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Research Article

Lutein delays photoreceptor degeneration in a mouse model of retinitis pigmentosa

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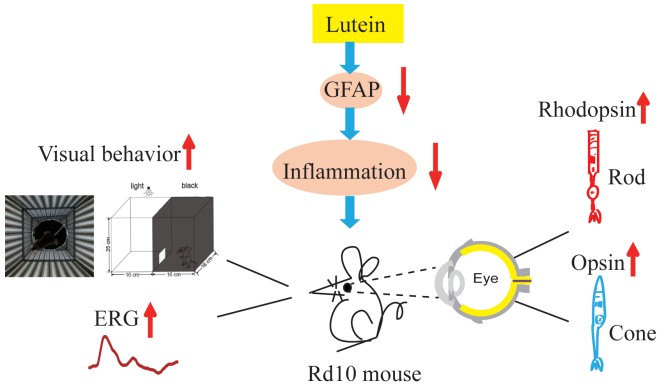
Graphical Abstract *Protective effects of lutein on photoreceptor degeneration*



Abstract

Retinitis pigmentosa is a retinal disease characterized by photoreceptor degeneration. There is currently no effective treatment for retinitis pigmentosa. Although a mixture of lutein and other antioxidant agents has shown promising effects in protecting the retina from degeneration, the role of lutein alone remains unclear. In this study, we administered intragastric lutein to Pde6b^{rd10} model mice, which display degeneration of retinal photoreceptors, on postnatal days 17 (P17) to P25, when rod apoptosis reaches peak. Lutein at the optimal protective dose of 200 mg/kg promoted the survival of photoreceptors compared with vehicle control. Lutein increased rhodopsin expression in rod cells and opsin expression in cone cells, in line with an increased survival rate of photoreceptors. Functionally, lutein improved visual behavior, visual acuity, and retinal electroretinogram responses in Pde6b^{rd10} mice. Mechanistically, lutein reduced the expression of glial fibrillary acidic protein in Müller glial cells. The results of this study confirm the ability of lutein to postpone photoreceptor degeneration by reducing reactive gliosis of Müller cells in the retina and exerting anti-inflammatory effects. This study was approved by the Laboratory Animal Ethics Committee of Jinan University (approval No. LACUC-20181217-02) on December 17, 2018.

Key Words: anti-inflammation; glial fibrillary acidic protein; lutein; microglia; Pde6b^{rd10} (rd10) mouse; photoreceptor; reactive gliosis; retinal degeneration; retinal disease; retinitis pigmentosa

Chinese Library Classification No. R452; R741; TQ611.5

Introduction

Retinal degenerative diseases include many ophthalmological disorders such as retinitis pigmentosa (RP) and age-related macular degeneration, of which RP is the most common, with a prevalence rate of nearly 1/4000 individuals worldwide (Hartong et al., 2006). Patients with RP show symptoms of night blindness and degradation of peripheral vision caused by the gradual loss of rod cells in the early stages, followed by the death of cone cells, eventually leading to the loss of central vision (Farrar et al., 2002; Mitamura et al., 2012). Currently, more than 1.5 million people with RP experience progressive visual deterioration, with most suffering from poor vision or blindness (Ikeda et al., 2014). Although there are several treatments for RP including drug therapy, gene therapy to replace photoreceptor cells, and stem cell therapy (Compagnone and Mellon, 2000; Plassart-Schiess and Baulieu,

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2001; Schumacher et al., 2012), there is currently no effective long-term treatment.

As a classic strategy, drug therapy has the advantages of convenience and safety, with strict regulations. Several antioxidant and anti-inflammatory agents have been shown to slow the degeneration of photoreceptor cells in the retina (Kang et al., 2016; Zhang et al., 2017; Liu et al., 2018, 2021; Fernández-Albarral et al., 2021), suggesting that inhibiting oxidative stress and the inflammatory response may play essential roles in preventing the pathogenesis of RP. Lutein is a dietary carotenoid that forms the macular pigment in the human eye (van de Kraats et al., 2008); however, it cannot be synthesized in the human body and needs to be obtained from the diet (Ranard et al., 2017). Combinations of lutein and other antioxidants (such as zeaxanthin, alpha-lipoic acid, glutathione, wolfberry extracts) have been applied in studies of various retinal diseases, such as RP (Sanz et al., 2007; Miranda et al., 2010), diabetic retinopathy (Gong and Rubin, 2015), and retinal ischemia (Li et al., 2012), and have been shown to protect retinal structure and function by reducing oxidative stress, reactive oxygen species, and inflammation (Sanz et al., 2007; Miranda et al., 2010; Sasaki et al., 2010; Li et al., 2012). A mixture of lutein and other carotenoids has also demonstrated promising effects on RP, cataracts, glaucoma, retinal ischemia, and diabetic retinopathy in clinical settings (Gong and Rubin, 2015), and has prevented the progression of age-related macular degeneration (Eisenhauer et al., 2017). However, different formulas have shown various effects (Sanz et al., 2007), and the ability of purified lutein alone to protect against retinal degeneration, especially in inherited diseases like RP, remains unclear.

In this study, we examined the ability of lutein to prevent the progression of photoreceptor cell death in Pde6b^{rd10} (rd10) mutant mice with RP, based on visual behavior, electroretinogram (ERG), and immunohistochemistry. We also explored the anti-inflammatory effect of lutein on the retina in rd10 mice to disclose the mechanism responsible for its protective effect. Rd10 mice have spontaneous missense mutations in the rod phosphodiesterase-6β (*PDE6β*) gene, which encodes the β subunit of PDE6, a protein complex necessary for light transduction (Garcia-Delgado et al., 2018). In humans, mutations in the *PDE6β* gene cause autosomal recessive RP (Cheng et al., 2016). The rd10 mouse model is thus an important animal model for examining photoreceptor cell degeneration in RP.

Materials and Methods

Animals

Breeding pairs of rd10 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and C57BL/6J (C57) mice were purchased from Guangdong Medical Lab Animal Center (license No. SYXK (Yue) 2017-0174). All the mice were provided with adequate water and food under standard laboratory conditions with 12/12-hour light/dark cycles. All experiments on animals were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and approved by the Laboratory Animal Ethics Committee of Jinan University on December 17, 2018 (approval No. LACUC-20181217-02). All efforts were taken to minimize animal suffering.

Experimental design

Lutein (CAS: 127-40-2, 99.9%; Sigma, Shanghai, China) was dissolved in phosphate-buffered saline (PBS) containing 0.1% dimethyl sulfoxide. The solvent was used as a vehicle control. A previous safety evaluation found no adverse effects of lutein at a maximum dose of 400 mg/kg/day (Ravikrishnan et al., 2011). We therefore tested lutein at doses of 1, 10, 100, 200, and 400 mg/kg body weight in our preliminary screenings, followed by 4′,6-diamidino-2-phenylindole (DAPI) staining of retinal slices. Mutations in the *PDE6β* gene cause rod cells to degenerate around postnatal day 18 (P18) in rd10 mice (Gargini et al., 2007), and a recent study showed an abnormal decrease in retinal vascular density in rd10 mice at P17 and significant vascular stenosis of the vascular layer appeared by P28 (Kim et al., 2018). We carried out a preliminary test at P16, which showed that although the photoreceptors were relatively normal, expression levels of the ionized calcium binding adaptor molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) increased, indicating reactive gliosis (**Additional Figure 1**). Based on this study (Xiang et al., 2018), we randomly orally fed rd10 littermates with lutein or vehicle daily from P17 to P25 and examined their visual behavior and ERG results. The retinas were then removed for immunostaining (**Figure 1**). We used C57 mice at the same age as a normal control group (**Figure 1**). The three groups in this study were C57 mice (n = 21), vehicle-treated mice (rd10 + vehicle; n = 35), and lutein-treated mice (rd10 + lutein; n = 47).

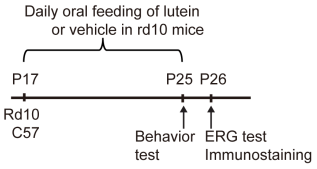

Figure 1 | **Experimental design.** Pde6b^{rd10} (rd10) mice were administered daily orally with lutein or vehicle from postnatal days 17 to 25. C57: C57BL/6J mice; ERG: electroretinogram.

Black-white transition test

A black-white transition system device was custom-made by Metronet Technology Ltd. (Beijing, China) containing white and black boxes of the same size (16 cm × 16 cm × 25 cm), separated by a light-proof baffle. A small hole underneath the baffle allowed the mouse to move freely. Two infrared cameras were mounted above the boxes to monitor mouse movement between the two boxes, and were connected to Noldus Etho Vision XT 8.0 analytical software (Doldus, Wageningen, Netherlands). In our study, the black-white transition test was conducted on the morning of P25 to assess the mouse's ability to detect light. Each mouse was tested in the box for 5 minutes, and the percentage of time spent in the black box was calculated using Noldus software.

Optomotor test

An optomotor test to determine visual acuity was performed on the afternoon of P25. Each mouse was placed freely on a central high platform surrounded by four computer screens that displayed vertical rotating sine gratings (100% contrast, moving speed 12 cycles/degree (cpd)) with different spatial frequencies (programmed by Matlab 7.0; MathWorks, Natick, MA, USA). When the mouse saw the moving grating, it tended to follow the rotation of the grating with its head in the same direction and the same speed, referred as the optomotor response. The maximum spatial frequency of the grating that the mouse could follow was defined as the visual acuity. Mouse head movements were recorded by a camera and analyzed manually (Wang et al., 2014; Huang et al., 2018). The tested spatial frequency ranged from 0.1 to 0.4 cpd for rd10, and 0.1 to 0.6 cpd for C57 mice, given that rd10 mice have a poorer response than C57 mice (Liu et al., 2021).

ERG

Animals were dark-adapted overnight prior to the experiment, and an ERG was recorded using a RETI-scan system (Roland Consult, Brandenburg, Germany), as described previously (Liu et al., 2018). Dark-adapted mice were stimulated with a green flashing light with increasing intensities of 0.03, 0.3, and 3.0 cd/m^2 to elicit scotopic ERG responses. The mice were then light-adapted for 5 minutes using a bright green background (20 cd/m^2) and photopic response to green flashes were recorded at 10.0 cd/m^2. The data were captured by the amplifier of the RETI-scan system at a sampling rate of 2 kHz and then processed by RETIport software (Roland Consult) after applying a 50 Hz low-pass filter. For each animal, the result with the best response in both eyes was used for statistical analysis.

Tissue processing

After ERG testing, the mice were sacrificed using an overdose of anesthetic (intraperitoneal injection of 100 mg/kg pentobarbital sodium; R&D Systems, Minneapolis, MN, USA), and both eyes were enucleated and fixed in 4% paraformaldehyde for 30 minutes. The tissues were then rinsed three times in PBS for 10 minutes each, and cryo-protected overnight at 4°C in 0.1 M phosphate buffer containing 30% sucrose. Samples were then embedded in optimal cutting temperature compound (Tissue Tek, Torrance, CA, USA) and cryo-sectioned longitudinally at a thickness of 15 µm, and slices with the optic disk were mounted on glass slides for further processing.

Immunocytochemistry

Retinal sections were rinsed three times with 0.1% Triton X-100 in PBS for 5 minutes each, followed by blocking buffer for 1 hour at room temperature. The blocking buffer was composed of 0.3% Triton X-100 in PBS containing 3% normal donkey serum, 1% bovine serum albumin, and 0.3% Triton X-100 (all Ruiqian Biotechnology Co., Ltd., Guangzhou, China). The sections were then washed and incubated with primary antibodies in blocking buffer overnight at 4°C, followed by the corresponding secondary antibodies for 2 hours at room temperature, after thorough washing. The sections were then washed and sealed with an anti-fluorescence-quenching media coverslip post-incubation. For DAPI staining, sections were incubated with DAPI (1:2000, Electron Microcopy Sciences, Hatfield, PA, USA) for 5 minutes at room temperature before mounting The primary antibodies used in the experiment were mouse anti-rhodopsin (1:1000, Millipore, Bedford, MA, USA; Cat# MAB5356, RRID: AB_2178961), mouse anti-opsin (1:1000, Millipore, Cat# AB5405, RRID: AB_177457), rabbit anti-postsynaptic density protein 95 (PSD95; 1:1000, Abcam, Cambridge, UK; Cat# ab18258, RRID: AB_444362), rabbit anti-Iba1 (1:1000, Wako, Osaka, Japan; Cat# 019-19741, RRID: AB_839504), and rat anti-GFAP (1:1000, Thermo Fisher, Waltham, MA, USA; Cat# 13-0300, RRID: AB_2748894). The secondary antibodies were donkey-anti-mouse or donkey-anti-rabbit IgG conjugated to Alexa Fluor-488 (1:1000; Invitrogen, Carlsbad, CA, USA; Cat# 150105, RRID: AB_1930716) or 594 (1:1000; Invitrogen; Cat# 150108, RRID: AB_1930716).

Image collection and processing

Fluorescent images were recorded using a Zeiss LSM700 confocal microscope (Carl Zeiss, Oberkochen, Germany) for opsin, rhodopsin, PSD95, GFAP, and Iba1-staining, or a fluorescence microscope (Carl Zeiss) for DAPI staining only. The thickness of the outer nuclear layer (ONL) including the rod and cone nuclei was measured as an estimate of photoreceptor survival. Because photoreceptor degeneration in rd10 mice was not synchronized from the center to the periphery, all measurements were determined in sections including optic nerve transection to ensure the same eccentricity. The thickness of the ONL was measured sequentially at ±400, ±800, ±1200, and ±1600 µm from the optic nerve head, and the readings from both sides of the optic nerve head were averaged to give the data points at various distance. Opsin, rhodopsin, PSD95, GFAP, and Iba-1 staining were quantified in an area located 800–1000 µm from the center of the optic disk. The mean length of the cone outer segments (OS) and Müller cell processes was measured by drawing a line along the axis of each cone OS and Müller cell process in each image. The number of activated Müller cells was counted by the GFAP-positive processes that vertically crossed the INL layer. To determine the thickness of the rhodopsin in the OS, five regions were measured at 30 µm intervals and the average value was taken as one data point for each image. To compare the fluorescence intensities of GFAP, PSD95, and Iba-1, retinal sections from different groups were processed using the same procedure and imaging parameters, and the mean fluorescence intensity from a Z-stack of 10 sections was measured. ImageJ software 1.41 (National Institutes of Health, Bethesda, MD, USA) was applied for all measurements. Calculations from three to five slices (image size: 160 µm × 160 µm) were averaged to get one data point for each animal, and these values were then averaged to produce a mean value for the group.

Statistical analysis

No statistical methods were used to predetermine the sample sizes; however, our sample sizes were similar to those reported in a previous publication (Xiang et al., 2018). No animals or data points were excluded from the analysis. The results were expressed as mean ± standard error of mean (SEM) and were analyzed using Prism 7 (GraphPad Software, San Diego, CA, USA) with Student's *t*-tests and one-way or two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. *P* values < 0.05 indicated a significant difference.

Results

Lutein protects retinal morphology and structure in rd10 mice at P26

We first estimated photoreceptor survival by measuring the thickness of the ONL of the retina, where the photoreceptor somas are located. We tested five doses of lutein (1, 10, 100, 200, and 400 mg/kg body weight) to screen for the optimal therapeutic concentration required to protect the retina. DAPI was used to label the nuclei in each layer of the retina. The thickness of the ONL in rd10 mice increased to varying degrees after oral gavage with lutein (**Figure 2A**). A dose of 200 mg/kg increased the thickness from the center to the periphery ($P < 0.001$, compared with vehicle-treated littermates), with the most significant difference at 400 µm from the optic nerve center (**Figure 2B**). Subsequent experiments were therefore carried out using lutein at 200 mg/kg, withC57 mice as the wild-type (WT) control. Lutein markedly delayed photoreceptor degeneration at day 9 after treatment, as demonstrated by the increased thickness of the ONL (**Figure 3A**). The increased ONL thickness was most evident in the central retina at ±400 µm from the optic nerve (**Figure 3A**). The ONL thickness in C57 mice was 60 ± 2.30 µm ($n = 9$) and this was reduced to 14 ± 1.94 µm in vehicle-treated rd10 mice ($n = 12$). However treatment with lutein significantly increased the thickness of the ONL in rd10 mice to 25 ± 3.68 µm ($n = 12$, $P < 0.001$, *vs*. vehicle control group; **Figure 3B**).

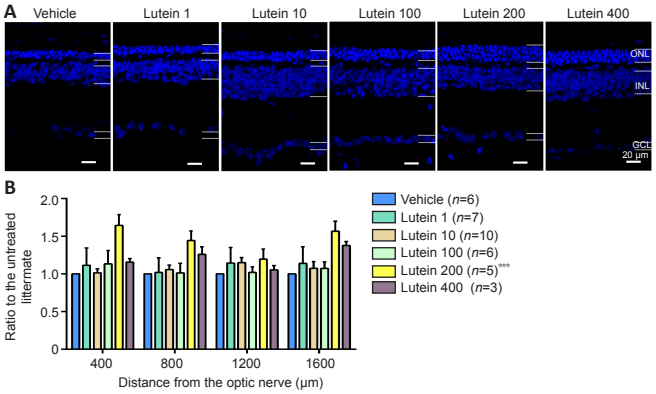

Figure 2 | **Effects of various lutein doses on photoreceptor cell layer in Pde6b^{rd10} (rd10) mice at postnatal day 26.**
(A) Images of retinal sections stained with DAPI from rd10 mice treated with increasing dose of lutein and vehicle control. The outer nuclear layer (ONL) was very thin in vehicle-treated rd10 retinas and was increased by various concentrations of lutein. Scale bars: 20 μm. (B) Quantification of dose-dependent effect of lutein on ONL thickness (normalized to vehicle-treated rd10 mice) in rd10 mice along the optic cup. Data are shown as mean ± SEM. Number of animals tested shown in brackets. ***P < 0.001, *vs.* vehicle-treated rd10 mice (one-way analysis of variance followed by Tukey's *post hoc* test). C57: C57BL/6J mice; DAPI: 4′,6-diamidino-2-phenylindole; GCL: ganglion cell layer; INL: inner nuclei layer; ONL: outer nuclei layer.

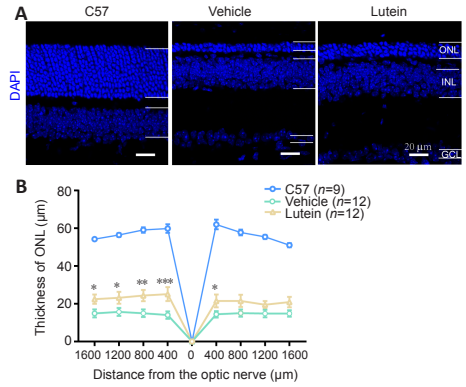

Figure 3 | **Lutein partially rescues photoreceptor cells in the retina in Pde6b^{rd10} (rd10) mice at postnatal day 26.**
(A) Images of retinal sections stained with DAPI in different groups. The outer nuclear layer (ONL) was much thinner in rd10 compared with C57 retinas and was increased by lutein. Scale bars: 20 μm. (B) Quantification of ONL thickness in different groups. Data are shown as mean ± SEM. Number of animals tested shown in brackets. *P < 0.05, **P < 0.01, ***P < 0.001 (two-way analysis of variance followed by Dunnett's *post hoc* test). C57: C57BL/6J mice; DAPI: DAPI: 4′,6-diamidino-2-phenylindole; GCL: ganglion cell layer; INL: inner nuclei layer; ONL: outer nuclei layer.

Rhodopsin and opsin are light-sensitive proteins expressed by the OS of rod and cone cells, respectively (Carroll et al., 2012; Makino et al., 2012). We therefore used rhodopsin immunostaining to confirm the molecular structure of the rods. The rod OS were clearly stained in control C57 mice, with a thickness of 17.56 ± 0.58 μm (n = 4) (**Figure 4A** top panel, left). In contrast, most of the rod OS had degenerated in vehicle-treated rd10 mice and the OS layer was reduced to 1.36 ± 0.12 μm (n = 5) (**Figure 4A** top panel, middle). Lutein treatment increased the expression of rhodopsin in the rod OS (**Figure 4A** top panel, right) and increased the OS thickness to 2.90 ± 0.23 μm (n = 5; P < 0.05, vs. vehicle control group; **Figure 4B**). These results were consistent with those of nuclear staining indicating more somas in the ONL. Lutein treatment thus slowed down the degeneration of rd10 rods.

Cones also degenerate in rd10 mice, although later than rods (Barone et al., 2014), and we therefore tested the protective effect of lutein on cone structure by opsin immunostaining. Opsin in the cone OS in C57 mice appeared as long strips (**Figure 4A** bottom panel, left) with an average length of 9.91 ± 0.51 μm (n = 4). The length of the cone OS in rd10 mice was significantly reduced to 1.35 ± 0.27 μm (n = 4) (**Figure 4A** bottom panel, middle), and this was significantly increased by lutein treatment (**Figure 4A** bottom panel, left) to 2.89 ± 0.36 μm (n = 5, P < 0.05, *vs.* vehicle control group; **Figure 4C**), indicating that lutein treatment also slowed the degeneration of rd10 cones.

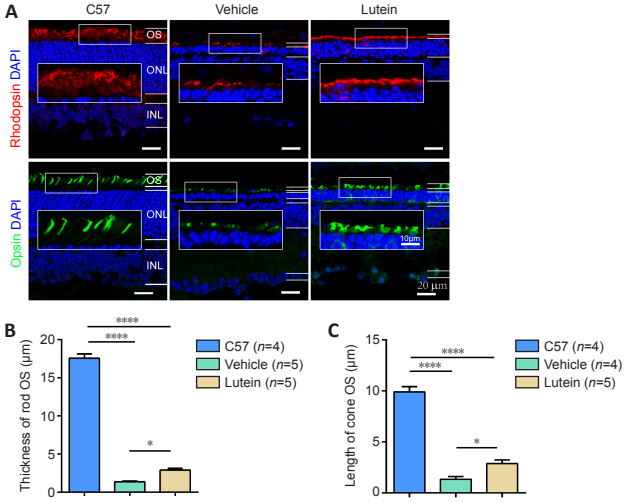

Figure 4 | **Lutein protects retinal structure in Pde6b^{rd10} (rd10) mice at postnatal day 26.**
(A) Images of opsin (green, Alexa Fluor-488), rhodopsin (red, Alexa Fluor-594), and DAPI (blue) staining in retinal sections. Rhodopsin labelled rod outer segment (OS), and opsin labelled cone OS. Inset shows enlarged area from OS layer. Scale bars: 20 μm, with 10 μm in enlarged areas. (B) Average thickness of rod OS. (C) Average length of cone OS. Data are shown as mean ± SEM. Number of animals tested shown in brackets. *P < 0.05, ****P < 0.0001 (one-way analysis of variance with Tukey's *post hoc* test). C57: C57BL/6J mice; DAPI: 4′,6-diamidino-2-phenylindole; INL: inner nuclei layer; ONL: outer nuclei layer; OS: outer segment.

We further examined the synaptic connections between photoreceptors and bipolar cells by immunostaining for PSD95. The expression of PSD95, indicated by the mean fluorescence intensity, was reduced in rd10 compared with C57 mice, and was largely unaffected by lutein (**Additional Figure 2**).

Lutein enhances visual behavior in rd10 mice

The morphological improvements induced by lutein were further testified by visual behavior. We used the black-white box system (**Figure 5A**) to estimate the tendency of mice to stay in the dark. Due to their nocturnal characteristics, C57 mice with normal vision tended to stay longer in in the black box compared with the white box. However, rd10 mice lost the ability to distinguish between light and dark, in line with the degeneration of their photoreceptor cells, causing them to spend more-equal amounts of time in the black and white boxes. Vehicle-treated rd10 mice (n = 14) spent 52.3 ± 1.5% of their time in the black box, compared with 59.9 ± 0.9% in the normal group (n = 12; P < 0.01). After oral administration of lutein, the time spent by rd10 mice in the black box increased to 58.9 ± 1.6% (n = 14, P < 0.01, *vs.* vehicle control group; **Figure 5B**). These results suggested that lutein could improve the ability of rd10 mice to distinguish between light and dark.

We also tested the visual acuity of the mice using an optomotor system, which encircled the mouse with computer screens displaying moving gratings at various spatial frequencies (**Figure 5C**). The maximum frequency of a grating

that a mouse could follow by its head movement was defined as the visual acuity. The visual acuity of C57 mice was 0.37 ± 0.01 cpd (n = 9), compared with 0.21 ± 0.02 cpd in vehicle-treated rd10 mice (n = 11; P < 0.0001). Lutein treatment significantly improved the visual acuity of rd10 mice to 0.27 ± 0.02 cpd (n = 12; P < 0.05 *vs*. vehicle control group), though it remained lower than in C57 mice (P < 0.01; **Figure 5D**). These visual acuity results confirmed that lutein improved visual function in rd10 mice.

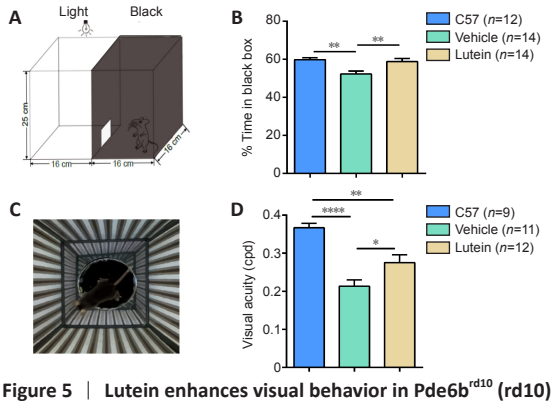

Figure 5 | Lutein enhances visual behavior in Pde6b^{rd10} (rd10) mice at postnatal day 25.

(A) Black-white transition system device for visual behavior test. (B) Average time mice stayed in the black box (percentage of the total time in the boxes). Lutein significantly increased the residence time of rd10 mice in the black box. (C) Optomotor system. (D) Average visual acuity of animals tested by optomotor system. Lutein significantly increased the visual acuity of rd10 mice. Data are shown as mean ± SEM. Number of animals tested shown in brackets. *P < 0.05, **P < 0.01, ****P < 0.0001 (one-way analysis of variance with Tukey's *post hoc* test). C57: C57BL/6J mice; cpd: cycle per degree.

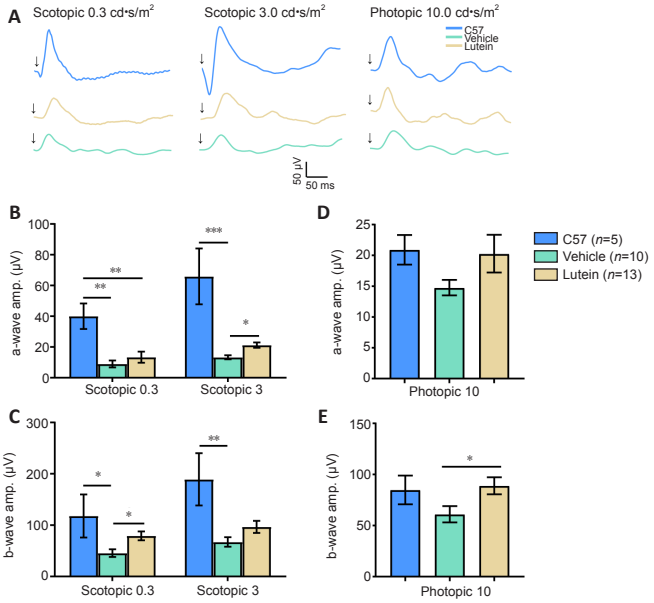

Figure 6 | Lutein improves the light responses of Pde6b^{rd10} (rd10) retina at postnatal day 26.

(A) Typical electroretinogram waveforms in response to flashes of increasing intensities under dark adaptation (scotopic) and light adaptation (photopic) conditions. Electroretinogram responses were smaller in rd10 than in C57 mice, and lutein treatment improved the rd10 responses. Arrowhead indicates onset of flash. (B, C) Average peak amplitudes of scotopic a- (B) and b-waves (C). (D, E) Average peak amplitudes (amp.) of photopic a- (D) and b-waves (E). Data are shown as mean ± SEM. Number of animals tested shown in brackets. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with Dunnett's *post hoc* test). C57: C57BL/6J mice.

Lutein promotes retinal light responses in rd10 mice

After confirming the protective effect of lutein on the visual behavior of rd10 mice, we further examined the functional characteristic of the retina by ERG. Retinal neurons responded strongly to light flashes under scotopic (dark-adapted) and photopic (light-adapted) conditions in C57 mice (black lines, **Figure 6A**). In contrast, light responses in rd10 mice decreased significantly for all tested flash intensities (blue lines), and lutein treatment partially rescued these response (red lines, **Figure 6A**).

Most vehicle-treated rd10 mice (7/10, 70%) failed to respond to light stimuli with a clear waveform under scotopic conditions with a low flash intensity at 0.03 cd·s/m^2, while lutein treatment reduced the non-responsive rate to 28.5% (4/14, 28.5%). Small ERG responses were observed in all rd10 mice at flash intensities of 0.3 and 3.0 cd·s/m^2, which were significantly smaller than those in C57 mice (**Figure 6B** and **C**). Under dark adaptation conditions, lutein enhanced both the a- and b-wave amplitudes in rd10 mice. Specifically, lutein significantly increased the b-wave amplitude from 45 ± 7.5 μV in vehicle-treated rd10 mice (38% of WT) to 79 ± 8.5 μV (67% of WT) at scotopic 0.3 cd·s/m^2 (P < 0.05, *vs*. vehicle control group; **Figure 6C**), and increased the a-wave amplitude from 13 ± 1.3 μV (20% of WT) to 22 ± 1.8 μV (32% of WT) at scotopic 3 cd·s/m^2 (P < 0.05, *vs*. vehicle control group; **Figure 6B**). After light adaptation, lutein increased the a-wave amplitude slightly but not significantly (P = 0.2596, *vs*. vehicle control group; **Figure 6D**), and significantly raised the b-wave amplitude from 55 ± 9.1 μV to 90 ± 10.0 μV (P < 0.05, *vs*. vehicle control group; **Figure 6E**).

Given that the responses of bipolar cells rely on the inputs from photoreceptor cells, our ERG data revealed that lutein rescued the functional responses in both rod- and cone-mediated light pathways in rd10 mouse retinas.

Lutein inhibits Müller gliosis in rd10 retinas

Given that lutein has an anti-inflammatory effect (Chung et al., 2017), we determined if it inhibited inflammation in the rd10 retina. Reactive gliosis in Müller cells and microglia in the retina is an indicator of inflammation, with increasing expression of GFAP in Müller cells and reactivity of microglia cells (Bringmann and Wiedemann, 2012; Genové et al., 2014). We first examined the immunopositivity of GFAP in Müller cells by counting the number of Müller glial cells and measuring the length of their processes. Müller cells were not activated in C57 mice and GFAP immunopositivity was limited to the ganglion cell layer, with extremely short dendrites (**Figure 7A** top panel, left). In contrast, Müller glial cells were much more active in vehicle-treated rd10 mice, with more processes expressing GFAP and the GFAP-positive processes extending to the ONL layer (**Figure 7A**). The mean fluorescence intensity of GFAP staining significantly increased 2.16 ± 0.16-fold compared with C57 mice (P < 0.05; **Figure 7B**). Lutein treatment significantly weakened the reactive gliosis of Müller cells, with fewer and shorter GFAP-stained processes (**Figure 7A**). The mean fluorescence intensity of GFAP was significantly reduced to 1.22 ± 0.17-fold that in C57 mice (P < 0.05 *vs*. vehicle; **Figure 7B**). We also analyzed the number and length of GFAP-positive processes, and found that both were significantly increased in the vehicle-treated group and both were significantly reduced by lutein (data not shown).

We measured the reactivity of microglial cells by Iba-1 staining. Iba-1-labelled microglia in C57 mice had branches mainly in the inner retina (**Figure 7A**), while the microglia extended all over the retina in vehicle-treated rd10 mice, with many in the outer layers, with thick branches and large somas (**Figure 7A**). The mean fluorescence intensity increased significantly to 3.26 ± 0.27-fold that in C57 mice (P < 0.01, *vs*. C57; **Figure 7C**). Lutein reduced the numbers of microglia in

the outer and inner retinas (**Figure 7A**), but only decreased the average Iba-1 fluorescence intensity slightly to 3.04 ± 0.21-fold of that in C57 mice ($P < 0.01$, *vs*. C57), which was not significantly lower than that in the vehicle group ($P = 0.53$, *vs*. vehicle group). These results imply that lutein inhibited the gliosis of Müller cells but had less effect on microglia in the rd10 retina.

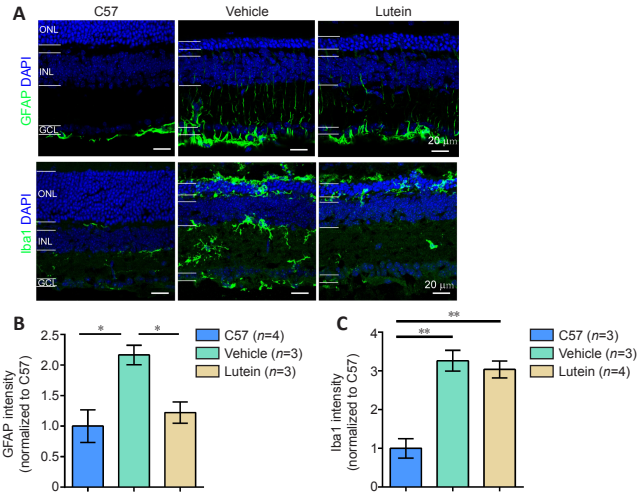

Figure 7 | Lutein prevents the progression of reactive gliosis in the Pde6b^{rd10} (rd10) retina at postnatal day 26.

(A) Retinal sections stained with GFAP (green: Alexa Fluor-488, top panel, single slice) or Iba1 (green: Alexa Fluor-488, bottom panel, Z-stacks) and DAPI (blue). In rd10 retinas, GFAP expression in Müller glial cells crossed the entire retina and lutein treatment reduced the GFAP signal from the outer retina. (B, C) Average fluorescence intensities of GFAP (B) and Iba1 (C) normalized to C57. Lutein treatment significantly reduced the GFAP fluorescence intensity in rd10 retinas. Data are shown as mean ± SEM. Number of animals tested shown in brackets. *$P < 0.05$ **$P < 0.01$ (Student's *t*-test). C57: C57BL/6J mice; DAPI: DAPI: 4′,6-diamidino-2-phenylindole; GCL: ganglion cell layer; GFAP: glial fibrillary acidic protein; INL: inner nuclei layer; ONL: outer nuclei layer.

Discussion

In the current study, we demonstrated that lutein 200 mg/kg promoted the survival of rods and cones in rd10 mice. Functionally, lutein improved the visual performance and visual acuity as well as the ERG responses, while structurally, it increased the expression of rhodopsin in rod cells and opsin in cone cells, but had little effect on synaptic connections, as indicated by PSD95 expression. These effects may be related to the reduction of reactive gliosis in the retina. Indeed, lutein decreased the fluorescence intensity of GFAP staining and the number and length of Müller glial cell processes in the rd10 retina. It also slightly reduced the Iba1 staining intensity. These data indicate that lutein preserved the retinal structure and function in rd10 mice, partly via an anti-gliosis effect.

The protective effects of lutein on retinal photoreceptor cells have been reported in a variety of animal models of retinal diseases, such as RP, light-induced retinal degeneration, and ischemia/hypoxia-induced retinopathy (Sanz et al., 2007; Miranda et al., 2010; Sasaki et al., 2010; Li et al., 2012; Kamoshita et al., 2016; Shivarudrappa and Ponesakki, 2020). However, the purity of the lutein used in those experiments differed from the current study, which used lutein alone rather than combined with other molecules. In addition, we administered the lutein to rd10 mice by oral administration, which is the most clinically favorable route of administration. The current results thus support future clinical applications of lutein. Indeed, in a previous clinic trial, lutein supplementation at 12 mg/day slowed loss of the mid-peripheral visual field among nonsmoking RP patients taking vitamin A, supporting the clinical use of lutein to treat RP (Berson et al., 2010). Mixtures of lutein and other carotenoids, such as zeaxanthin, are also being tested for the clinical treatment of retinal diseases (Aziz et al., 2020).

RP is a hereditary eye disease resulting in gradual loss of vision as a result of the progressive degeneration of photoreceptor cells (Mitamura et al., 2012). Early symptoms usually appear during puberty, but severe visual dysfunction occurs around 40–50 years of age (Sahni et al., 2011). Most patients with RP are already in the middle or even late stages of the disease when they develop clinical symptoms, highlighting the need to develop treatments for RP patients in the advanced stage of the disease. The only previous study of lutein and zeaxanthin isomers in rd10 mice started treatment from P6 (Yu et al., 2018), i.e., before the eyes were open and before the development of symptoms. In our study, we administered lutein at P17, a week after the occurrence of reactive gliosis (Arroba et al., 2014) at the time of an abnormal decrease in retinal vascular density (Kim et al., 2018) and when the rod cells began to degenerate in rd10 mice (Gargini et al., 2007). The current study was thus more applicable to the clinical situation than previous studies. When administered at this stage, lutein alone significantly improved the structure and function of the retina in rd10 mice, and we speculated that its protective effect might be increased if treatment was started earlier. However, to the best of our knowledge, the current study provides the first evidence for the protective effects of purified lutein administered after the appearance of symptoms in rd10 mice.

Our study further confirmed the anti-inflammatory effect of lutein in rd10 mice. Previous studies showed that lutein exerted its antioxidant and anti-inflammatory activities by targeting reactive oxygen species and down-regulating inflammatory proteins and pro-inflammatory cytokines (Phan et al., 2018). Increased microglial activity and gliosis are observed in the rd10 retina before the onset of photoreceptor loss (Wang et al., 2016). Reactive gliosis is characterized by increased expression of GFAP in Müller cells and increased reactivity of microglia, which provide a sensitive indicator of the early retinal stress response (Bringmann and Wiedemann, 2012). Indeed, our experiments showed that GFAP expression in Müller glial cells and the reactivity of microglia were increased in the rd10 retina, while lutein treatment significantly decreased GFAP fluorescence intensity, indicating inhibition of Müller gliosis by lutein in rd10 mice. Lutein also slightly reduced microglia reactivity. Microglia–Müller glia crosstalk has been implicated in the initiation of gliosis, and microglia cell reactivity is a hallmark of neuroinflammation in RP animal models (Zeiss and Johnson, 2004) and human patients (Gupta and Yücel, 2003). Our data therefore suggest that lutein can protect photoreceptor cells in rd10 mice by reducing the inflammatory response.

This study had several limitations and biases. For example, we did not investigate other possible protective mechanisms of lutein acting on the rd10 retina. A previous study showed that the combined use of lutein and zeaxanthin, both antioxidants, could slow down photoreceptor degeneration in rd10 mice, and this protective effect may occur by reducing endoplasmic reticulum stress (Yu et al., 2018). An *in vitro* study of retinal pigment epithelial cells also revealed that lutein and zeaxanthin could protect the proteasome from oxidative inactivation and resist the inflammatory response caused by photo-oxidation (Bian et al., 2012). Lutein can also exert its retinal-protective effect by inhibiting the c-Jun NH2-terminal kinase-mediated autophagy pathway and activating

transcription factor nuclear factor erythroid2-related factor 2 in human retinal pigment epithelial cells (Frede et al., 2017). Lutein may thus also act through these alternative pathways to protect the rd10 retina, in addition to inhibiting inflammation. In addition, our experiments only monitored the effect of lutein on rd10 retinal degeneration from P17 to P26, and its long-term protective effect in rd10 mice remains unknown. Examination of rd10 retinas at later stages may help to elucidate this. Similarly, the protective effect of lutein may be improved if lutein is administered at P14 or even earlier.

In conclusion, we demonstrated that lutein protects retinal structure and function in an inherited animal model of RP. Lutein may delay photoreceptor degeneration through its anti-inflammatory effect by reducing Müller cell gliosis in the retina. These results thus provide new information to support the clinical use of lutein to treat RP.

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Additional files:
Additional Figure 1: *Structure of rd10 retina at P16 is relative normal.*
Additional Figure 2: *Effect of lutein on the expression of PSD95 in rd10 retina.*

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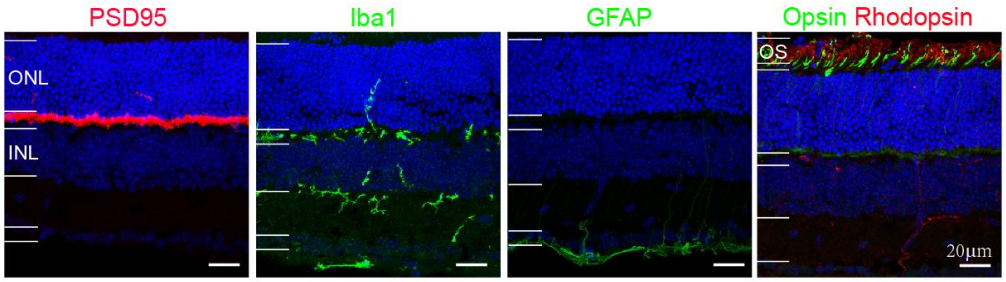
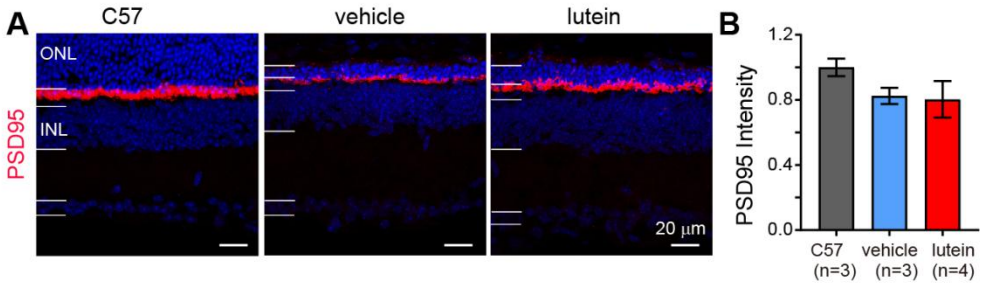


Additional Figure 1 Structure of rd10 retina at P16 is relative normal.

Retina was immunostained with PSD95 (red, Alexa Fluor-594), Iba1 (green: Alexa Fluor-488), GFAP (green: Alexa Fluor-488), Opsin (green: Alexa Fluor-488), and rhodopsin (red, Alexa Fluor-594). The ONL was thick at P16, and photoreceptors showed normal outer segment. But there is an increased expression of Iba1 and GFAP, indicating reactive gliosis is happening. Scale bars: 20 μm. GFAP: glial fibrillary acidic protein; Iba1: ionized calcium binding adaptor molecule 1; INL: inner nuclei layer; ONL: outer nuclei layer; OS: outer segment; P16: postnatal day 16; PSD95: postsynaptic density protein 95; rd10: Pde6b rd10 mice.



Additional Figure 2 Effect of lutein on the expression of PSD95 in rd10 retina.

(A) Immunostaning of PSD95 (red, Alexa Fluor-594) in retinal slices. The expression of PSD95 decreased in rd10 retina, lutein hardly improved it. Scale bars: 20 μm. (B) Average fluorescent intensity of PSD95, normalized to that of C57. Data are shown as mean ± SEM, and were analyzed by Student's t-test. INL: inner nuclei layer; ONL: outer nuclei layer; PSD95: postsynaptic density protein 95; rd10: Pde6b rd10 mice.