in *FoxO1* and *Caspase3* mRNA and protein in the lacrimal gland (Figs. 7A, 7B); the expression of *FoxO3A* and *PPARγ* mRNA and protein were downregulated in the lacrimal gland (Figs. 7A, 7C).

Discussion

MGD is a chronic, diffuse abnormality characterized by obstruction of the terminal ducts of the MG or qualitative or quantitative abnormalities in meibum secretion.[23] It has been reported in many studies that isotretinoin can cause meibomian gland dysfunction, but the mechanism has not been clarified.

When the effects of isotretinoin in animal models were explored, it was shown that oral administration of low-dose isotretinoin (2 mg/kg/d) to rabbits for 60 days reduced goblet cells, increased thickening and keratinization of the meibomian gland ducts, and reduced meibomian gland acini size and lipid secretion. No evidence of an inflammatory response in the meibomian gland.[24] Meibomian gland morphology was also analyzed in a hamster model, where oral administration of isotretinoin over 30 days resulted in a 75% reduction in the mean acinar volume of the meibomian gland. In addition, hamsters developed eyelid crusting and conjunctival erythema.[25] Two recent studies evaluated the effects of isotretinoin on the rat meibomian gland. Ibrahim et al.[26] treated male albino rats with 0.5 mg/kg/d isotretinoin (dissolved in 0.5 mL distilled water) for three months, morphometric analysis of the mean area of the acinar tissue in the isotretinoin-treated group showed a highly significant decrease by high magnification images. In addition to this, thickening and keratinization of the epithelial lining of the ducts and a significant increase in collagen fiber content were found. Karadag et al.[11] treated Wistar rats by oral administration of isotretinoin (dissolved in glycerol) in two different concentrations: 7.5 mg/kg and 15 mg/kg for four weeks. A significant reduction in acini size of meibomian gland and the number of goblet cells in both 7.5 mg/kg/d and 15 mg/kg/d groups compared with the control group.[11] Although statistically significant differences were observed between the isotretinoin and control groups, the change of glandular vesicle size was not dramatic. And there was no significant difference between the 15 mg/kg/d high-dose group and the 7.5 mg/kg/d low-dose isotretinoin-treated group.

In clinical studies, the contrast of the meibomian gland was the first to change significantly during isotretinoin treatment. Subsequently, Morphological changes such as

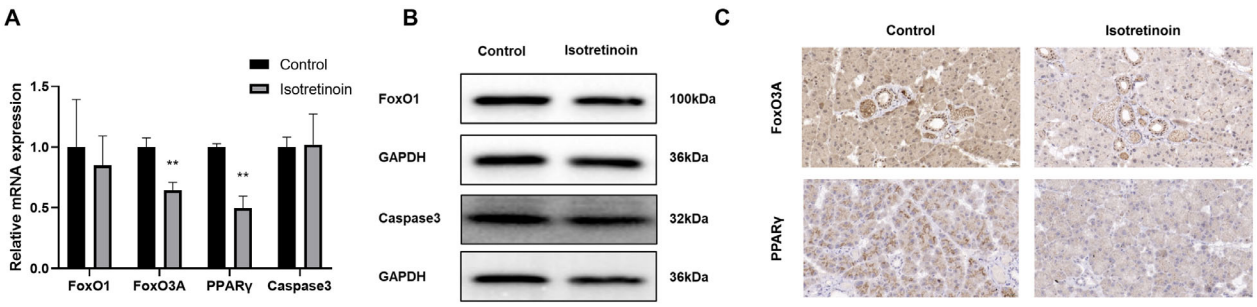

Figure 7. (**A**) *FoxO1*, *FoxO3A*, *PPARγ*, and *Caspase3* mRNA levels in the lacrimal gland were measured by qPCR (n = 6). (**B**) Western blot assay of indicated FoxO1, and Caspase3 proteins in the lacrimal gland homogenates (n = 6). (**C**) Immunohistochemical staining to detect lacrimal gland *FoxO3A*, *PPARγ* expression (n = 3). Data are presented as the mean ± SD, $^{**}P < 0.01$ versus control group. *Scale bars*: 20 µm.

Investigative Ophthalmology & Visual Science

dropout, distortion and shortening occur, and some studies have also shown a significant decrease in contrast after five months of treatment after receiving isotretinoin, but the gland length remained stable during this process, and no atrophy was seen.[18,27–29] This suggests that isotretinoin may first significantly cause a decrease in the secretory capacity of the meibomian gland. Subsequently, changes in glandular morphology occurred.[18]

In the present experiment, our results showed that isotretinoin 20 mg/kg/d for five months resulted in significant thickening of the MG duct epithelium, but no significant atrophy of the acinus, eyelid inflammation, or obstruction of the MG orifice was observed. We also observed the eyelids as a whole by slit lamp and infrared imaging and found that the MG did not show significant atrophy, but mainly a significant decrease in gland contrast after isotretinoin treatment, similar to the results of some previous clinical studies. In addition, lipid production was significantly decreased after isotretinoin treatment by oil red O staining. The effect of isotretinoin resulted mainly in a decrease in meibum production.

Differences in dissolution method, the analysis method of meibomian gland area, animal species and sex may be responsible for the differences that occur. First of all, the dissolution method is different. As far as we know, isotretinoin is insoluble in water and suspended in glycerol. Secondly, the dose of 7.5 mg/kg isotretinoin in the rat model is equivalent to the dose of 550 to 600 ng/mL in humans. The dose of 7.5 mg/kg isotretinoin applied in rats is consistent with human serum after the administration of isotretinoin in acne therapy (0.5-1 mg/kg).[30] This concentration has also been used in other studies of isotretinoin.[31–33] Thirdly, we scanned the sections using whole slide scanners, and performed an overall analysis of the meibomian gland acini area. This gives a more comprehensive picture of the changes in acini area compared to localized high magnification sections in the study of Ibrahim et al.[26] And last, but not least, it may also be species-related.[34,35] The low teratogenic doses of isotretinoin in rats and mice far exceed the teratogenic doses in humans. Rats and mice belonging to an insensitive species can rapidly eliminate isotretinoin by detoxification into β-glucuronide.[35] The rabbit and hamster are more sensitive to isotretinoin than the rat. The variation in the drastic species difference of isotretinoin is due in large part to differences in toxicokinetics, placental transfer, and metabolism.[35] This may be the reason why we did not observe significant meibomian gland acinar atrophy in the rat.

Among patients with acne, nuclear deficiency and cytoplasmic expression of *FoxO1* are increased in the sebaceous glands compared to the healthy control group,[36] and expression of nonphosphorylated *FoxO1* in the nucleus was upregulated and the nuclear-cytoplasmic ratio was significantly increased during isotretinoin treatment, involved in isotretinoin-induced pro-apoptotic signaling in sebaceous glands.[9] Isotretinoin treatment of acne by affecting the sebaceous glands through the isotretinoin→*ATRA*→*RAR*→*FoxO1* pathway, and it is speculated that possibly isotretinoin affects the MG also via *FoxO1*.[10] In our study, the expression level did not significantly increase in the MG by systemic isotretinoin treatment. This may be related to the disease state. In acne patients, sebocytes show a deficiency of *FoxO1* in the nucleus, which is transferred to the cytoplasm. Normal individuals are mainly expressed in nuclear or nuclear with faint cytoplasmic expression of *FoxO1*.[9,36] After isotretinoin treatment, the nuclear/cytoplasmic ratio was significantly increased, but it did not exceed the nuclear/cytoplasmic ratio of normal human *FoxO1*.[9] We used normal rats for our experiments and in normal specimens itself *FoxO1* expression made strong positive expression in the nucleus and weak expression in the cytoplasm. The action of isotretinoin did not significantly promote the upregulation of *FoxO1* intranuclear expression in normal samples. Isotretinoin does not affect the basal expression level of *FoxO1* in the meibomian gland of normal rats. In addition to this, it may be related to the sensitivity of the species to isotretinoin. In the present study, we did not cause significant atrophy of the meibomian gland by isotretinoin.

The MG is a modified holocrine sebaceous gland, and its secretion requires constant destruction of MG acinus cells and therefore requires constant differentiation of MG stem cells located in the basal layer of the acinus to allow renewal.[37] Literature revealed the presence of a stem cell-like population around the circumference and the presence of transient amplifying cells in the basal layer of the acinus.[38] Before adipogenesis and holocrine secretion, the basal acinar cells of the MG differentiate and move toward the center of the acinus. In the literature, peroxisome proliferator-activated receptor (*PPAR*γ), a lipid-sensitive nuclear receptor that regulates differentiation and lipid synthesis in MG cells, is significantly downregulated in aging human and mouse MGs.[19–21] In the MG cells, *PPAR*γ was expressed in both the nucleus and cytoplasm, whereas in the lacrimal gland it was expressed only in the cytoplasm, which may be related to the cell type and cell function. In both tissues, the expression level of *PPAR*γ was downregulated after isotretinoin treatment. Its downstream adaptor *ADRP* is a storage protein involved in adipocyte maintenance and development. It is found in lipid droplets of different cell types and is thought to be a molecule expressed early in lipid accumulation and early adipocyte differentiation. *ADRP* protein decreases as adipocyte differentiation progresses. *ADRP* protein is expressed on the surface of lipid droplets in the early stages of adipocyte differentiation, whereas *ADRP* is absent from the surface of lipid droplets in late differentiation and mature adipocytes.[39] The presence of *ADRP* has been reported in rat MG tissue by Northern and Western blot analysis and immunohistochemistry.[40] In our study, *ADRP* was strongly expressed in the cytoplasm of acinar cells at the margins of the glandular vesicles in the control group. In contrast, *ADRP* expression was weak in the isotretinoin group. This indicates that isotretinoin significantly influenced the early differentiation of acinar cells.

The lipids of the meibomian gland consist mainly of neutral lipids. Neutral lipids, such as wax esters and cholesterol esters, form a nonpolar lipid sublayer in the tear film that is in contact with the external environment.[41] A significant decrease in wax esters in the lipids of the sebaceous glands after isotretinoin action has been documented.[42,43] In addition to this, meibum differ from sebum in that they contain more extremely long-chain fatty acids such as (O-Acyl)-ω-hydroxy fatty acids (OAHFAs).[44] OAHFAs are the main components of the amphiphilic lipid sublayer and are thought to have a role in stabilizing the tear film by producing an interface between the water-immiscible nonpolar lipid sublayer and the aqueous layer beneath it.[45] All OAHFA-based lipids and their derivatives contain extremely long-chain fatty acids with residues $>C_{28}$. The elongase of long chain fatty acid family (*ELOVL1–ELOVL7*)

are the key rate-limiting enzymes for extremely long chain fatty acid synthesis. Of these, *ELOVL4* shows activity toward substrates $\geq C_{26}$.[46] Fatty acid binding proteins (*FABPs*) are proteins that bind long-chain fatty acids and are involved in fatty acid uptake and transport.[47] Therefore we examined the expression level of *ELOVL4* and *FABP4* after the action of isotretinoin and showed that the expression of *ELOVL4* and *FABP4* were significantly reduced after the action of isotretinoin, which may lead to a reduction in the synthesis, uptake, and transport of extremely long-chain fatty acids such as OHAFA, affecting the stability between the lipid and aqueous layers of the tear film and leading to evaporative dry eye. *ELOVL4* and *FABP4* are also regulated by *PPARγ*.[48,49]

In the present study, we demonstrate for the first time that isotretinoin regulates MG acinar cell differentiation and lipogenesis through the *PPARγ* pathway. This conclusion is supported by substantial evidence. The results showed a significant decrease in the expression of the lipid-related genes *PPARγ*, *ADRP*, *ELOVL4* and *FABP4*, this change was paralleled by a decreased lipid production, as verified by the infrared imaging system and oil red O staining. This may reflect qualitative or quantitative changes in meibum secretion.

In addition, the number of conjunctival goblet cells was also decreased in rat conjunctiva after isotretinoin treatment. This is consistent with previous findings from animal studies and clinical studies.[11,50] We did not observe significant changes in tear secretion and lacrimal gland morphology in rats after the action of isotretinoin. The results of tear secretion after isotretinoin treatment have been inconsistently reported in the literature. Some literature reports a significant decrease in tear production, but tear production remains within the normal value.[51,52] Other literature shows no significant change in tear secretion.[6,53–56] Barbosa et al.[57] reported a white female with lacrimal gland agenesis, and after excluding Sjogren's syndrome, a review of the medical history revealed a possible association with Isotretinoin use. But in previous animal studies, rabbits were given doses five to 10 times higher than those used clinically for more than five months, there were no significant changes in tear secretion compared with the control group. Furthermore, histological analysis showed no significant changes in lacrimal gland morphology.[58] No significant differences were also seen in the expression of MMP9 in the ocular tissues after isotretinoin treatment. Moreover, the expression of inflammatory factor IL6 was downregulated. Species differences may be an important reason for the inconsistency of inflammation with previous cellular experiments. In our study, because of the insensitivity of the rats to isotretinoin treatment, no significant meibomian gland atrophy was induced similar to that observed clinically in humans, while the large difference in inflammatory response between rodents and humans may have contributed to the inconsistent observations with previous experiments with human meibomian gland epithelial cells.[35,59–62]

In general, our findings further support existing data showing that isotretinoin can induce MGD. These results were consistent with those in the ocular surfaces in patients receiving prolonged systemic isotretinoin treatment. Isotretinoin action was mainly followed by a marked thickening of the MG duct epithelium and a decrease in meibum secretion and conjunctival goblet cells. *FoxO1* was not significantly altered by isotretinoin treatment. Rather, there was a marked decrease in lipid genes, probably related to the *PPARγ* pathway, reflecting qualitative and quantitative changes in the meibum. Similar to the clinical hyposecretory form of MGD, it is suggested that clinical attention should not only be focused on the manifestation of acinus atrophy shown on infrared imaging of MG, but also on the altered contrast of MG and decreased secretory capacity. In particular, it is important to monitor the condition of the MG regularly during the period of medication and to adjust dose when it is appropriate to reduce the ocular impact of long-term use of isotretinoin.

Acknowledgments

Supported by the National Natural Science Foundation of China (81970764) and Youth Beijing Scholar 2020 (NO.022).

Disclosure: **P. Zhang**, None; **L. Tian**, None; **J. Bao**, None; **S. Li**, None; **A. Li**, None; **Y. Wen**, None; **J. Wang**, None; **Y. Jie**, None

References

1. Lynn DD, Umari T, Dunnick CA, Dellavalle RP. The epidemiology of acne vulgaris in late adolescence. *Adolesc Health Med Ther*. 2016;7:13–25.
2. Bettoli V, Guerra-Tapia A, Herane MI, Piquero-Martin J. Challenges and solutions in oral isotretinoin in acne: reflections on 35 years of experience. *Clin Cosmet Invest Dermatol*. 2019;12:943–951.
3. Layton A. The use of isotretinoin in acne. *Dermatoendocrinol*. 2009;1:162–169.
4. Nast A, Dreno B, Bettoli V, et al. European evidence-based (S3) guidelines for the treatment of acne. *J Eur Acad Dermatol Venereol*. 2012;26(Suppl 1):1–29.
5. Zaenglein AL, Pathy AL, Schlosser BJ, et al. Guidelines of care for the management of acne vulgaris. *J Am Acad Dermatol*. 2016;74:945–973.e33.
6. Caglar C, Senel E, Sabancilar E, Durmus M. Reduced ocular surface disease index (OSDI) scores in patients with isotretinoin treatment. *Int Ophthalmol*. 2017;37:197–202.
7. Ruiz-Lozano RE, Hernandez-Camarena JC, Garza-Garza LA, Bustamante-Arias A, Colorado-Zavala MF, Cardenas-de la Garza JA. Isotretinoin and the eye: a review for the dermatologist. *Dermatol Ther*. 2020;33(6):e14029.
8. Moy A, McNamara NA, Lin MC. Effects of Isotretinoin on Meibomian Glands. *Optom Vis Sci*. 2015;92:925–930.
9. Agamia NF, Hussein OM, Abdelmaksoud RE, et al. Effect of oral isotretinoin on the nucleo-cytoplasmic distribution of FoxO1 and FoxO3 proteins in sebaceous glands of patients with acne vulgaris. *Exp Dermatol*. 2018;27:1344–1351.
10. Melnik BC. Isotretinoin and FoxO1: A scientific hypothesis. *Dermatoendocrinol*. 2011;3:141–165.
11. Karadag R, Karadag AS, Ozlu E, et al. Effects of different doses of systemic isotretinoin on eyes: a histopathological and immunohistochemical study in rats. *Cornea*. 2020;39:621–627.
12. Li X, Kang B, Woo IH, et al. Effects of topical mucolytic agents on the tears and ocular surface: a plausible animal model of mucin-deficient dry eye. *Invest Ophthalmol Vis Sci*. 2018;59:3104–3114.
13. Bu J, Zhang M, Wu Y, et al. High-fat diet induces inflammation of meibomian gland. *Invest Ophthalmol Vis Sci*. 2021;62:13.
14. Zhang R, Park M, Richardson A, et al. Dose-dependent benzalkonium chloride toxicity imparts ocular surface epithelial changes with features of dry eye disease. *Ocul Surf*. 2020;18:158–169.

15. Yao YL, Han X, Li ZM, Lian LH, Nan JX, Wu YL. Acanthoic acid can partially prevent alcohol exposure-induced liver lipid deposition and inflammation. *Front Pharmacol*. 2017;8:134.
16. Messmer EM, von Lindenfels V, Garbe A, Kampik A. Matrix metalloproteinase 9 testing in dry eye disease using a commercially available point-of-care immunoassay. *Ophthalmology*. 2016;123:2300–2308.
17. Aragona P, Aguennouz MH, Rania L, et al. Matrix metalloproteinase 9 and transglutaminase 2 expression at the ocular surface in patients with different forms of dry eye disease. *Ophthalmology*. 2015;122:62–71.
18. Tanriverdi C, Nurozler Tabakci B, Donmez S. Longitudinal assessment of meibomian glands and tear film layer in systemic isotretinoin treatment [published online ahead of print May 20, 2021]. *Eur J Ophthalmol*, doi:10.1177/11206721211018361.
19. Nien CJ, Paugh JR, Massei S, Wahlert AJ, Kao WW, Jester JV. Age-related changes in the meibomian gland. *Exp Eye Res*. 2009;89:1021–1027.
20. Nien CJ, Massei S, Lin G, et al. Effects of age and dysfunction on human meibomian glands. *Arch Ophthalmol*. 2011;129:462–469.
21. Jester JV, Potma E, Brown DJ. PPARgamma regulates mouse meibocyte differentiation and lipid synthesis. *Ocul Surf*. 2016;14:484–494.
22. Hwang HS, Parfitt GJ, Brown DJ, Jester JV. Meibocyte differentiation and renewal: Insights into novel mechanisms of meibomian gland dysfunction (MGD). *Exp Eye Res*. 2017;163:37–45.
23. Nelson JD, Shimazaki J, Benitez-del-Castillo JM, et al. The international workshop on meibomian gland dysfunction: report of the definition and classification subcommittee. *Invest Ophthalmol Vis Sci*. 2011;52:1930–1937.
24. Kremer I, Gaton DD, David M, Gaton E, Shapiro A. Toxic effects of systemic retinoids on meibomian glands. *Ophthalmic Res*. 1994;26:124–128.
25. Lambert RW, Smith RE. Effects of 13-cis-retinoic acid on the hamster meibomian gland. *J Invest Dermatol*. 1989;92:321–325.
26. Ibrahim MAA, Elwan WM. Role of topical dehydroepiandrosterone in ameliorating isotretinoin-induced Meibomian gland dysfunction in adult male albino rat. *Ann Anat*. 2017;211:78–87.
27. Yeh TN, Lin MC. Repeatability of meibomian gland contrast, a potential indicator of meibomian gland function. *Cornea*. 2019;38:256–261.
28. Gurlevik U, Kemeriz F, Yasar E. The effect of isotretinoin on meibomian glands in eyes: A pilot study [published online ahead of print Jan 03, 2022]. *Int Ophthalmol*, doi:10.1007/s10792-021-02205-1.
29. Yeh TN, Lin MC. Meibomian gland contrast sensitivity and specificity in the diagnosis of lipid-deficient dry eye: a pilot study. *Optom Vis Sci*. 2021;98:121–126.
30. Cisneros FJ, Gough BJ, Patton RE, Ferguson SA. Serum levels of albumin, triglycerides, total protein and glucose in rats are altered after oral treatment with low doses of 13-cis-retinoic acid or all-trans-retinoic acid. *J Appl Toxicol*. 2005;25:470–478.
31. Ferguson SA, Cisneros FJ, Gough BJ, Ali SF. Four weeks of oral isotretinoin treatment causes few signs of general toxicity in male and female Sprague-Dawley rats. *Food Chem Toxicol*. 2005;43:1289–1296.
32. Ferguson SA, Berry KJ. Oral Accutane (13-cis-retinoic acid) has no effects on spatial learning and memory in male and female Sprague-Dawley rats. *Neurotoxicol Teratol*. 2007;29:219–227.
33. Ilić I, Oršolić N, Rođak E, et al. The effect of high-fat diet and 13-cis retinoic acid application on lipid profile, glycemic response and oxidative stress in female Lewis rats. *PLoS One*. 2020;15:e0238600.
34. Kamm JJ. Toxicology, carcinogenicity, and teratogenicity of some orally administered retinoids. *J Am Acad Dermatol*. 1982;6:652–659.
35. Nau H. Teratogenicity of isotretinoin revisited: species variation and the role of all-trans-retinoic acid. *J Am Acad Dermatol*. 2001;45:S183–187.
36. Agamia NF, Abdallah DM, Sorour O, Mourad B, Younan DN. Skin expression of mammalian target of rapamycin and forkhead box transcription factor O1, and serum insulin-like growth factor-1 in patients with acne vulgaris and their relationship with diet. *Br J Dermatol*. 2016;174:1299–1307.
37. 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. *Invest Ophthalmol Vis Sci*. 2011;52:1938–1978.
38. Olami Y, Zajicek G, Cogan M, Gnessin H, Pe'er J. Turnover and migration of meibomian gland cells in rats' eyelids. *Ophthalmic Res*. 2001;33:170–175.
39. Itabe H, Yamaguchi T, Nimura S, Sasabe N. Perilipins: a diversity of intracellular lipid droplet proteins. *Lipids Health Dis*. 2017;16:83.
40. Kutsuna M, Kodama T, Sumida M, et al. Presence of adipose differentiation-related protein in rat meibomian gland cells. *Exp Eye Res*. 2007;84:687–693.
41. Green-Church KB, Butovich I, Willcox M, et al. The International Workshop on Meibomian Gland Dysfunction: report of the Subcommittee on Tear Film Lipids and Lipid-Protein Interactions in Health and Disease. *Invest Ophthalmol Vis Sci*. 2011;52:1979–1993.
42. Strauss JS, Stranieri AM, Farrell LN, Downing DT. The effect of marked inhibition of sebum production with 13cis-retinoic acid on skin surface lipid composition. *J Invest Dermatol*. 1980;74:66–67.
43. Robosky LC, Wade K, Woolson D, et al. Quantitative evaluation of sebum lipid components with nuclear magnetic resonance. *J Lipid Res*. 2008;49:686–692.
44. Butovich IA. Meibomian glands, meibum, and meibogenesis. *Exp Eye Res*. 2017;163:2–16.
45. Miyamoto M, Sassa T, Sawai M, Kihara A. Lipid polarity gradient formed by omega-hydroxy lipids in tear film prevents dry eye disease. *Elife*. 2020;9:e53582.
46. McMahon A, Lu H, Butovich IA. A role for ELOVL4 in the mouse meibomian gland and sebocyte cell biology. *Invest Ophthalmol Vis Sci*. 2014;55:2832–2840.
47. Storch J, Thumser AE. Tissue-specific functions in the fatty acid-binding protein family. *J Biol Chem*. 2010;285:32679–32683.
48. Kim SW, Xie Y, Nguyen PQ, et al. PPARgamma regulates meibocyte differentiation and lipid synthesis of cultured human meibomian gland epithelial cells (hMGEC). *Ocul Surf*. 2018;16:463–469.
49. You W, Xu Z, Sun Y, Valencak TG, Wang Y, Shan T. GADD45alpha drives brown adipose tissue formation through upregulating PPARgamma in mice. *Cell Death Dis*. 2020;11:585.
50. de Queiroga IB, Antonio Vieira L, Barros Jde N, Melo Diniz Mde F, de Morais LC. Conjunctival impression cytology changes induced by oral isotretinoin. *Cornea*. 2009;28:1009–1013.
51. Karalezli A, Borazan M, Altinors DD, Dursun R, Kiyici H, Akova YA. Conjunctival impression cytology, ocular surface, and tear-film changes in patients treated with systemic isotretinoin. *Cornea*. 2009;28:46–50.

Investigative Ophthalmology & Visual Science

52. Polat M, Kükner Ş. The effect of oral isotretinoin on visual contrast sensitivity and amount of lacrimation in patients with acne vulgaris. *Cutan Ocul Toxicol*. 2017;36:35–38.
53. Bozkurt B, Irkeç MT, Atakan N, Orhan M, Geyik PO. Lacrimal function and ocular complications in patients treated with systemic isotretinoin. *Eur J Ophthalmol*. 2002;12:173–176.
54. Bayhan SA, Bayhan HA, Colgecen E, Gurdal C. Effects of topical acne treatment on the ocular surface in patients with acne vulgaris. *Cont Lens Anterior Eye*. 2016;39:431–434.
55. Duzgun E, Ozkur E. The effect of oral isotretinoin therapy on meibomian gland morphology and dry eye tests. *J Dermatolog Treat*. 2020;1–7.
56. Elhamaky TR. Efficacy of omega-3 fatty acids and punctal plugs in the prevention of isotretinoin-associated ocular surface disease. *Eur J Ophthalmol*. 2021;31:2339–2345.
57. Barbosa AP, FRd Oliveira, FJd Rocha, Muglia VF, Rocha EM. Lacrimal gland atrophy and dry eye related to isotretinoin, androgen, and prolactin: differential diagnosis for Sjogren's syndrome. *Arq Bras Oftalmol*. 2021;84:78–82.
58. Rismondo V, Ubels JL, Osgood TB. Tear secretion and lacrimal gland function of rabbits treated with isotretinoin. *J Am Acad Dermatol*. 1988;19:280–285.
59. Ding J, Kam WR, Dieckow J, Sullivan DA. The influence of 13-cis retinoic acid on human meibomian gland epithelial cells. *Invest Ophthalmol Vis Sci*. 2013;54:4341–4350.
60. Perlman RL. Mouse models of human disease: an evolutionary perspective. *Evol Med Public Health*. 2016;2016:170–176.
61. Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA*. 2013;110:3507–3512.
62. Chen X, Sullivan BD, Darabad RR, Liu S, Kam WR, Sullivan DA. Are BALB/c mice relevant models for understanding sex-related differences in gene expression in the human meibomian gland? *Cornea*. 2019;38:1554–1562.