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Topical Ketotifen Fumarate Inhibits Choroidal Mast Cell Degranulation and Loss of Retinal Pigment Epithelial Cells in Rat Model for Geographic Atrophy

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Purpose: This study evaluates whether topical ketotifen fumarate (KTF) can prevent geographic atrophy (GA)-like phenotypes in a rat model.

Methods: Pharmacokinetics (PKs) of KTF after topical administration twice daily for 5 days was analyzed in rat retina, retinal pigment epithelium (RPE)/choroid/sclera, and in plasma by an liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Rats were then given hydrogel implants +/- 48/80 in the superior subconjunctival space and topically treated with 1% and 0.25% of KTF or phosphate buffer saline (PBS) twice daily. Rats were euthanized at 1, 2, 4, and 8 weeks postinjection. Choroidal mast cells (MCs) were stained with nonspecific esterase and the RPE monolayer was labeled with RPE65 and ZO-1 in whole mount choroids. Retinal and choroidal areas were determined in cryosections stained with picrosirius red. Dark-adapted electroretinogram (ERG) was also performed to evaluate retinal function.

Results: PK results showed the highest level of KTF (average 5.6 nM/mg) in the RPE/choroid/sclera in rats given topical 1% KTF. Topical 1% KTF significantly reduced choroidal MC degranulation at 1 week and 2 weeks (both $P < 0.001$) and RPE loss at 4 weeks ($P < 0.001$) as well as retinal and choroidal thinning (both $P < 0.001$) and reduction in ERG amplitude at 8 weeks ($P < 0.05$) compared to PBS. Similar results were obtained with 0.25% KTF.

Conclusions: Both 1% and 0.25% KTF eye drops effectively reduced MC degranulation, RPE loss, and retinal and choroidal thinning while preventing the decline of ERG amplitude in a GA-like rat model. These data suggest that topical KTF might be a new therapeutic drug for treating GA.

Translational Relevance: The results of this study demonstrate that topical KTF successfully reduced GA-like phenotypes in a rat model and may provide a novel therapy for GA.

Introduction

Geographic atrophy (GA) is the advanced form of nonexudative age-related macular degeneration (AMD) and is characterized by loss of retinal pigment epithelium (RPE), photoreceptors, and underlying choriocapillaris.[1] Although GA affects nearly 1 million people in the United States and accounts for approximately one-quarter of cases of legal blindness,[1,2] to date, there is no proven treatment to reduce its progression and experimental animal models are very limited.[1,3] Therefore, new therapies and reliable animal models to evaluate the drug efficacy for GA are still needed.

Mast cells (MCs) are key effector cells of innate immunity and are present in connective tissue and at mucosal surfaces throughout the body.[4] Normally, mast cells are known to regulate vasodilation, vascular homeostasis, innate and adaptive immune responses, angiogenesis, and venom detoxification.[5] When activated, MCs degranulate releasing a vast array

of proinflammatory mediators (e.g. TNFα, IL6), proteases (e.g. tryptase and chymase), cytokines (e.g. VEGF and TGFβ), proteoglycans, and biogenic amines (e.g. histamine).[6,7] Proteases, glycosaminoglycans, and histamine are preformed and released in the initial burst of degranulation, which can alter vascular permeability.[8] Repeated or long-term stimulation of MCs leads to tissue dysfunction and damage. Consequently, MCs are implicated in the pathophysiologic aspects of numerous diseases, including asthma, abdominal aortic aneurysms, anaphylaxis, malignancies, mastocytosis, and cardiovascular diseases.[5,8,9] With regard to the eye tissues, MCs are abundant in the conjunctiva and uveal tract, which includes the choroid along arteries and arterioles in rats and throughout choroid in humans.[10–12]

The complement factors C3a, C5a, IL33, C-reactive protein, and advanced glycosylation end products play roles in activation and degranulation of MCs[13–16] and are all elevated in AMD choroid.[17–19] It has recently been demonstrated that choroidal MC number and percent of MC degranulated are increased in early AMD and GA.[12] Furthermore, tryptase is released into Bruch's membrane and choroidal stroma in GA.[20] Moreover, we have reported that subconjunctival slow-released 48/80, a snake venom-like compound and well-established MC stimulator, causes continuous degranulation of choroidal MCs leading to loss of RPE, reduced electroretinography (ERG), and thinning of retina and choroid (i.e. GA-like phenotypes in the rat).[21] In addition, quiescing MCs with oral administration of the MC stabilizer, ketotifen fumarate (KTF) prevented these changes.[21]

KTF is a US Food and Drug Administration (FDA)-approved generic MC stabilizer that has been used for many years to treat both adults and children with asthma[22] and mastocytosis.[23] KTF can inhibit mast cell release of allergy mediators by stabilizing the membrane, preventing lysis and degranulation. By preventing degranulation, KTF reduces the release of various inflammatory mediators, such as histamine and tryptase. KTF has been marketed for many years in different preparations, including eye drops,[24] nasal spray,[25] tablets,[26] and syrup[27] for adult and pediatric indications. Recently, KTF has been widely used for the treatment of allergic diseases, such as bronchial asthma, allergic rhinitis, atopic dermatitis, and chronic urticaria.[28] Repurposing these FDA-approved drugs targeting mast cells may represent a potentially rapid avenue for treating GA-like phenotypes.

Topical administration is one of the most commonly used delivery routes to treat eye diseases. Although the bioavailability is low because pre-corneal anatomic barriers and the nature of the drug formulation itself, this delivery form is advantageous due to its easy, noninvasive application, which allows for self-administration of the drug.[29] Moreover, many elderly patients struggle with oral drug administration. Some patients have a risk of vomiting, while others find swallowing pills a near-impossible task. Additionally, antihistamines should be avoided to prevent some at-risk patients from aspiration pneumonia, which is known to interfere with swallowing.[30] Therefore, it is useful for elderly patients to have topical route of drug administration as an alternative to oral administration.

The purpose of this study was to evaluate whether topical administration of KTF has the potential to prevent the 48/80-induced GA-like changes in a rat model. Because KTF already has multiple delivery routes available clinically, the consideration of the efficacy of the ophthalmic form will lead to more options in delivery form for patients and caregivers, and the possibility of shortening the distance to clinical application in GA.

Methods

Study Design

Male Sprague Dawley rats (200–250 g) were purchased from Envigo, Frederick, MD, USA. They received a subconjunctival injection of hydrogel with or without compound 48/80 (hereafter called 48/80) and were treated with topical eye drops of KTF or phosphate buffer saline (PBS) until being euthanized. Rats were divided in groups as follows: group 1 = Empty + KTF; the rats received subconjunctival injections of blank hydrogel (called empty herein) and were treated with topical eye drops of KTF (1% or 0.25%) and were considered as a control group. Group 2 = 48/80 + PBS; the rats received subconjunctival injections of 48/80 in hydrogel and were treated with PBS, and group 3 = 48/80 + KTF; the rats received subconjunctival injections of 48/80 in hydrogel and were treated with topical eye drops of KTF (1% or 0.25%). In addition, rats received subconjunctival injections of blank hydrogel and were treated with PBS (considered as the empty + PBS group). These rats were evaluated to certify the empty + KTF group as a control. Rats were pretreated twice 1 day prior to the subconjunctival injection (implantation) with either topical eye drop of KTF or PBS. Treatments were continued until rats were euthanized. Rats were housed in a 12-hour light and 12-hour dark cycle and fed water and dried ration ad libitum. For all procedures, anesthesia was performed by intraperitoneal injection of a ketamine (100 mg/mL) and xylazine (20 mg/mL) cocktail.

All animal experimental procedures were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, following approval from the Animal Care and Use Committee at the Johns Hopkins University.

Pharmacokinetic Study

In a previous study, we have shown that oral administration of KTF (15 mg/kg) adequately reached the retina and choroid,[21] but topical administration of KTF has not been evaluated. To assess whether topical eye drops of KTF could reach the posterior segment of the rat eye, the pharmacokinetic (PK) evaluation of (topically and orally) KTF was performed. Rats were given KTF twice daily by topical eye drops (ketotifen fumarate 10 mg solubilized in 1 mL 1 × PBS; 1% KTF, 10 uL/eye) or gavage (15 mg/kg of KTF) until euthanization. The rats were euthanized within 30 to 60 minutes after the last administration of KTF on the fifth day. The rats were anesthetized and the eyes were rapidly enucleated, anterior segments removed, and retinas dissected from RPE/choroid. At the same time, the blood sample was collected in a heparinized syringe from the inferior vena cava. The plasma samples were prepared by centrifuging of the whole blood at 4°C for 10 minutes at 3000 rpm (Beckman Coulter, Inc, Indianapolis, IN, USA). The tissues and plasma samples were frozen until analyzed. The quantification of KTF was conducted using high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS), as reported previously.[21]

Hydrogel Preparation and Injection

Hydrogels with or without 48/80 were prepared as reported previously.[21] A hydrogel which slowly released the compound 48/80 (Millipore Sigma, St. Louis, MO, USA) was formulated using thiolated hyaluronic acid and 4-arm poly (ethylene glycol) acrylate crosslinked using thiolene click chemistry.[31] The gel consisted of 48/80 (20 mg/mL), 4-arm PEG-acrylate (100 mg/mL, PSB-421), 8-arm PEG SH (150 mg/mL, PSB-851), and hyaluronic acid-SH (200 mg/mL, HA-371) added to Irgacure-2959 photo initiator (10 mg/mL; Ciba-Geigy, Tarrytown, NY, USA). All components of poly (ethylene glycol) PEG products were purchased from Creative PEGWorks (Chapel Hill, NC, USA). All gel components were then mixed together by vortex and placed on wet ice. The final solution was loaded into an insulin syringe with a 31-gauge needle (BD Biosciences, San Jose, CA, USA) and exposed to UV light (Analytik Jena, Upland, CA, USA) for 3 minutes. After confirming the gel formation by its consistency, 35 µL of hydrogel with or without 48/80 (48/80 or empty hydrogel) was implanted into the superior subconjunctival space. The hydrogel solidified in the subconjunctival space and is called an implant herein.

Formulation of KTF as Topical Eye Drops

The 10 mg of KTF solubilized in 1 mL of 1 × PBS, which is maximum solubility in water, was used as high dose treatment (1% KTF eye drop) and 2.5 mg of KTF solubilized in 1 mL of 1 × PBS was used as low dose treatment (0.25% KTF eye drop), which is closer to the concentration of clinical use.

Choroidal Flat Mount for Mast Cell Count and Degranulation

Rats were euthanized 1 and 2 weeks after implantation. After enucleating the eyes, the anterior segments were removed and the retina was carefully separated from the RPE/choroid complex. The RPE/choroids were soaked in 1% Ethylenediamine tetra acetic acid (EDTA; Thermo Fisher Scientific, Waltham, MA, USA) in 1 × PBS for 2 hours at room temperature (RT). Then the RPE cells were removed by shooting EDTA solution from a syringe with a blunted 25-gauge needle. RPE denuded choroids were briefly washed with 0.1 M cacodylate buffer. After washing, tissues were fixed with 2% paraformaldehyde (PFA) in 0.1 M cacodylate buffer at 4°C overnight. The following day, choroids were incubated for nonspecific esterase (NSE) staining using a naphthol AS-D chloroacetate kit (91C-1KT; Millipore Sigma, St. Louis, MO, USA) as reported previously.[12] Four pie cuts were made to allow flattening of the choroid/sclera eyecup, which clearly separated superior and inferior quadrants (shown in Figs. 1A–C). The pie cut choroid was mounted, cover slipped on glass slides, and a photomontage taken at 10 times magnification using a light microscope (Accu-Scope 3000 LED; Accu-Scope Inc., Commack, NY, USA). The total number of MCs in two quadrants of the superior choroid (called gel side) and two quadrants of the inferior choroid (called non-gel side) were counted as follows: rat choroid montage images were opened in Adobe Photoshop (version 21.2.4; Adobe Systems, San Jose, CA), using magnetic lasso tool, the edge of each quadrant of choroid was traced and saved as photoshop PDF file. Under the image menu, brightness adjustments were made, and images were saved for analysis. The saved images were opened in a new document and thresholded to have an accurate

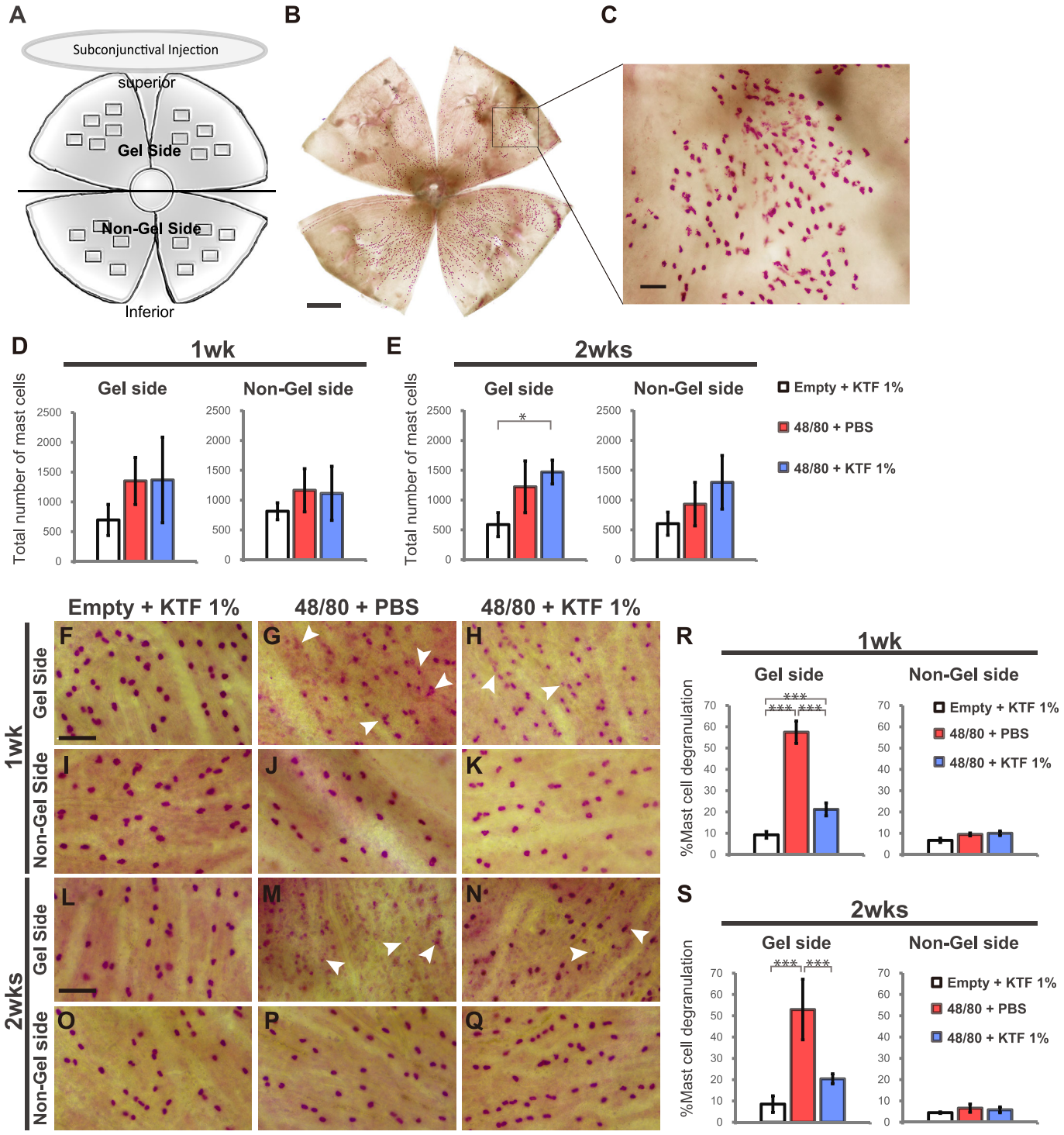

Figure 1. **Effect of topical 1% ketotifen fumarate on 48/80-induced mast cell degranulation.** (**A**) The schematic of a choroidal flat mount showing four pie cut quadrants. The superior two quadrants are called "gel side" where the hydrogel with or without 48/80 compound was implanted and the inferior two quadrants are called "non-gel side" herein. The square boxes in each quadrant show the areas of the higher magnification images for evaluating mast cell degranulation. (**B**) Representative image of the choroidal flat mount in low magnification showing NSE stained MCs in pink for counts (scale bar: 1 mm). (**C**) MCs at high magnification in the box area of **B** (scale bar: 100 µm). (**D, E**) The bar graphs show the total number of MCs in the gel and non-gel side of the choroids in the rats treated with topical 1% KTF or PBS at 1 week and 2 weeks after implantation (*white*: empty + KTF, *red*: 48/80 + PBS, and *blue*: 48/80 + KTF). (**F–Q**) Representative high magnification images of the choroidal flat mount stained with NSE showing MCs at 1 week and 2 weeks (scale bar: 100 µm). Degranulated MCs (*white arrowheads*) are obvious in the gel side at 1 week **G** and 2 weeks **M** in 48/80 + PBS rats. At 2 weeks, the degranulated MCs are very small in →

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size in the 48/80 + PBS choroid **M** compared to the 48/80 + KTF choroid **N**. (**R, S**) The bar graphs show the percentage of MC degranulation in the gel and non-gel sides of the choroid in the rats treated with topical 1% KTF or PBS at 1 week and 2 weeks ($n = 6$ at 1 week, and $n = 3$ at 2 weeks per group) *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, 1-way ANOVA with Tukey post hoc test.

representation of only the MCs without any nonspecific choroidal background. The threshold image was flattened and saved as a separate tiff image. The images were converted to binary using NIH ImageJ (version 1.50) and noise reduction was applied before counting cells using the analyze particle function. Pixels greater than one were counted. For MC degranulation, 5 random fields in each quadrant were captured at 25 times using Zeiss Photomic II microscope coupled with an attached camera (Progress Gryphax Naos; Jenoptik, Jena, Germany). At higher magnification, the number of degranulated and non-degranulated MCs in each quadrant were counted manually. Ten fields were counted and documented per the two quadrant area (gel and non-gel side). Manual hand counts were done by three independent reviewers to verify the accuracy of counts in ImageJ software. The percentage of MCs degranulated was averaged per choroid.

Fluorescence Immunohistochemistry of RPE/Choroidal Flat Mounts for Measurement of RPE Degeneration and/or Loss

Rats were euthanized at 2 weeks and 4 weeks after implantation. After enucleating the eyes, the anterior segments were removed and the retina was carefully separated from the RPE/choroid complex. Tissues were fixed with 2% PFA in Tris-buffered saline (TBS) at 4°C overnight. The choroid was prepared for immunohistochemistry (IHC) as previously described.[32] In brief, the choroid was blocked with 2% normal donkey serum (Jackson ImmunoResearch Inc., West Grove, PA, USA) at 4°C overnight, washed in 0.1% Triton X-100 in TBS, and then incubated with a primary antibody cocktail containing: mouse anti-RPE65 (clone: 401.8B11.3D9, 1:200; Novus Biologicals, LLC, Centennial, CO, USA), and polyclonal rabbit anti-ZO-1 (1:100, cat. # 61-7300; Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight. After washing, they were incubated with a secondary antibody cocktail containing: Cy3-conjugated donkey anti-mouse (1:200; Jackson ImmunoResearch Inc.), Cy5-conjugated donkey anti-rabbit (1:200; Jackson ImmunoResearch Inc.), and DAPI (1:1000; Invitrogen, Thermo Fisher Scientific, Inc, Waltham, MA, USA) at 4°C overnight. After washing, the choroid/sclera eyecup was flattened by making four pie cuts. Ten random fields from the gel and non-gel sides of each choroid were captured at 20 times magnification using optimized z-stacks settings with Zeiss LSM880 with Airyscan (Carl Zeiss AG, Jena, Germany). Degeneration of RPE and/or loss of RPE areas were determined based on RPE65 staining only as reported previously.[21] ZO-1 antibody was used to determine the deterioration of RPE junctions. The confocal microscopic images were exported and saved as tiffs. The tiff images were exported to ImageJ software, converted into 8-bit gray color and thresholded. The dark areas lacking RPE65 staining but intact ZO-1 staining was considered as RPE degeneration, whereas complete cell loss area without RPE65 and ZO-1 staining was considered as RPE loss. To exclude the area of RPE nucleus (unstained with anti-RPE65 Ab, therefore dark) from the total dark area, particle sizes ranging from 400-infinity (pixels^2) were analyzed. The percentage of RPE loss or degeneration was averaged per choroid.

Measurement of Retinal and Choroidal Thickness

Rats were euthanized at 8 weeks after implantation. Before freezing the tissue, the eye cups were hemisected through the optic nerve with one side being the gel side where the hydrogel was implanted (superior) and the other side as a non-gel side (inferior). The eyecups were cryopreserved as previously described.[20] Cryoblocks were cut at 10 µm thickness using a Microm HM500M cryostat (Microm, Walldorf, Germany) through the optic nerve from ora serrata to ora serrata and sections were stained with Picrosirius Red Stain Kit (Polysciences, Inc., Warrington, PA, USA) as recommended by the manufacturer.

The panoramic images through the optic nerve from ora serrata to ora serrata were captured with a light microscope (Accu-Scope 3000 LED; Accu-Scope Inc, Commack, NY, USA) at 10 times magnifications for measuring retinal and choroidal areas. ImageJ was used to precisely hand-trace the entire retina and choroid of each image and the area was quantified. After setting the scale bar, the entire retina or choroid were precisely traced in each panoramic image using the freehand selection tool. The analyze to measure tool was used

to calculate the selected area. The gel and non-gel side retina and choroid were individually measured and analyzed. The serial sections were also stained with hematoxylin and eosin (H&E; Sigma-Aldrich, Inc., St. Louis, MO, USA and Polysciences, Inc., Warrington, PA, USA) for morphologic analysis as previously described.[21]

Dark-Adapted Full-Field Scotopic ERG

Rats were dark adapted overnight (more than 12 hours) before recording. The procedure was done under a dim-red light. The animals were deeply anesthetized followed by pupil dilation with 1% tropicamide and 2.5% phenylephrine eye drops. The cornea was anesthetized with a drop of 0.5% proparacaine hydrochloride and moisturized with a hypromellose ophthalmic demulcent solution 2.5% (Gonak; Akron Inc., Lake Forest, IL, USA). The rats were placed on a heated platform, light guide electrodes were positioned at the centered cornea and full-field scotopic ERGs were elicited by white light flashes with corneal electrodes at intensities ranging from 0.01 to 1 cd.s/m^2 using the Celeris ERG system (Diagnosys LLC, Lowell, MA, USA).

Statistics

Statistical analyses were performed with JMP version 14.2.0 software (SAS Institute Inc., Cary, NC, USA) using a 2-tailed paired *t*-test for the ERG amplitude, whereas 2-way analysis of variance (ANOVA) was applied for the comparison between gel and non-gel side, or 1-way ANOVA and Tukey's honestly significant difference tests was used to compare the gel to gel and non-gel to non-gel sides separately among all groups in all parameters. The results are presented as the mean ± standard deviations (SDs). The *P* values of less than 0.05 were considered statistically significant.

Results

Pharmacokinetic Study

The PK results showed higher levels of KTF (1% and 0.25%) in RPE/choroid/sclera following topical administration compared to oral (5.6 ± 3.5 nmol/g and 2.5 ± 1.2 nmol/g, respectively; shown in the Table). Therefore, the current study was conducted using topical administration of KTF.

The Comparison Between the Empty + KTF and Empty + PBS groups

To assess the impact of KTF eye drop alone on MC numbers/degranulation or RPE morphology as well as retinal/choroidal thickness and retinal function, rats were injected with empty hydrogel and treated with 0.25% KTF (empty + KTF) for up to 8 weeks. At the same time, another group of rats were also injected with empty hydrogel and treated with PBS alone (empty + PBS) to determine the differences, if any, between these two groups. There was no significant difference in any parameters evaluated (i.e. the percent degranulation of MCs, [gel and non-gel side], $P = 0.98$ and $P = 0.34$ at 1 week, and $P = 0.14$ and $P = 0.08$ at 2 weeks, respectively), RPE65$^+$ cell loss area ($P = 0.66$ and $P = 0.42$ at 2 weeks, and $P = 0.75$ and $P = 0.07$ at 4 weeks, respectively), retinal and choroidal thickness ($P = 0.21$ and $P = 0.72$, and $P = 0.99$ and $P = 0.23$, respectively), and no significant decline in the a- and b-wave amplitude of ERG between empty + KTF and empty + PBS group (shown in Supplementary Fig. S1). Based on the results obtained, empty + KTF was used as a control in this study.

The Number of Mast Cells and Mast Cell Degranulation

At 1 week and 2 weeks after the implantation, the total number of MCs and percentage of degranulated

Table. Pharmacokinetic Levels of Ketotifen Fumarate in RPE/Choroid/Sclera, Retina, and Plasma

	Topical Administration		Oral Administration
Tissues	1% KTF	0.25% KTF	15 mg/kg
RPE/choroid/sclera	5.6 ± 3.5 (nmol/g)	2.5 ± 1.2 (nmol/g)	0.9 ± 1.0 (nmol/g)
Retina	1.8 ± 1.0 (nmol/g)	1.6 ± 0.8 (nmol/g)	1.7 ± 1.7 (nmol/g)
Plasma	0.03 ± 0.01 (nmol/mL)	0.05 ± 0.02 (nmol/mL)	0.3 ± 0.1 (nmol/mL)

KTF, ketotifen fumarate; RPE, retinal pigment epithelium.

The PK analysis shows the highest levels of KTF of both concentration in RPE/choroid/sclera among the tissues and plasma in topical administration than the oral administration (mean ± SD, $n = 5$, each group).

MCs in the gel and non-gel sides were evaluated in choroidal flat mounts stained for NSE activity. The total number of MCs (the gel and non-gel sides) in all three groups treated with 1% KTF (high dose) or PBS are shown in Figure 1D. Although there was a tendency for the total number of MCs in choroids (the gel and non-gel sides) to be higher in 48/80 hydrogel implantation (48/80 + KTF and 48/80 + PBS) groups compared to the empty hydrogel (empty + KTF) group, the difference was not significant. However, at 2 weeks, a significant increase in MCs was observed in the gel side of 48/80 + KTF compared to empty + KTF ($P = 0.03$; see Fig. 1E). Moreover, there was no statistically significant difference between 48/80 + KTF and 48/80 + PBS in the gel side at 1 week and 2 weeks ($P = 0.99$ and $P = 0.60$, respectively). Furthermore, there was no statistically significant difference in the number of MCs between the gel and non-gel sides at 1 week and 2 weeks (2-way ANOVA, $F_{1, 32} = 0.53$, $P = 0.47$, and $F_{1, 16} = 0.94$, $P = 0.35$, respectively). At higher magnification, the degranulated MCs appeared small in size, but were still countable.

The degranulated MCs in choroids of rats treated with topical 1% KTF or PBS are shown in Figures 1F–1Q. In 48/80 + PBS choroids, most of the MCs were degranulated and reduced in size in the gel side compared to the empty hydrogel choroid. By contrast, in 48/80 + KTF treated choroids, MCs retained their shape and size and resembled those seen in empty + KTF treated choroids. The percentage of MC degranulation in the high dose of KTF treatment is shown in Figures 1R and 1S. The percent MC degranulation in the gel side was significantly higher in the 48/80 + PBS group compared to the empty + KTF group at 1 week and 2 weeks (both $P < 0.001$). Furthermore, the percent degranulation in the 48/80 + KTF group was significantly lower compared to the 48/80 + PBS group at 1 week and 2 weeks (both $P < 0.001$). In addotion, there was a statistically significant difference between the gel and non-gel sides at 1 week and 2 weeks (2-way ANOVA, $F_{1, 32} = 546.8$, $P < 0.0001$, and $F_{1, 16} = 55.0$, $P < 0.0001$, respectively).

The total number of MCs and the percentage of degranulated MCs in the low dose (0.25% KTF) treatment are shown in Supplementary Figure S2. The rats treated with low dose KTF showed similar results to the high dose KTF treated rats. There was no significant difference in the total number of MCs among the three groups at 1 week and 2 weeks. However, the percentage of the degranulated MCs in the gel side of 48/80 + PBS rats was significantly higher than both the empty + KTF and the 48/80 + KTF groups at 1 week and 2 weeks (both $P < 0.001$ at 1 week, and $P < 0.001$ and $P < 0.01$ at 2 weeks, respectively). Thus, both concentrations of KTF (1% and 0.25%) significantly prevented MC degranulation.

Loss of RPE cells

The representative images of flat mount RPE/choroids of rats from all groups treated with the high dose of KTF (1% KTF) or PBS are shown in Figure 2. At 2 weeks, few dark areas of single-cell size which lacked RPE65 staining were observed (see Fig. 2B), but kept junctional integrity (ZO-1$^+$ staining) and nuclei (stained with DAPI) in the gel side of 48/80 + PBS and 48/80 + KTF choroid (Supplementary Fig. S3). These were considered RPE degeneration. By contrast, in the non-gel side of all groups, RPE changes were rarely observed (see Figs. 2D–F). The percentage of RPE degeneration at 2 weeks is shown in Figure 2M. Although there was a trend for RPE degeneration on the gel side in the 48/80 + PBS and 48/80 + KTF groups, there were no statistically significant differences among the three groups ($P = 0.07$, $P = 0.30$, and $P = 0.53$, respectively). At 4 weeks, confluent absence of RPE65$^+$ cells (see Fig. 2H) combined with the disruption of RPE junctions and the absence of nuclei (see Supplementary Fig. S3), which was considered RPE loss, was apparent in the gel side of the 48/80 + PBS group. Nevertheless, these confluent areas of RPE65$^+$ cell loss were not seen in 48/80 + KTF choroid. The percentage of RPE loss area in the gel side of 48/80 + PBS were significantly higher compared to empty + KTF and 48/80 + KTF (both $P < 0.001$; see Fig. 2N). There was no significant difference between empty + KTF and 48/80 + KTF ($P = 0.22$). Additionally, there was a statistically significant increase between gel and non-gel side at 2 weeks and 4 weeks (2-way ANOVA, $F_{1, 16} = 12.6$, $P = 0.007$, and $F_{1, 34} = 467.8$, $P < 0.0001$, respectively).

Similarly, in the rats treated with 0.25% KTF (low dose), there were no significant differences in the percentage of RPE65$^+$ cell loss area in the gel and non-gel sides in the three groups at 2 weeks. At 4 weeks, the percentage of RPE loss in the gel side of 48/80 + PBS were significantly higher compared to empty + KTF and 48/80 + KTF (both $P < 0.001$). There was no significant difference in the percentage RPE65$^+$ loss in the gel side between empty + KTF and 48/80 + KTF ($P = 0.15$; Supplementary Fig. S4).

Retinal and Choroidal Thickness

The retinal and choroidal thickness in the gel side of 48/80 + PBS was significantly thinner than that in the empty + KTF (both $P < 0.001$) and the 48/80 + KTF ($P < 0.01$ in the retina and $P < 0.001$ in the choroid) groups (Figs. 3A, 3B). The 1% KTF

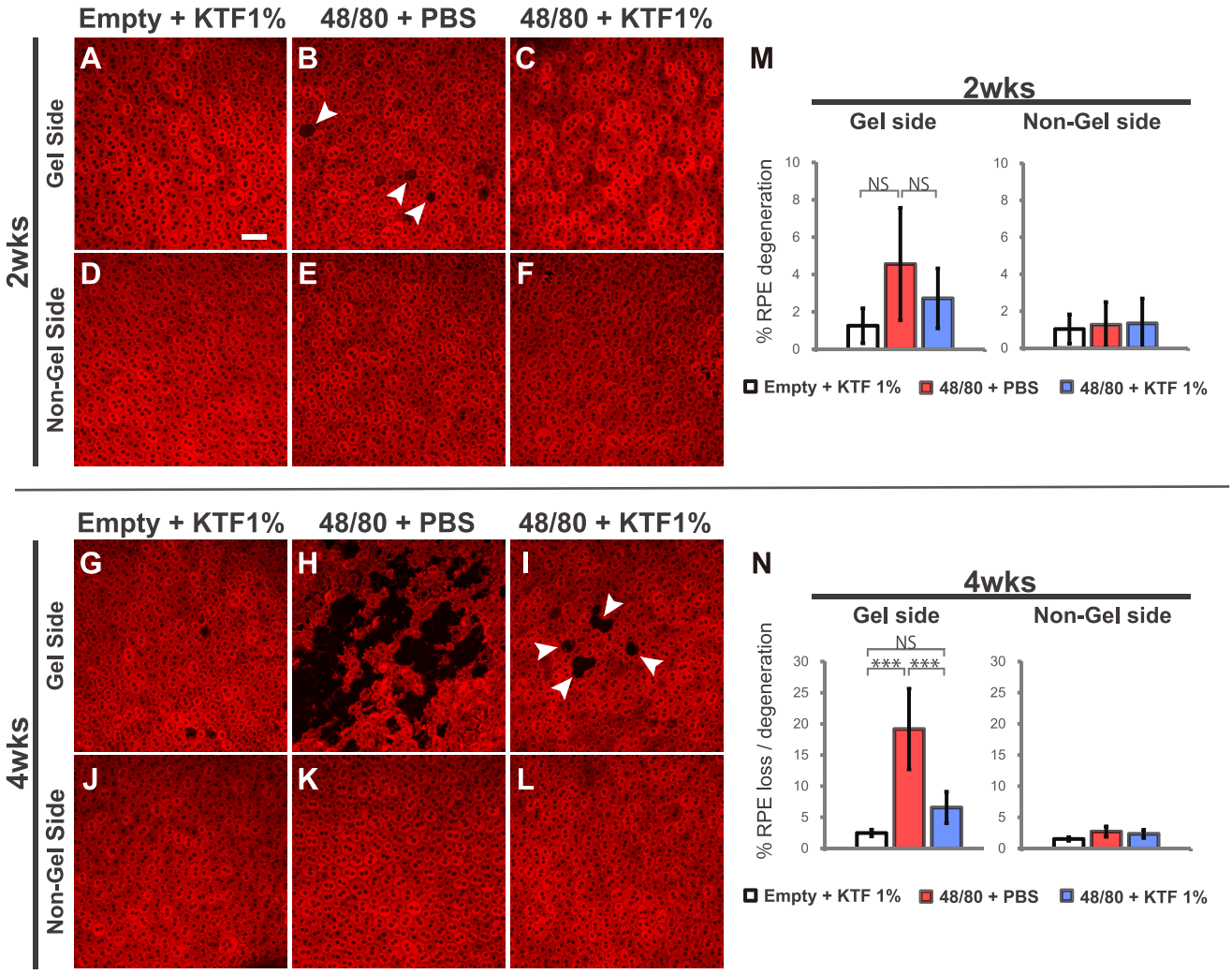

Figure 2. **RPE/choroid flat mounts showing the effect of topical 1% ketotifen fumarate at 2 weeks and 4 weeks.** (**A–L**) Representative images from the gel and non-gel side of the RPE/choroid flat mounts labeled for RPE65 in all three groups treated with 1% KTF or PBS at 2 weeks and 4 weeks (scale bar: 100 µm). Single-cell sized RPE areas lacking staining with RPE65 are seen in the gel side of 48/80 + PBS at 2 weeks **B** as well as 48/80 + KTF at 4 weeks **I** (*white arrowheads*). Confluent absence of RPE65 staining (RPE loss) is seen in the gel side of the 48/80 + PBS group at 4 weeks **H**. In the non-gel side choroids of all groups, RPE changes were rarely observed. (**M, N**) The bar graphs show the quantification of RPE degeneration or loss in the gel and non-gel sides among the three groups at 2 weeks and 4 weeks post implantation. Topical 1% KTF treatment prevented the 48/80-induced RPE loss at 4 weeks post implantation ($n = 3$ at 2 weeks and $n = 6$ at 4 weeks per group). ***$P < 0.001$, 1-way ANOVA with Tukey post hoc test.

treatment significantly prevented the thinning of retina and choroid compared to 48/80 + PBS. However, the thickness was still reduced compared to that of the empty + KTF group. H&E staining of cryosections showed normal morphology of retina and choroid in the empty + KTF group, and confirmed the retinal and choroidal thinning in the 48/80 + PBS group compared to the 48/80 + KTF and empty + KTF groups (see Figs. 3C–H). In addition, the retinal and choroidal thickness were statistically significantly thinner in the gel side compared to the non-gel side (2-way ANOVA, $F_{1, 34} = 11.2$, $P = 0.002$, and $F_{1, 34} = 48.7$, $P < 0.0001$, respectively).

Similar results were obtained with 0.25% KTF (low dose) or PBS treatment. Both the retinal and choroidal thickness were significantly thinner in 48/80 + PBS rats than that in empty + KTF and 48/80 + KTF (all $P < 0.001$; Supplementary Fig. S5).

Dark-Adapted Full-Field Scotopic ERG

The ERG graphs at baseline and 8 weeks among the three groups treated with 1% KTF or PBS are shown in Figures 4A–C. At 8 weeks post implantation, the amplitude of a- and b-waves with the all stimuli in the 48/80 + PBS rats were statistical significantly declined

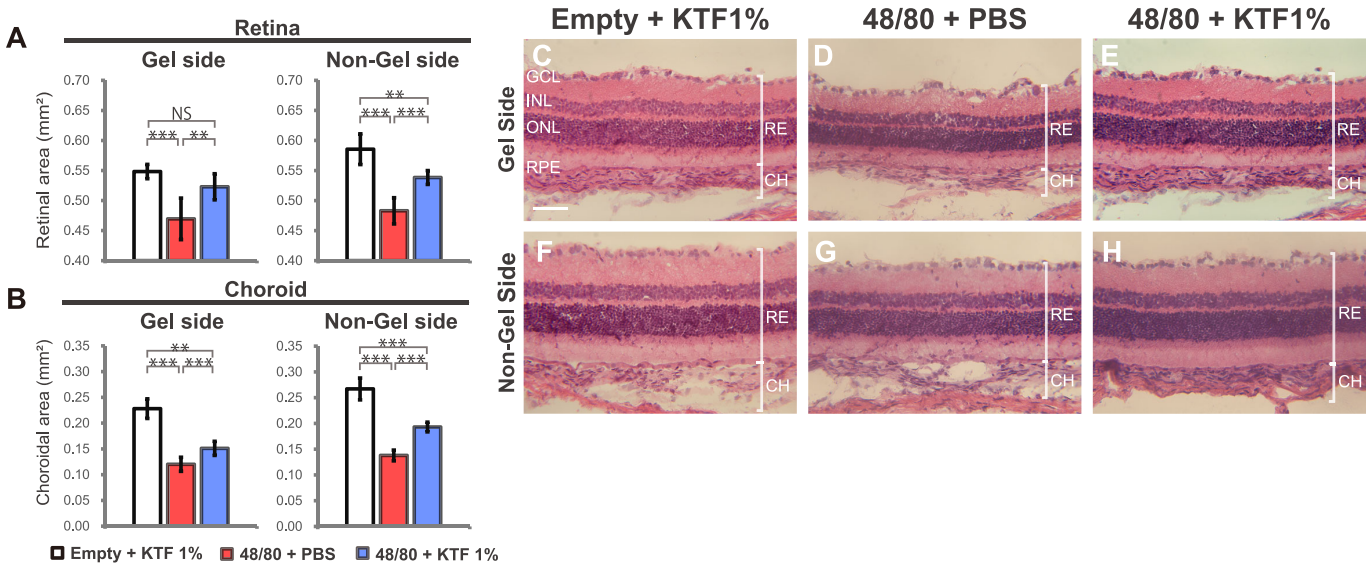

Figure 3. **Measurement of retinal and choroidal thinning**. The bar graphs show the total area (mm^2) of the retina (**A**) and choroid (**B**) in the gel and non-gel sides among the three groups treated with 1% KTF or PBS at 8 weeks post implantation. The areas of retina and choroid were significantly thinner in the 48/80 + PBS group compared to the empty + KTF and 48/80 + KTF groups. (**C–H**) Cryosections stained with H&E confirms that topical 1% KTF prevents the retinal and choroidal thinning in the 48/80 + KTF group ($n = 6$ per group) $^{**}P < 0.01$. $^{***}P < 0.001$, 1-way ANOVA with Tukey post hoc test. Scale bar: 50 µm. CH, choroid; RE, retina; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelial cell.

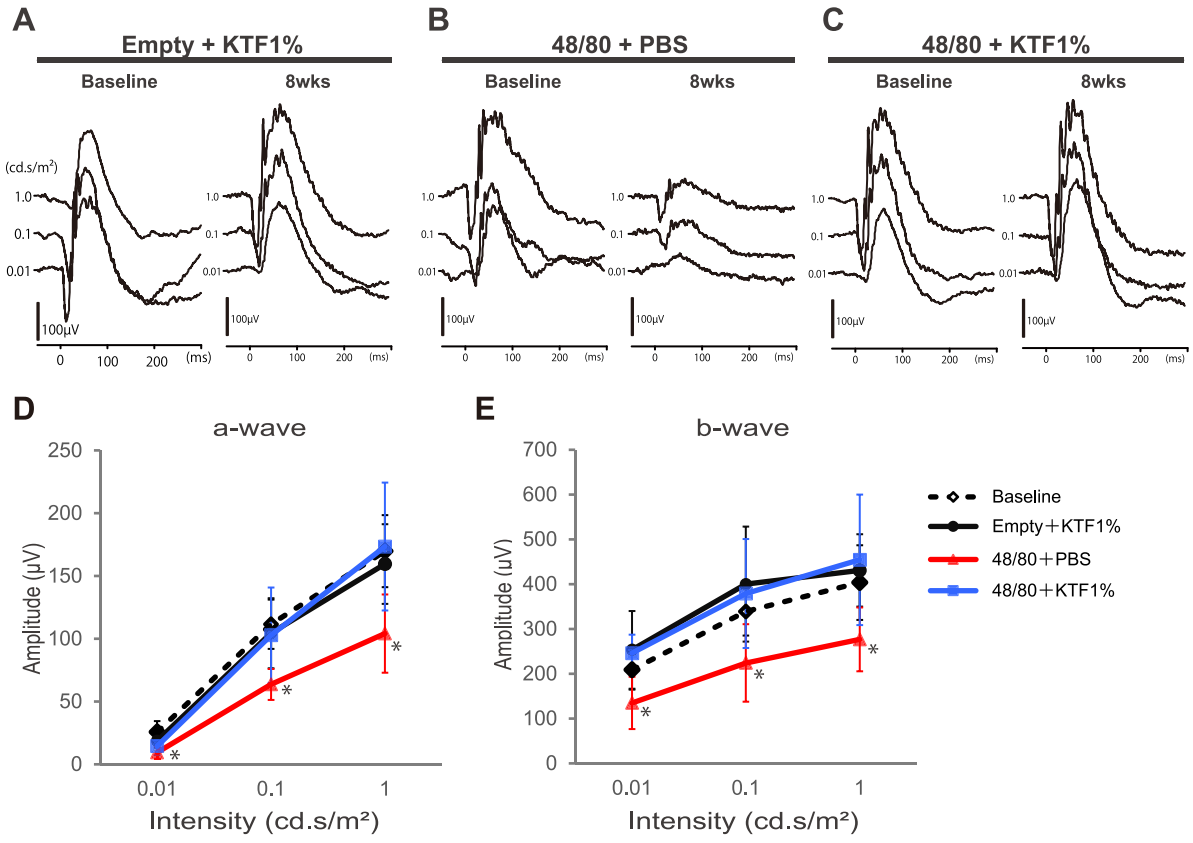

Figure 4. **The dark-adapted full-field scotopic ERG amplitudes in all groups.** (**A–C**) Representative dark adapted full-field scotopic ERG amplitudes with the stimuli of 0.01, 0.1, and 1.0 cd.s./m^2 among the three groups at the baseline and 8 weeks post implantation. The line graphs show a-wave (**D**) and b-wave (**E**) amplitude with the stimuli of 0.01, 0.1, and 1.0 cd.s./m^2 in the rats treated with topical 1% KTF or PBS at baseline and 8 weeks post implantation ($n = 6$, per group) $^{*}P < 0.05$, 2-tailed paired *t*-test.

compared to the baseline (all $P < 0.05$). Whereas in the 48/80 + KTF group, there were no significant differences in the amplitude of a- and b-waves with all stimuli between the baseline and 8 weeks (see Figs. 4D, 4E). The amplitude of a- and b-waves with all the stimuli at 8 weeks in 48/80 + KTF were significantly higher than that in 48/80 + PBS, except that of a-wave at 0.01 cd.s/m^2 stimuli (all $P < 0.05$).

Similarly, in all three groups treated with 0.25% KTF (low dose) or PBS, the amplitude of a- and b-waves with the all stimuli in 48/80 + PBS were significantly declined compared to the baseline (all $P < 0.05$ or $P < 0.01$) except that of a-wave at 0.01 cd.s/m^2 stimuli, but had a downward trend ($P = 0.09$). In the 48/80 + KTF group, there were no significant differences in the ERG amplitude between the baseline and 8 weeks (see Supplementary Fig. S5). In addition, the amplitude of a- and b-waves with the all stimuli at 8 weeks in 48/80 + KTF were significantly higher than that in 48/80 + PBS, except that of a-wave at 0.01 cd.s/m^2 stimuli (all $P < 0.05$).

Discussion

The current study demonstrated that the subconjunctival injection of 48/80-slow released hydrogel caused MC degranulation in the choroid at 1 week and 2 weeks, followed by RPE loss at 4 weeks, retinal and choroidal thinning at 8 weeks, and the decline of the ERG amplitude at 8 weeks after implantation, as previously reported.[21] Furthermore, daily treatment with topical 1% and 0.25% KTF effectively reduced MC degranulation, loss of RPE, and retinal and choroidal thinning, and prevented decline of ERG amplitude in a GA-like rat model. Quiescing MCs with not only oral but also topical administration of KTF, both high and low dose, showed the potential to prevent these GA-like changes in the rat model. These data suggest that topical KTF eye drops might be a new therapeutic drug for treating GA, and the presence of effectively topical administrated therapy would be advantageous for elderly patients.

We propose the hypothesis that choroidal MCs degranulate and release MC-derived tryptase, resulting in degradation of Bruch's membrane and choroidal stroma, which ultimately progresses to RPE degeneration as well as retinal and choroidal thinning, hallmarks of GA.[21] The 48/80 rat model reported herein in which 48/80 is slow-released from a hydrogel pellet allowing continuous activation and degranulation of choroidal MCs without acute uveitis, was responsible for RPE degeneration.[21,33] In the current study, 48/80-induced MC degranulation in the gel side at 1 week and 2 weeks, RPE loss in the gel side at 4 weeks, both retinal and choroidal thinning at 8 weeks, and decline of the ERG amplitude at 8 weeks were observed. These results were consistent with our previous report.[21] In the current study, however, at the longer time point (8 weeks), retinal and choroidal thinning in the gel and non-gel side as well as ERG amplitude in the 48/80 + PBS and 48/80 + KTF groups were observed. As we have previously reported, the percent of MC degranulation in the gel side peaked at 7 days and continued to increasingly degranulate until day 36 post implantation, which suggested gradual interaction with the non-gel choroid.[21] Therefore, this may explain why the retinal and choroidal thinning was noted not only in the gel side but also the non-gel side as well as decline of the full-field ERG amplitude at 8 weeks in the current study.

KTF, a serotonin and histamine antagonist, is derived from cyproheptadine and is known to have three independent pharmacological mechanisms established in humans: inhibition of histamine H1-receptor, mast cell stabilization, and prevention of eosinophil accumulation.[34,35] Although the mechanism of mast cell-stabilizing property of KTF has not yet been well-established in the rat, it may be attributed to their ability to counteract the plasma membrane deformation in degranulating mast cells.[36] The current study demonstrated that topical KTF can reach the posterior eye tissues and the drug inhibits the exocytotic process of MC, and that topical KTF prevented MCs degranulation, followed by RPE loss, and retinal and choroidal thinning, and decline of ERG amplitude in rat. In terms of drug delivery, the highest level of KTF was found in the RPE/choroid/sclera with topical administration in the PK study. The level of KTF in plasma delivered topically was much lower compared to that in the choroid/sclera/RPE, suggesting the KTF eye drops can reach the posterior tissues via systemic circulation partly[37,38] but mainly through the conjunctiva/cornea surface. With regard to the conjunctival/cornea surface, KTF can be transported by three different routes; the transvitreal route, the uvea–scleral route, and the periocular route.[39] The PK results reported herein were supported by the report that topical eye drops of VEGF receptor inhibitors showed efficient ocular delivery at 0.5 hours after administration to the choroid/sclera of the dosed eye from the instillation site in rats.[40] However, the amount of drug that permeates from the cornea/conjunctiva surface to the posterior segment depends on the nature of the drug, the pharmaceutical form, and the anatomic characteristics of the eye, and also species differences.[40] These differences should be taken into consideration

in developing future studies and further studies are needed in order to consider the PKs and pharmaceutical activities in the multiple animals and also in humans.

The pharmaceutical benefit of KTF was well-established in vitro, ex vivo, and in vivo experiments.[21] KTF is available in many forms, such as eye drops,[24] nasal spray,[25] and tablets,[35] which have been used for many years in clinical settings. In humans, with the oral form, only minor side effects, such as drowsiness, have been reported.[35] On the other hand, the application and handling of the eye drops is generally straightforward, although some people poorly apply eye drops, and the systemic side effects are limited for most users.[26] In addition, KTF ophthalmic drops that are commercially available (0.035% KTF in the United States and 0.069% KTF in Japan) have a favorable safety and tolerability profile.[41] These concentrations were mainly applied for superficial ocular disorders, such as allergic conjunctivitis. However, the absorption and distribution of these to the deeper ocular tissues, such as the retina and choroid, is unknown. Seven times higher concentrations than the commercially available KTF ophthalmic solution were used in this study to evaluate the efficacy of KTF in retinal and choroidal disease. The lower dose is considered to be within the maximum recommended human ocular dose. Although the highest concentration of KTF was used, the PK results showed the highest level of PKs in RPE/choroid/sclera and successfully prevented GA-like phenotypes changes and maintained visual function in rats. In addition, it did not cause any extraocular or intraocular changes and did not show any cytotoxic effects.

In conclusion, topical KTF eye drops (1% and 0.25% KTF) had efficacy in preventing MC degranulation, loss of RPE, retinal choroidal thinning, and ERG amplitude decline in our GA-like rat model. These data suggest that topical KTF eye drops might be a new therapeutic drug for treating GA.

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Author Contributions

The study conception and design: T.N., I.A.B., and G.A.L. Data acquisition: T.N., I.A.B., A.T., and R.R.G. (pharmacokinetic study) J.A. and R.R. Data analysis: T.N., I.A.B., J.A., and R.R. Data interpretation: T.N., I.A.B., M.M.E., and G.A.L. Drafting: T.N. Revising: IA.B., M.M.E., and G.A.L. Final approval of the version: G.A.L.

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