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Kaempferol Protects Against Retinal Photoreceptor Degeneration in a Mouse Model of Light-Induced Retinal Injury

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Abstract

Purpose: Age-related macular degeneration (AMD) is a leading cause of blindness in developed countries with little in the way of treatment that prevents progression to end-stage disease. Kaempferol (KF) is a plant-derived dietary flavonoid that has demonstrated as a strong antioxidant showing neuroprotection in stroke models. We hypothesize that KF has protective effects against retinal degeneration and may serve as a therapeutic agent against AMD.

Methods: BALB/c albino mice were assigned to 1 of 2 groups: control-treated or KF-treated retinal light injury mice. Mice were exposed to 8,000 lux cool white fluorescent light for 2 h to induce light injury. Control or KF was injected intraperitoneally after light injury for 5 days. Scotopic electroretinography (ERG) was recorded before light injury and 7 days after light injury. The retinal morphology and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed after light injury.

Results: ERG a- and b-wave amplitudes were significantly reduced in the retinal light injury group compared with the nonretinal light injury group. Retinal light injury produced markedly thinning of the outer nuclear layer along with significant TUNEL-positive signals. In contrast KF treatments significantly attenuated reduction of ERG a- and b- wave amplitudes and the loss of the outer nuclear layer.

Conclusions: KF protects retinal photoreceptors and preserves retinal function against retinal degeneration caused by light injury. These initial findings suggest that KF may represent a novel therapy for retinal degenerative conditions such as AMD.

Keywords: retinal light injury, AMD, kaempferol, electroretinogram, apoptosis

Introduction

Age-related macular degeneration (AMD) is a progressive retinal disorder that is a leading cause of blindness in developed countries.[1] AMD is characterized by the loss of central vision due to damage to the macula and choroid. Although the pathogenesis of AMD is not completely understood, the disease is pathologically characterized by the formation of drusen between the retinal pigmented epithelium (RPE) and Bruch's membrane concomitant with persistent activation of the complement cascade and inflammation leading to thickening and decreased

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permeability of the membrane.[2,3] These pathological lesions are caused by the degeneration of the retinal photoreceptors, RPE, and Bruch's membrane, as well as alterations in choroidal capillaries. The apoptotic pathway appears to be main course of photoreceptors cell death in AMD.[4–6] The ''dry'' form of AMD, characterized by RPE and photoreceptor atrophy, is the more prevalent form, accounting for 85% of cases worldwide.[7] This non-neovascular form of AMD can subsequently progress to the ''wet'' form of AMD, which is characterized by choroidal neovascularization and, in turn, is responsible for the majority of vision loss from AMD.[8]

Kaempferol (KF; 3,4',5,7-tetrahydroxyflavone), one of the most commonly found dietary flavonoids, has been isolated from grapefruit, tea, broccoli, and other plant sources.[9] KF has a wide range of pharmacological activities including antioxidant, antiangiogenic, and neuroprotective effects.[9] A recent study suggested neuroprotective effects of KF through downregulation of the aberrantly activated signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) pathways in a transient focal stroke model.[10] These 2 pathways can promote the transcription and expression of many genes that encode proinflammatory mediators, including cytokines, chemokines, adhesion molecules, and inflammatory enzymes.[11]

Excessive light exacerbates human AMD.[12] Studies have shown that after retinal light damage, the retina presents some key features of dry AMD, including thinning of the outer nuclear layer and loss of photoreceptor cells.[13–15] Furthermore, the apoptotic pathway appears to be the main course of light-induced photoreceptor cell death.[16] In this study, we investigated whether KF could mitigate retinal light injury in mice both structurally through changes in retinal morphology and functionally through changes in electroretinographic (ERG) responses.

Methods

Institutional Animal Care and Use Committee statement

All procedures involving mice were performed in accordance with the association for research in vision and ophthalmology statement for the use of animal in ophthalmic and vision research and were approved by the institutional animal care and use committee of the Edward Hines, Jr. VA Hospital.

Induction of retinal light injury

Six-week-old BALB/c albino mice (Jackson Laboratory, Bar Harbor, ME) were used for this study. They were randomly assigned to 2 groups: control-treated or KF-treated retinal light injury mice. The mice were dark adapted for 24 h before light exposure and the pupils were dilated with 1% cyclopentolate hydrochloride 1 h before light exposure. Nonanesthetized mice were exposed to 8,000 lux cool white fluorescent light for 2 h to induce light injury. All exposure to light began at 9 AM. The temperature during exposure to light was maintained at 25 ± 1.5°C.

Control (4% v/v dimethyl sulfoxide in normal saline) or KF [20 mg/(kg·d) in vehicle solution] (MW 286.24; Sigma-Aldrich) was injected intraperitoneally (i.p.) once per day for 5 days after light injury. The experimentation time frame as well as the dose of 20 mg/(kg·d) (i.p.) for KF was predetermined in a pilot study, in which retinal lesions (on days 7–14 after light injury) in response to light exposures and the effects of KF [ranging from 5 mg to 30 mg/(kg·d)] were tested on mice for the retinal function.

Electroretinography

Retinal function was evaluated before retinal light injury induction as well as 7 days after the injury. Mice were dark adapted overnight. They were anesthetized and the pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride (Bausch & Lomb Inc., Rochester, NY). Using a stainless-steel electrode coated with 1% methylcellulose, the ERG was recorded from the corneal surface with a series of stimulus luminances. Needle electrodes were subcutaneously inserted in the cheek and the tail served as reference and ground leads, respectively. The ERG responses were differentially amplified (0.3–1,500 Hz), averaged, and stored using a UTAS E-3000 signal averaging system (LKC Technologies, Gaithersburg, MD). A notch filter at 60 Hz was used during recording. Stimuli ranged from −3.6 to 2.1 log cd s/m^2 and were presented in increasing order in the dark, and at least 2 successive responses were averaged together for each stimulus presented. Intervals between the stimuli were increased from 4 to 61 s. The body temperature of anesthetized mice was kept at 37°C using a temperature-regulated heating pad. The a-wave amplitude was measured from the baseline to the trough. The amplitude of the b-wave was measured from the a-wave trough to the peak of the b-wave. If no a-wave was present, the b-wave was measured from the prestimulus baseline to the peak of the b-wave.[17,18]

Retinal histology

Mice were euthanized by carbon dioxide inhalation and eyes from all groups were collected 7 days after retinal light injury. Enucleated eyes were fixed in phosphate-buffered 2.5% glutaraldehyde–2% paraformaldehyde solution, transferred to rinsing buffer, and embedded in epoxy resin. Sections of 1-μm thickness were cut along the vertical meridian of the eye and passed through the optic nerve head. Sections were then stained with 1% toluidine blue. Three separate morphometric measurements of the outer nerve layer (ONL) from the superior and inferior retina within 1,000 μm from the optic disk were averaged together. The histological changes were evaluated by measuring the thickness of the ONL.[19]

Terminal deoxynucleotidyl transferase dUTP nick end labeling staining

Apoptosis was examined using the *In Situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). The euthanized mice eyes were enucleated 1 day after retinal light injury. The eyes were immediately embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), frozen over ice-cold isopentane on dry ice, and stored at –80°C. Frozen sections (7 μm) were cut along the sagittal plane, passing through the optic nerve head. The sections were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min and then rinsed 3 times with PBS. The

retinal sections (passed through or near the optic nerve head) were incubated with ice-cold permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 2 min, then rinsed 3 times with PBS. The sections were incubated with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture or solution only for 60 min at 37°C in the dark and then rinsed 3 times with PBS. The sections were mounted with VECTA-SHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and visualized with the fluorescence microscope (ECHO REVOLVE, San Diego, CA).[20] The total number of TUNEL-positive cells of the ONL in each section was counted using NIH ImageJ software.[21]

Statistical analysis

For ERG data analysis, 2-way repeated measures analysis of variance (ANOVA) was used. The power analysis was conducted by the *F*-test of 1-way ANOVA, where we considered numbers as the outcome and groups as the factor. For all other comparisons, unpaired Student's *t*-tests were used. For all statistical comparisons, $P < 0.05$ was considered significant.

Results

Electroretinography

In control-treated retinal light injury mice, scotopic ERG a- and b-waves were significantly diminished 7 days after the light injury (Fig. 1A, postlight injury control). However, mice treated with KF demonstrated notable preservation of functional responses, including oscillatory potentials with increasing light intensity (Fig. 1A, postlight injury KF). The a- and b-wave amplitudes were significantly different ($P < 0.01$) between KF- and control-treated mice 7 days after retinal light injury at log 1.4 and 2.1 cd s/m^2 light intensity, demonstrating that KF was able to mitigate loss of retinal function after retinal light injury (Fig. 1B).

Histological analysis

The retinal light injury induced distinct histological changes in the retina. The ONL of the retina was particularly sensitive to light injury. At 7 days postlight injury, there was notable thinning of the ONL in control retinal light injury. However, there was demonstrably less thinning of the ONL in the KF-treated light injury group compared with the control-treated

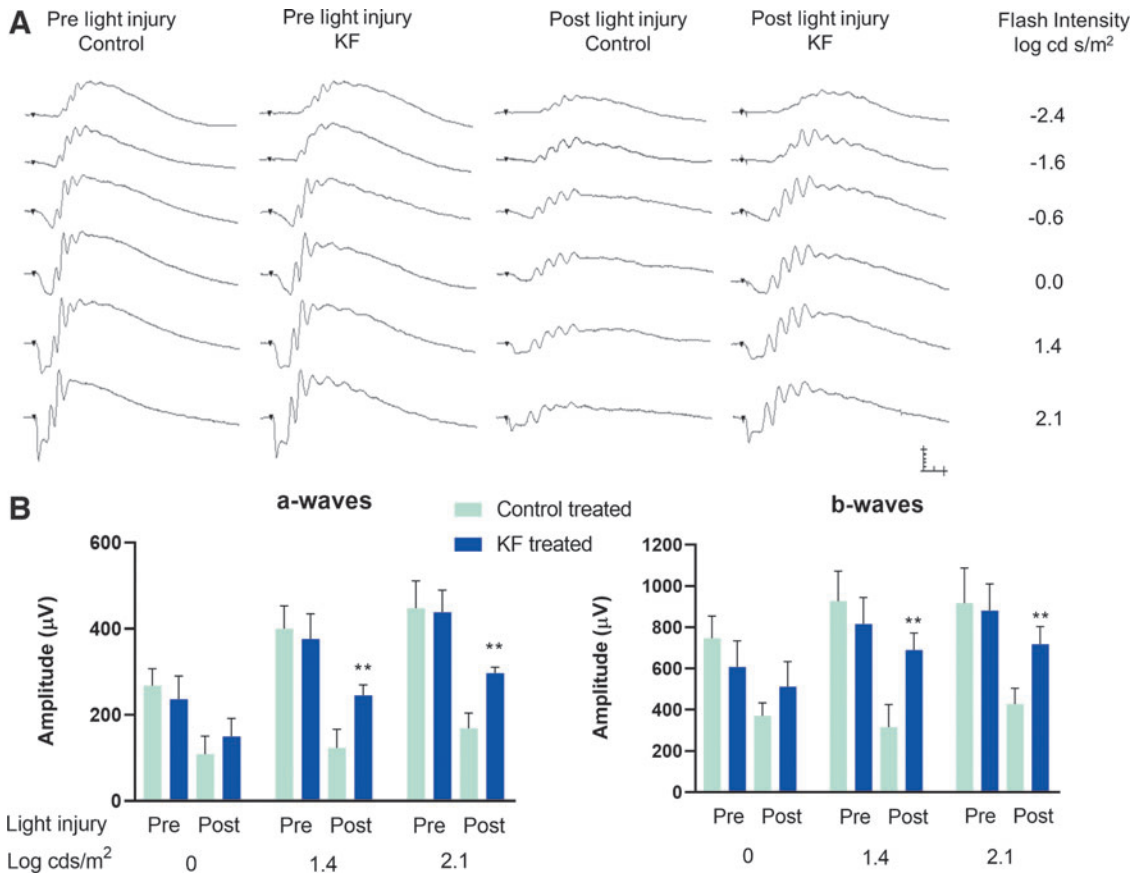

FIG. 1. Protective effect of KF on retinal function after retinal light injury. **(A)** Representative dark-adapted ERG a- and b-waves obtained from prelight injury control, prelight injury KF, postlight injury control, and postlight injury KF mice. Scale bars, 20 ms (*x*-axis) and 250 μV (*y*-axis). **(B)** Quantitative changes in ERG a- and b-wave amplitudes at 0, 1.4, and 2.1 log cd s/m^2 flash intensity. $P < 0.01$ for post-a- and post-b-wave compared between control- and KF-treated light injury group at 1.4 and 2.1 log cd s/m^2 flash intensity (represented by *double asterisk* **). Two-way ANOVA with Bonferroni *post hoc* analysis. The data given are the means ± SD, ($N = 8$). ANOVA, analysis of variance; ERG, electroretinography; KF, kaempferol; SD, standard deviation.

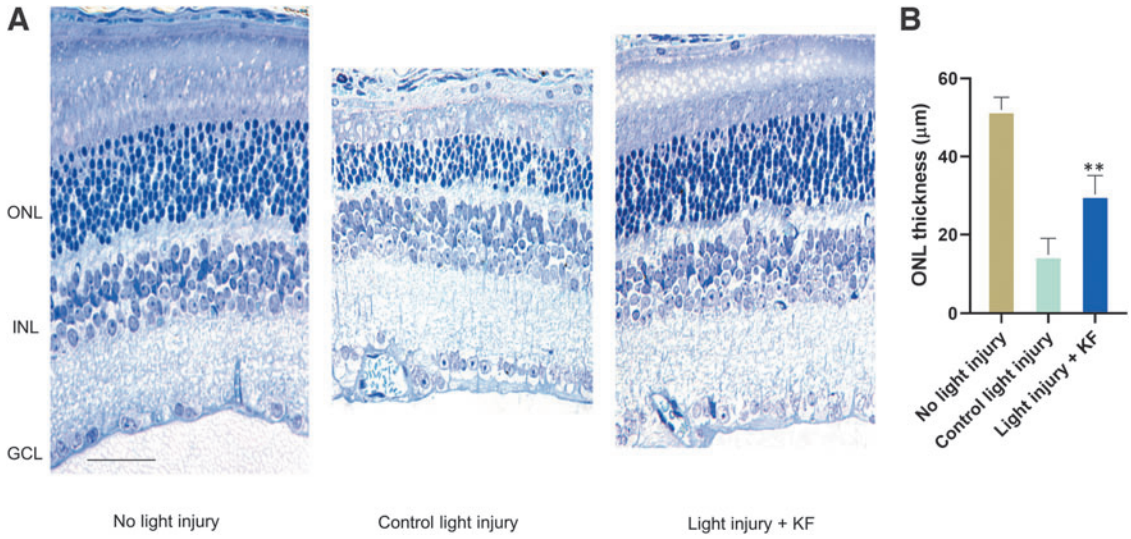

FIG. 2. Effect of KF on retinal histology after retinal light injury. **(A)** Representative toluidine *blue*-stained retinal sections from no light injury, control-treated light injury, KF-treated light-injured retinal tissues. Scale bar, 40 μm. **(B)** Morphometry quantification of the ONL thickness, data shown are the means ± SD ($n = 6$), $^{**}P < 0.01$; unpaired Student's *t*-test. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, out nuclear layer.

light injury group (Fig. 2A). The morphometric differences in the mean thickness of the ONL between the control- and the KF-treated light injury groups were statistically significant ($P < 0.01$) (Fig. 2B).

TUNEL analysis

TUNEL analysis was used to test for cellular apoptosis. Figure 3A displays representative images of TUNEL staining in nonlight injured retinas as well as 24 h after retinal light injury in control-treated light-injured and KF-treated light-injured retinas. In these representative images, TUNEL-positive cells appear as green spots. The no light-injured retinas demonstrated no visible TUNEL-positive cells.

There were numerous TUNEL-positive cells in the control-treated light-injured retinas, but much less TUNEL-positive cells in KF-treated light-injured retinas. The majority of TUNEL-positive cells were in the ONL of both control- and KF-treated retinas (Fig. 3A). The number of ONL TUNEL-positive cells was significantly higher in control-treated light-injured retinas than KF-treated light-injured retinas ($P < 0.01$) (Fig. 3B).

Discussion

AMD is a degenerative retinal disorder that continues to be the leading cause of blindness in developed countries.[1] The advent of intravitreal antivascular endothelial growth factor injections has revolutionized the way we treat the wet

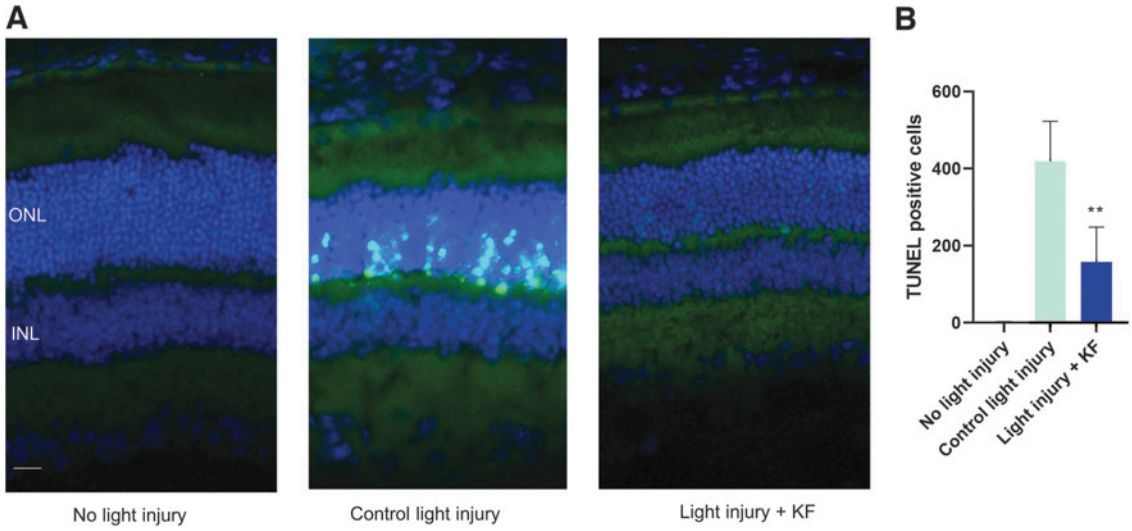

FIG. 3. KF treatment protects against retinal cell apoptosis after retinal light injury. **(A)** Representative images of TUNEL assay in no light injury, control light injury, and KF light injury. The *green* hyperfluorescent spots are TUNEL-positive cells of the ONL entering the apoptotic pathway. Scale bar, 20 μm. **(B)** The average number of total apoptotic cells in the ONL per retinal section with no light injury, control light injury, and KF light injury. Data shown are the means ± SD ($N = 6$). $^{**}P < 0.01$, control light injury versus KF light injury. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

form of AMD; however, we are left with fairly rudimentary treatment options, namely the Age-Related Eye Disease Study (AREDS) and AREDS2 vitamins, for the more predominant dry form of AMD.[7] Despite the widespread use of these vitamins, there remains a paucity of evidence supporting the efficacy of either of these formulations in preventing the development of chronic geographic atrophy, the severe end-stage form of dry AMD.[22] Recent studies using retinal light damage have allowed us to test new agents that could yield evidence-based treatments for AMD.[12–16,23]

In this study, we report that i.p. treatment with KF protects mouse retinas against light injury. The functional (ERG) and structural (histology) studies were used to confirm this protective effect. The results of our functional studies indicate a significant protective effect of KF treatment by attenuating light-induced loss of ERG a- and b-wave amplitudes compared with control treatment. We demonstrated that retinal light injury produced marked thinning of the ONL in control-treated mice. We also observed that there was significantly less thinning of the ONL in the KF treatment group compared with control treatment. Furthermore, we determined that KF protects retinal photoreceptors from light injury-induced apoptosis, as demonstrated by the fewer number of TUNEL-positive ONL cells in KF-treated mice relative to the number of TUNEL-positive ONL cells seen in control-treated mice after retinal light injury. Both the decreased number of apoptotic ONL cells and the preserved thickness of ONL in KF-treated light-injured retinas indicate that KF prevents ONL cells from undergoing apoptosis, thus maintaining ONL thickness after light injury.

Although the exact mechanism of this protective effect remains unknown, these results are not surprising given KF's wide range of pharmacological activities, including antioxidant, antiangiogenic, and neuroprotective effects.[9] Previous studies on transient focal stroke models reported downregulation of the aberrantly activated STAT3 and NF-κB pathways by KF, which may be centrally responsible for its neuroprotective effects.[10]

KF protected RPE cells from oxidative stress-induced retinal damage, KF exhibited stronger antioxidative efficacy than lutein and resveratrol on hydrogen peroxide-induced ARPE-19 cells.[24] A recent study also reported that KF protected RPE cells from hydrogen peroxide-induced inflammation and apoptosis.[25] The mechanisms underlying these antioxidative and anti-inflammatory effects apparently include downregulation of transcription and expression of genes encoding for various cytokines, adhesion molecules, and inflammatory enzymes, as well as possible suppression of the apoptosis pathway.

Although our data presented here confirmed the therapeutic efficacy of KF for the amelioration of retinal light injury in a mouse model, which suggests that KF may be a promising pharmacotherapeutic option for the prevention and treatment of dry AMD, the broad spectrum of anti-degeneration efficacy could also make it as a potential drug candidate for the treatment of other degenerative retinal disorders such as glaucoma, diabetic retinopathies, and traumatic retinal injury. Nevertheless, more extended studies are warranted to test these hypotheses.

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Authors' Contributions

All authors meet the criteria for authorship.

Author Disclosure Statement

The authors declare no competing financial interests exist.

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