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Intraperitoneal injection of (−)-Epigallocatechin-3-gallate protects against light-induced photoreceptor degeneration in the mouse retina

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Purpose: (−)-epigallocatechin-3-gallate (EGCG), a major catechin component of green tea, is reported to delay or prevent certain forms of cancer, arthritis, cardiovascular disease, and neurodegenerative disorders. In this study, we determined if systemically administered EGCG could protect the retina against light damage (LD) in mice.
Methods: BALB/cJ mice were treated with either EGCG or saline via intraperitoneal (IP) injection, and then placed under constant cool white light-emitting diode (LED) light (10,000 lux) for 5 h. Retinal structure and function were evaluated using optical coherence tomography (OCT), histology, and electroretinography (ERG) 7 days after LD. In addition, the mRNAs of several oxidative stress genes were quantified by qPCR before LD and 24 h after LD.
Results: OCT and photomicrographs of mouse retinas showed morphologic protection of photoreceptors. Mice in the EGCG group had significantly higher ERG amplitudes for all three wave types compared with mice in the saline control group, which indicated that EGCG protected retinal function. Furthermore, qPCR results showed that EGCG administration can increase the mRNA level of the antioxidant gene *Sod2* before LD and 24 h after LD.
Conclusions: The IP injection of EGCG attenuated the detrimental effects of bright light on the retinas of BALB/cJ mice by protecting the structure and function of the retina.

Age-related macular degeneration (AMD) is a common cause of irreversible and severe loss of vision. It is one of the major causes of blindness in developed countries, and has caused a significant social and economic burden [1]. The degeneration of photoreceptors is one of the main characteristics of atrophic AMD. The exact mechanism of AMD is not completely understood and the treatment methods are insufficient. Several studies have suggested that light exposure and oxidative stress play important roles in AMD [2,3]. Because the light damage (LD) mouse model involves oxidative stress, it can be used to test antioxidants for their retinal protective ability [4,5].

The multifunctional properties of (−)-epigallocatechin-3-gallate (EGCG), a major catechin component of green tea, include antioxidant activity and anti-inflammatory, anti-apoptotic, and neuroprotective effects. EGCG has been reported to delay or prevent certain forms of cancer, cardiovascular disease, and neurodegenerative disorders [6-10]. Chu et al. [11] found that EGCG can cross the blood–brain barrier and blood–retinal barrier, which suggests that systemic EGCG has the potential to protect against retinal degenerative or neurodegenerative diseases.

In the field of ophthalmology, Chen et al. [12] showed that the intraperitoneal (IP) injection of EGCG protected retinal ganglion cells against N-methyl-D-aspartate (NMDA)-induced death. Silva et al. [13] found that green tea is neuroprotective in diabetic retinopathy (DR) via an antioxidant mechanism. Zhang et al. [14] demonstrated that intravitreal and systemic injections of EGCG provide protection to retinal neurons against oxidative stress and ischemia/reperfusion injury. In the present study, we determined if an IP injection of EGCG protects the retina against LD in mice, and also determined the effect of EGCG on antioxidant genes before and after LD.

METHODS

Animal care: All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Experimental procedures were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Adult male albino BALB/cJ mice (20–30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Thirty mice that were 8 weeks of age were used in the

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study. All mice were fed a standard laboratory diet and given free access to water; they were maintained in a temperature-controlled room at 21–23 °C under dim cyclic light (12 h: 12 h light-dark cycle) during the experiments. The mice were given either LD or no light damage (NLD). Some mice were also given either IP saline or IP EGCG.

Drug administration and LD paradigm: EGCG (Sigma-Aldrich Corp., E4268, St. Louis, MO) was dissolved in 0.9% sodium chloride injection (10 mg/ml). EGCG was freshly prepared, and mice were given an IP injection at a dose of 50 mg/kg. An equal volume of 0.9% sodium chloride was used as a control. EGCG was given daily for 7 days to some mice before they were sacrificed. For some mice, daily IP injections of EGCG were given for 7 days before LD and also after LD until the mice were sacrificed.

Mice in the LD group were exposed to 10,000 lux of cool white LED light in a well-ventilated room continuously for 5 h. After exposure to the light, mice were either sacrificed or placed in a normal light/dark cycle for 24 h or for 7 days. Eyes were enucleated immediately after sacrifice 24 h after LD for analysis by quantitative PCR (qPCR), and 7 days following LD for morphologic analysis.

Morphologic analysis: Eye cups were made by dissecting away the cornea and lens, then were dehydrated in increasing concentrations of ethanol for 30min periods; the concentrations used were: 50%, 75%, 95% and 100%. Then, eye cups were immersed in infiltration solution (0.625 g Benzoyl Peroxide in 50 ml of JB-4 solution A, Polysciences Inc., Warrington, PA) overnight. A mixture of 1ml of JB-4 solution B and 25 ml of infiltration solution was used for embedding. For standard histology, 3 µm thick plastic sections were cut in the sagittal plane and were stained with toluidine blue by incubation of the sections in 1% toluidine blue O and 1% sodium tetraborate decahydrate (Sigma-Aldrich Corp., St. Louis, MO) for 5 seconds. Stained sections were observed and photographed using brightfield illumination (model TE300; Nikon, Inc., Tokyo, Japan). The number of nuclei per column of outer nuclear layer (ONL) photoreceptors was counted in triplicate at 200 µm intervals from the optic nerve (ON) to 2,000 µm from the ON, using image analysis software (ImagePro Plus4.1; Media Cybernetics, Rockville, MD) to calculate the distances from manually set lengths.

Optical coherence tomography (OCT) imaging: Mice were anesthetized with a cocktail containing (in mg/kg bodyweight): 80 ketamine (Par Pharmaceutical, Spring Valley, NY), 10 xylazine (LIoyd Inc., Shenandoah, IA), and acepromazine (Boehringer Ingelheim Vetmedica, Inc.St. Joseph, MO). One drop of 1% tropicamide ophthalmic solution (Mydriacil; Alconox, New York, NY) was administrated to the eyes before examination. As described previously [15], we performed OCT using a Bioptigen imager (Bioptigen INC., Durham, NC). One standard horizontal line scan was used, which was approximately 0.4 mm above the superior edge of the optic disc. Corresponding ONL thicknesses for the different groups were compared at the same location.

Electroretinography (ERG): Flash electroretinograms were recorded from both eyes of each mouse. The electroretinography (ERG) recordings followed procedures described previously [16,17]. Briefly, mice were dark-adapted overnight and then anesthetized with a cocktail containing (in mg/kg bodyweight): 80 ketamine, 10 xylazine, and 2 acepromazine. The pupils were dilated with 1% tropicamide saline solution (Mydriacil; Alconox, New York, NY), and the mouse was placed on a stage maintained at 37 °C. Two miniature cups made of ultraviolet (UV)-transparent plastic with embedded platinum wires that served as recording electrodes were placed in contact with the corneas. A platinum wire loop placed in the mouth served as the reference and ground electrode. The ERGs were then recorded (Espion Electrophysiology System; Diagnosys LLC, Lowell, MA). The apparatus was modified by the manufacturer for experiments with mice by substituting LEDs with a maximum emission wavelength of 365 nm for standard blue LEDs. The stage was positioned in such a way that the mouse's head was located inside the stimulator (ColorDome; Diagnosys LLC), thus ensuring full-field uniform illumination. Methods for light stimulation and calibration of light stimuli have been described previously. Briefly, the flash intensities for recordings of rod a- and b-waves were 500 and 0.01 scot cd m^{-2} s delivered by the white xenon flash and green (maximum emission wavelength of 510 nm) LED, respectively. The white flash intensity of the cone b-wave was 500 scot cd m^{-2} s with a rod-suppressing steady green background of 30 scot cd m^{-2} s. The intensities of the UV flashes used for cone stimulation in the current study were (rounded to tens) 1650 photons/μm^2, 1780 photons/μm^2, and 2820 photons/μm^2.

Real-time qPCR: Gene expression was analyzed in the neurosensory retina (NSR) samples by quantitative RT–PCR as we have described [18]. Briefly, samples were obtained from IP EGCG and saline-treated mice with NLD, IP EGCG and saline-treated mice with LD at the indicated time points, and no IP NLD mice. The probes used were superoxide dismutase 1 (*Sod1*, Mm01700393_g1), superoxide dismutase 2 (*Sod2*, Mm01313000_m1), and glutathione peroxidase 4 (*Gpx4*, Mm00515041_m1); glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, Mm99999915_g1) was used as an endogenous control. Real-time qPCR (TaqMan; Applied Biosystems, Carlsbad, CA) was performed on a sequence detection system

(Prism Model 7500; Applied Biosystems) using the delta delta cycle threshold ($^{\Delta\Delta}C_T$) method, which describes the relative fold change. The amount of target mRNA was compared among the groups of interest. All reactions were performed in biological and technical triplicates; in other words, there were three qPCR replicates per biological sample.

Statistical analysis: The mean and the standard error were calculated for each comparison group. Statistical analysis was performed using the Student's two-sided unpaired *t* test for two group comparisons. We used one-way ANOVA with post hoc pairwise comparisons using the Tukey method to correct for multiple comparisons. All statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

RESULTS

OCT in vivo retinal imaging 7 days after LD: OCT imaging provided in vivo images showing the thickness and reflectivity of the ONL region (Figure 1). Images from approximately the same location in the superior retina were compared. In mice not exposed to LD, OCT images showed a region of low reflectivity corresponding to the ONL (Figure 1A). At 7 days after LD, OCT images showed severe thinning in the ONL in mice treated with saline, combined with a relatively increased reflectivity of the choroid (Figure 1B). EGCG-treated mice had a relatively thicker ONL, which was similar to the images of the retinas in mice without LD (Figure 1C). A quantitative comparison of the thickness of the ONL in the IP and no IP groups showed a loss of nearly 70% thickness in the IP saline LD group compared with the no IP NLD group ($p < 0.01$), but only a 14% thickness loss in the IP EGCG LD group ($p < 0.01$; Figure 1D).

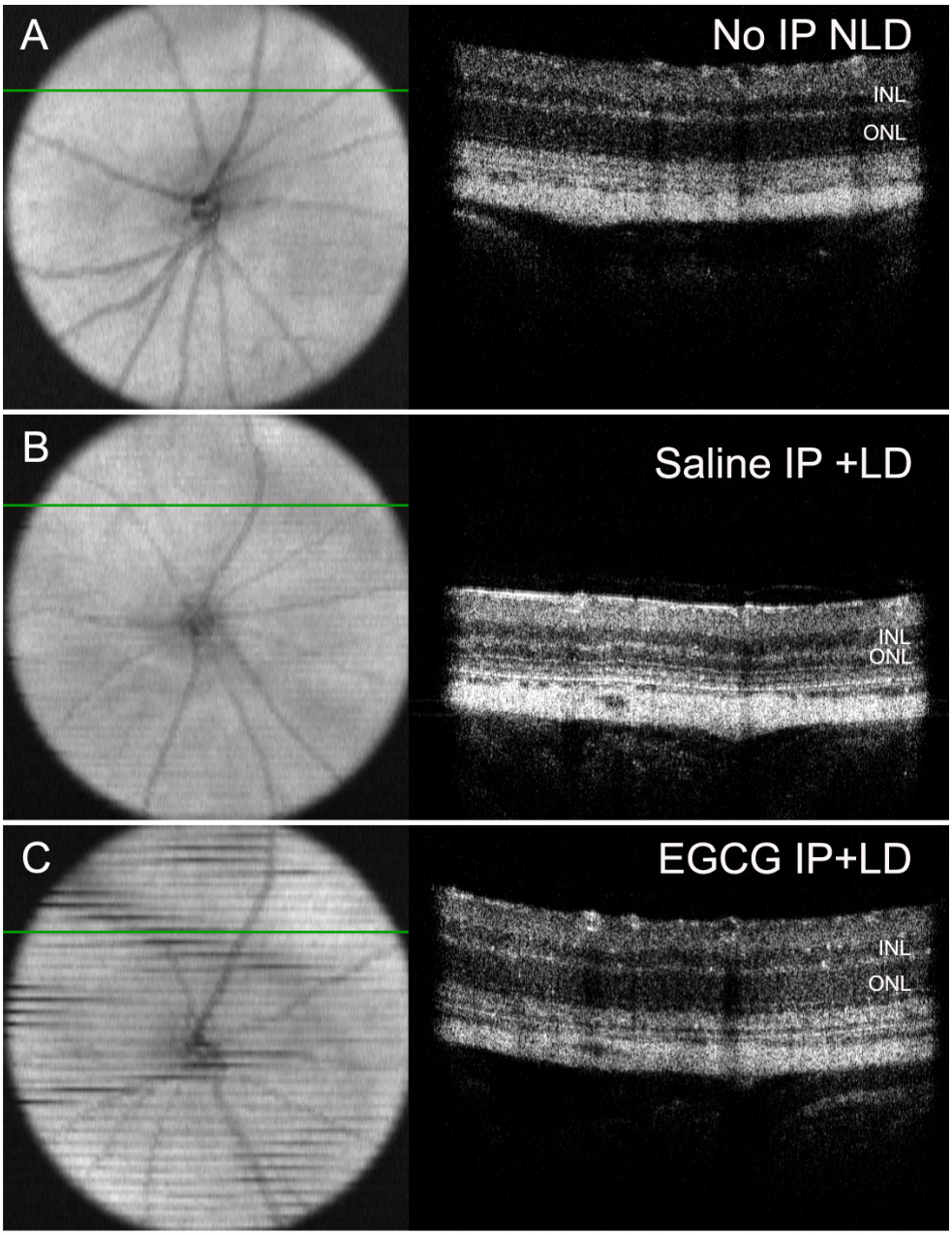
Preservation of photoreceptor morphology by EGCG: OCT images indicated the morphological preservation of retinal structure. To confirm the preservation of the photoreceptors by EGCG, a morphometric analysis (Figure 2) was performed 7 days after LD; the numbers of photoreceptor nuclei were counted in sagittal sections through the ONL ($n = 4$). The retinal morphology in the saline control group showed severe loss of ONL nuclei after LD. The retinal morphology in the EGCG group (Figure 2C) was similar to that of the group with NLD (Figure 2A). The mice treated with EGCG had a thicker ONL and better preserved photoreceptor inner and outer segments compared with LD mice that received IP saline (Figure 2B). Figure 2D-F show high magnification images of Figure 2A-C. The graph of the thickness of the ONL showed that the most severely damaged part of the retina was supero-central, although this region was well protected by the administration of EGCG. For example, 600 µm from the ON, the average ONL nuclei number in the NLD group was 9.5; after LD, the number of nuclei in the saline control group decreased to 2.4, while the average number was 6.7 in the EGCG treatment group (Figure 2G).

Retinal function assessed by ERG 7 days after LD: The maximum ERG amplitude of the cone-b, rod-a, and rod-b waves were measured. The amplitudes of the three waves were significantly attenuated by the LD treatment. However, EGCG protected against this attenuation. All three wave amplitudes were significantly higher in the EGCG-treated LD mice compared with the saline-treated LD controls. Quantitative comparison of the maximum ERG amplitudes showed a nearly 59% (rod b), 72% (rod a), and 70% (cone b) decrease in the IP saline LD group compared with NLD group: $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively. There was only a 4% (rod b), 32% (rod a), and 7% (cone b) decrease in the IP EGCG LD group: $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively (Figure 3A-C). Representative ERG recordings are shown in Figure 3D-F.

EGCG affected expression of antioxidant genes: To investigate the effect of EGCG on photoreceptor protection, antioxidant gene expression was analyzed in the NSR samples obtained from EGCG and saline-treated mice with and without LD. In the NSR samples from mice with NLD, IP EGCG treatment led to higher *Sod2* (1.1 fold, $p < 0.01$; Figure 4B). In addition, *Sod2* mRNA levels in the NSR of EGCG-treated mice were upregulated significantly 24 h after LD compared with IP saline LD controls (1.1 fold, $p < 0.05$). There was no significant difference between the EGCG and the saline groups for *Sod 1* and *Gpx 4* mRNA levels ($p > 0.05$; Figure 4A,C).

DISCUSSION

Prior studies demonstrated that EGCG has beneficial properties in several disease models [19]. In this study, we investigated whether IP injection of EGCG can protect the photoreceptors against light-induced damage in BALB/cJ mice. In our study, one week after light exposure, morphologic analysis demonstrated that EGCG administration can effectively protect the retina against damage caused by light exposure, which was evidenced by relative thicker ONL in OCT images, photoreceptor morphology in plastic sections, and quantification of photoreceptor nuclei. Furthermore, ERG results also indicated that retinal function was well protected by EGCG. Gene expression changes, while small, indicated that EGCG can upregulate the antioxidant gene *Sod2* before LD, which, in addition to the direct antioxidant function of EGCG, could contribute to retinal protection.



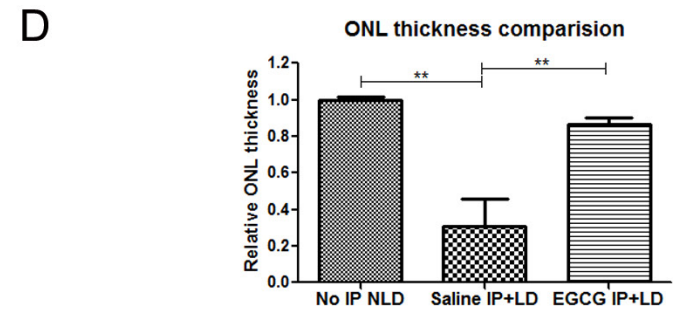


Figure 1. OCT images of mouse retinas. **A**: The retina of a mouse that received no IP injection and NLD. **B**: The retina of a mouse that received IP saline and LD showed ONL thinning 7 days after LD. **C**: The retina of a mouse that received IP EGCG and LD showed nearly normal thickness ONL 7 days after LD. **D**: A comparison of relative ONL thickness: No IP NLD (n = 4); saline IP + LD (n = 4), and EGCG IP + LD (n = 4). Numbers represent mean values (± SEM). ** $p < 0.01$. ONL = outer nuclear layer, INL = inner nuclear layer, and SEM = standard error of the mean.

Prior studies have shown that a higher dose of EGCG may result in hepatic toxicity and pro-oxidation [11,20,21]. Moreover, the tolerance for EGCG varies among the different mouse strains [22]. We chose the 50 mg/kg dose because this concentration has been shown to be efficacious in several other rodent models [23,24].

Costa et al. [25] found that orally administered EGCG attenuated light-induced photoreceptor damage in albino rats as shown by the protection against the reduction of the a- and b-waves and photoreceptor specific mRNAs/proteins caused by light. EGCG also significantly reduced the light-induced increase in some apoptosis-associated protein levels, such as caspase-3, B-cell lymphoma 2-associated death promoter (Bad), and poly ADP-ribose polymerase (PARP), although it did not modify the level of B-cell lymphoma 2 (Bcl-2) [25]. Our study complements that study by providing different morphological, functional, and molecular evidence of the protective effects of EGCG in a different animal species. We also provide evidence of antioxidant gene upregulation by EGCG. Together, these reports illustrate the protective properties of EGCG against light-induced retinal degeneration.

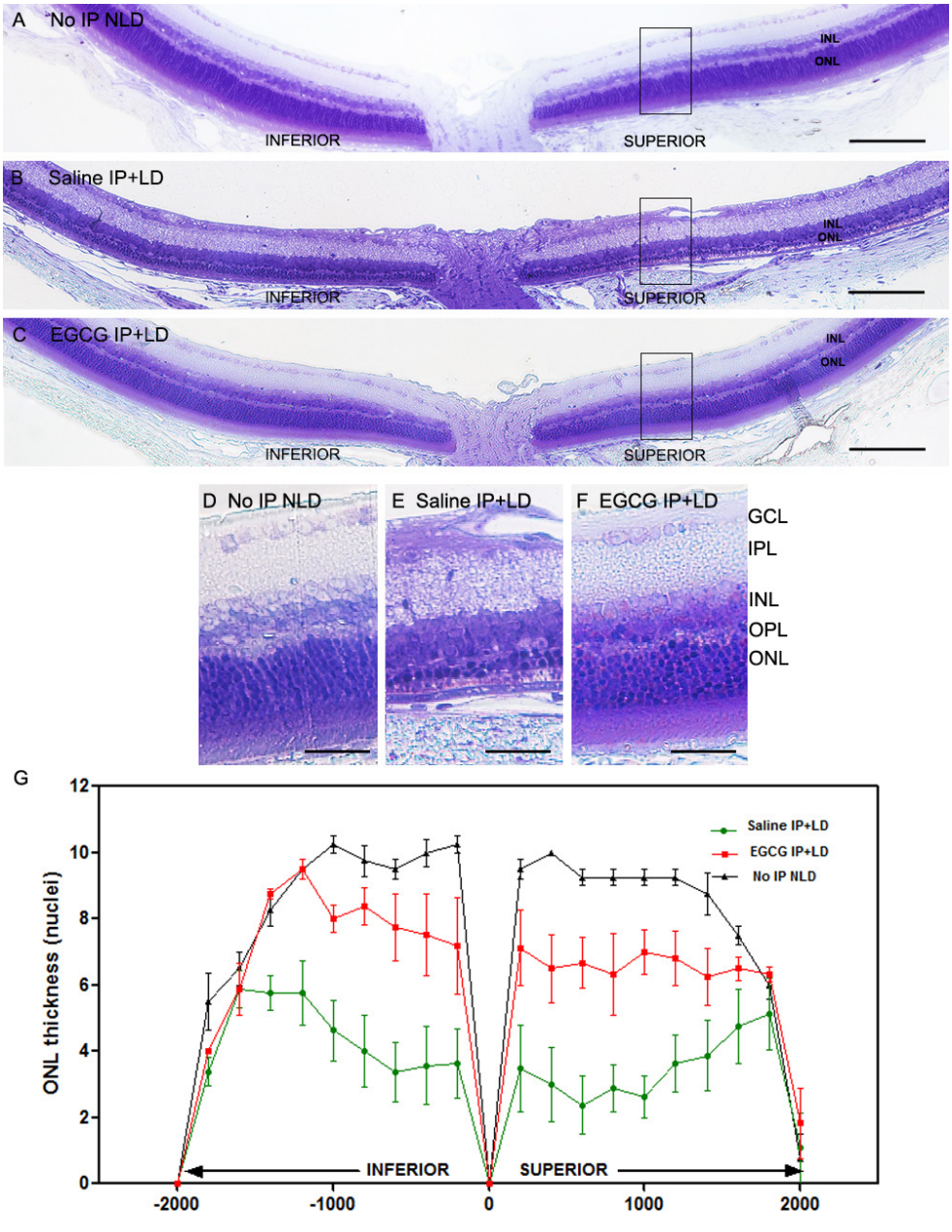


Figure 2. Photomicrographs of plastic sections of mouse retinas 7 days after LD. The sagittal plane sections pass through the optic nerve. **A**: Posterior retina from a mouse that received NLD. **B**: The retina of a mouse in the saline plus LD group showed ONL thinning 7 days after LD. **C**: In the retina of a mouse in the EGCG plus LD group, the photoreceptor nuclei in the ONL were well protected from light-induced damage, especially in the superior area (scale bars = 100 µm). **D**-**F**: High magnification images of **A**-**C** (scale bars = 25 µm). **G**: Plots of the thickness of the ONL, measured in numbers of photoreceptor nuclei per column. Measurements were made in triplicate at intervals of 200 µm from the optic nerve, which is reference point 0 on the x-axis. No IP NLD (n = 4, black), saline IP + LD (n = 4, green), and EGCG IP + LD (n = 4, red). Numbers represent mean values (± SEM). GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, and SEM = standard error of the mean.

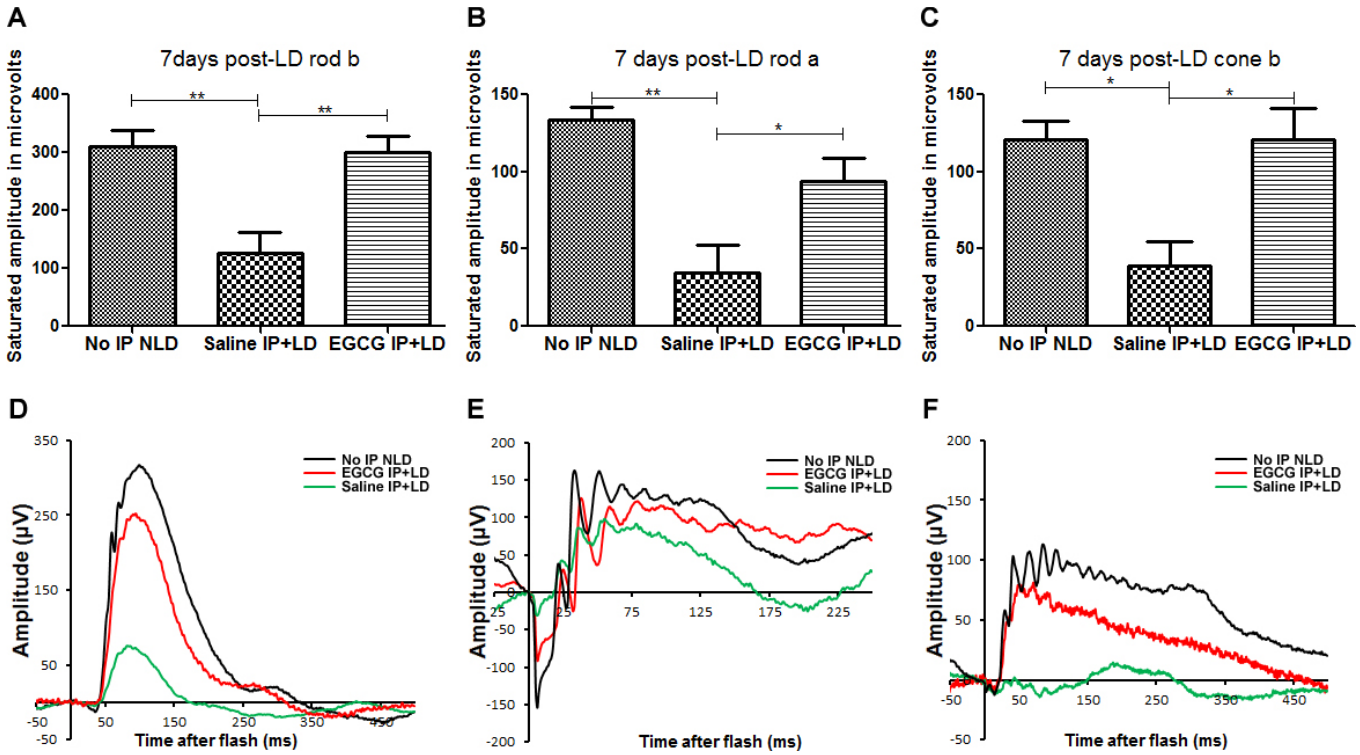


Figure 3. Full-field ERG responses of mice in the EGCG-treated and saline control groups 7 days after light exposure. The maximum amplitude ERG responses were compared. The amplitudes of three wave types were significantly higher in the LD EGCG-treated group compared with the LD saline control group (**A**-**C**). Numbers represent mean values (± SEM). * $p < 0.05$ and ** $p < 0.01$. Representative ERG traces from each group were plotted in **D** (rod b), **E** (rod a), and **F** (cone b).

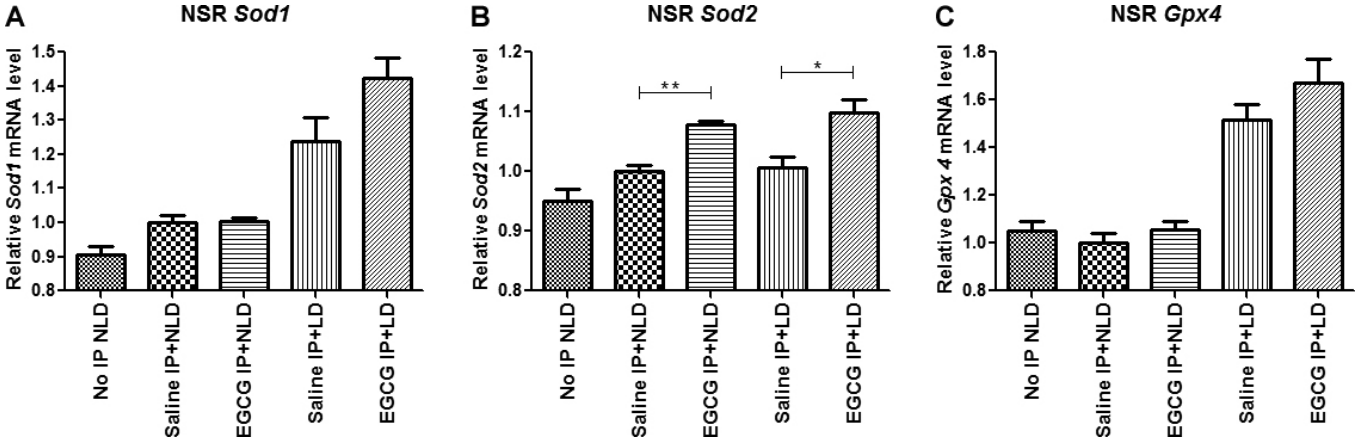


Figure 4. Graphs showing relative antioxidant mRNA levels measured by qPCR. Relative mRNA levels were measured after mice received IP injections for 7 days but no LD, or 24 h after LD. *Sod1* and *Gpx4* mRNA levels were not significantly changed in the neural retinas of mice in the daily IP EGCG group versus mice in the saline groups (**A**, **C**). *Sod2* mRNA levels in the neural retina increased after 7 days of daily IP EGCG administration compared with the saline control. *Sod2* mRNA levels in the NSR of mice receiving daily IP EGCG versus IP saline were upregulated 24 h after LD (**B**).The mRNA levels for the indicated genes in the retinas from mice in the no IP NLD (n = 4), saline IP + NLD (n = 5), EGCG IP + NLD (n = 5), saline IP + LD (n = 4), and EGCG IP + LD (n = 4) groups are displayed as mean values (± SEM). * $p < 0.05$ and** $p < 0.01$. In **A**, (Saline IP+LD) vs (EGCG IP+LD) (Unpaired t test, p= 0.0914); in **C**, (Saline IP+NLD) vs (EGCG IP+NLD; Unpaired t test, p= 0.3101), Saline IP+LD vs EGCG IP+LD (Unpaired t test, p= 0.2304).

Prior studies on cancer, inflammatory disease, and neurodegenerative disease focused on the antioxidant, radical scavenging, metal chelating, anti-apoptotic, and anti-inflammatory properties of EGCG [19,26]. The antioxidant function is one of the important potential therapeutic activities of EGCG for the retina. An in vitro study by Cia et al. [27] showed that EGCG pretreatment can protect primary rat retinal pigment epithelium (RPE) cells from hydrogen peroxide (H_2O_2)-induced death. Furthermore, several in vivo studies provided evidence supporting the antioxidant properties of EGCG in neurodegeneration or retinal degeneration promoted by different oxidants [28,29]. A study from Yang et al. [30] found that retinal lesions generated by sodium iodate-induced oxidative stress in the adult rat retina were ameliorated by tea extract and its catechin constituents containing EGCG. Retinal protective effects were associated with diminished oxidative stress indicated by reduced levels of 8-iso-prostaglandin F2α in the retina [30].

Due to its chemical structure, EGCG is a radical scavenger and metal chelator, which enables it to execute antioxidant effects directly [31-33]. Some studies demonstrated that EGCG can induce endogenous antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase. EGCG upregulated the gene expression or elevated the bioactivities of these antioxidants. Thus, EGCG could directly or indirectly regulate the antioxidant levels or activity to reduce oxidative stress [34-36]. In our study, elevated *Sod2* mRNA levels were detected by qPCR in the retina one week after EGCG administration with NLD and 24 h after LD. This indicates that the retinal protective effect may be in part from the EGCG-induced endogenous antioxidants. In conclusion, our data demonstrate the protective effect of EGCG in the murine LD model. The mechanism may be due to the multiple biological and chemical activities of EGCG, including its antioxidative function. Because EGCG is a major catechin in green tea, additional studies on the potential retinal protective effects of drinking green tea or taking EGCG supplements are warranted.

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