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Published in:
Experimental Eye Research

DOI:
10.1016/j.exer.2016.04.015

A Model of Progressive Photo-oxidative Degeneration and Inflammation in the Pigmented C57BL/6J Mouse Retina

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ABSTRACT

Light–induced degeneration in rodent retinas is an established model for of retinal degeneration, including the roles of oxidative stress and neuroinflammatory activity. In these models, photoreceptor death is elicited via photo-oxidative stress, and is exacerbated by recruitment of subretinal macrophages and activation of immune pathways including complement propagation. Existing light damage models have relied heavily on albino rodents, and mostly using acute light stimuli. These albino models have proven valuable in uncovering the pathogenic mechanisms of such pathways in the context of retinal disease. However, their inherent albinism hinders comparability to normal retinal physiology, and also makes gene technology analyses time-consuming due to the predominance of the pigmented mouse strains in these applications. In this study, we characterise a new light damage model utilising C57BL/6J mice over a 7 day period of chronic light exposure. We use high-efficiency LED technology to deliver a sustained intensity of 100k lux with negligible modulation of ambient temperature. We show that in the C57BL/6J mouse, chronic light exposure elicits the cardinal features of light damage including photoreceptor degeneration, atrophy of the choriocapillaris, decreased retinal function and increases in oxidative stress markers 4-HNE and 8-OHG, which emerge progressively over the 7 day period of exposure. These changes are accompanied by robust recruitment of IBA1+ and F4/80+ macrophages to the ONL and subretinal space, followed the strong up-regulation of monocyte-chemoattractants *Ccl2, Ccl3,* and *Ccl12,* as well as increases in expression of complement component *C3*. These findings are in agreement with prior damage models conducted in albino rodents such as Balb/c mice, and support the use of this new model in further investigating the causative features of oxidative stress and inflammation in retinal disease.

1 INTRODUCTION

The capacity for exposure to bright light to induce retinal degeneration has been known in a laboratory context for nearly 50 years. A seminal study by Noell (1966) demonstrated that exposure to light of a sufficient intensity and duration induces degeneration of photoreceptors and the RPE, resulting in severe vision loss in rats (Noell et al., 1966). Since this initial discovery, a vast amount of literature has been generated regarding the histological and functional implications of exposure to bright light on the retina (reviewed in (Glickman, 2002; Organisciak and Vaughan, 2009; Wu et al., 2006)). Light-induced degeneration induces photoreceptor apoptosis through oxidative damage in the photoreceptor outer segments, which stems from excessive photo-bleaching of the rhodopsin chromophore (Grimm et al., 2000; Grimm et al., 2001; Hao et al., 2002; Wenzel et al., 2005; Yamashita et al., 1992). These features are accompanied by atrophy of the retinal pigment epithelium (RPE) and choriocapillaris (Best et al., 1997; Marc et al., 2008; Rutar et al., 2010), as well as the induction of inflammatory responses such as macrophage recruitment (Langmann, 2007; Ni et al., 2008; Rutar et al., 2012b) and complement activation (Karlstetter et al., 2014; Rohrer et al., 2007; Rutar et al., 2011a).

Light damage in albino rodents has become a well-established model in the study of outer retinal degeneration, where it affords high reproducibility, rapid induction, ease of use, and flexibility through modulation of light duration and intensity (reviewed in (Wenzel et al., 2005)). Many investigations – including our own – have utilised the model to study of immunological responses including subretinal macrophage recruitment (Gordon et al., 2002; Joly et al., 2009; Levy et al., 2015; Rutar et al., 2015; Rutar et al., 2011b; Rutar et al., 2012b; Sennlaub et al., 2013), chemokine signalling, and complement activation (Karlstetter et al., 2014; Rohrer et al., 2007; Rutar et al., 2011a), all of which are implicated in the propagation

of inflammation in human diseases, including age-related macular degeneration (AMD) (Anderson et al., 2010; Penfold et al., 1984; Penfold et al., 1985; Penfold et al., 2001).

Despite the advances gleaned from these light-damage models, a recognised disadvantage is the reliance on albino strains. Albino rodents have been preferred because the lack of melanin means that retinal damage is elicited relatively easily (Sanyal and Zeilmaker, 1988; Zhu et al., 2010). Pigmented mouse strains such as C57Bl/6 also commonly possess the Leu450Met polymorphism in the *Rpe65* gene, which slows its recycling of rhodopsin chromophores and further reduces susceptibility to light damage. (Grimm et al., 2000; Wenzel et al., 2005; Wenzel et al., 2001). Although convenient for eliciting damage, albinism induces alterations in retinal physiology and other abnormalities including delayed neurogenesis and altered retinal ganglion cell projections (RGC) that may confound interpretations of findings (Bhansali et al., 2014; Rachel et al., 2002; Roffler-Tarlov et al., 2013). Moreover, the prevalence of the C57BL/6J background among knockout strains necessitates time consuming backcrossing onto albino mice strains (such as BALB/c) in order for them to be utilised in current light damage paradigms.

Here, we report the development of a new light damage apparatus using high efficiency LED technology to induce chronic retinal degeneration in the pigmented C57BL/6J retinas. We find substantial degeneration of the outer retina after 7 days of light exposure, correlating with robust recruitment of subretinal microglia/macrophages, and up-regulation of potent inflammatory and oxidative damage markers, which are known to be associated with retinal inflammation, including AMD. With the use of pigmented mice, this model is more closely aligned with human physiology and greatly cuts down on the time frame of knockout experiments which may offer clues to mechanisms that underpin retinal disease.

2 METHODS

2.1 Animal Experimentation

All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and approved by the Australian National University (ANU) Animal Experimentation Ethics Committee (Application ID: 2014/56). A colony of C57BL/6J mice used for the experiments, and were born and raised in a 12 hrs light/dark cycle of 5 lux with free access to food and water. For rigorousness the colony was genotyped for presence of either the *Rpe65*^{450Met} polymorphism or the deleterious *Crb1*^{Rd8} mutation using previously published primer sets (Kim et al., 2004; Mattapallil et al., 2012); sequencing for these was conducted at the ACRF Biomolecular Resource Facility, ANU. As expected, our C57BL/6J mice possessed the *Rpe65*^{450Met} polymorphism but were free of the Rd8 mutation.

Age-matched adult mice (8-10 weeks) were randomly assigned to light damage and dim-reared control (non-light damage) groups. Animals of the light damage group were continuously exposed to 100k lux white LED light for 1 day, 3, 5, and 7 days (Figure 1A). Pupils were dilated twice daily at 10am and 6pm with a single drop of 1% atropine sulfate (8.3 mg of atropine). Dim-reared control animals were also pupil dilated twice each day, however were returned to dim cyclic light (12 hrs light/dark, 5 lux).

2.2 Light Exposure Device

During bright-light exposure, animals were housed in plastic boxes (two per box), with free access to food and water. The floors of the cages were coated with a reflective Perspex surface and illuminated by a 100-W 65000k natural white LED (high CRI LED, Yuji, Beijing)

mounted 18 cm above the plastic boxes (Figure 1B-D). The LED light has an emission spectrum which more closely resembles daylight than halogen or incandescent bulbs (Figure 1E). Temperature in the cages was maintained at ~23±2 ^{o}C (Figure 1F) with a dual exhaust system to alleviate any heat generated from the LED, with one exhaust fan mounted next to the LED light source, and another one on the side of the cage (Figure 1A-C). In order to regulate accurate illumination each box was equipped with a dimmer and adjusted to 100k lux (Figure 1G) using a light meter data logging device (Extech HD450). Animals were provided with bedding, food and water during the time course of light exposure, and their behaviour was monitored daily.

2.3 Tissue collection and preparation

Animals were euthanized with CO_2. The left eye from each animal was enucleated with the superior surface marked, then immersed in 4% paraformaldehyde for 3 hrs, washed in 0.1M PBS and cryopreserved in 15% sucrose solution overnight. The eyes were embedded in OCT medium (Tissue Tek, Sakura, JP), and frozen in acetone with dry ice. Sections of 12 μm were cut in parasagittal plane (superior-inferior) at a vertical axis on Leica CM 1850 Cryostat, placed onto superfrost+ glass slides (ThermoScientific), dried overnight at 37°C and stored at -20°C. To enable comparison of histological sections across animals, only sections containing the optic nerve (ON) head were used for analysis. The retina from the right eye of each animal was excised through a corneal incision and placed in RNAlater solution (Ambion Biosystems, Austin, TX, USA), stored at 4°C overnight to allow penetration of the preservatives, and then stored at -80°C until required. Total RNA was extracted from the retinal samples in micro-scale according to the manufacturer's protocol (RNAqueous-Micro, Biosystems, Austin, TX, USA). The concentration and purity of the RNA samples was determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Only samples with a 260/280

ratio between 2.0 and 2.2 were considered for analysis. The quality of the RNA samples was examined with an RNA analyser (model 2100 Bioanalyser; Agilent Technologies, CA). The RNA samples were stored at -80°C (n=6 per experimental group).

2.4 TUNEL staining and quantification of TUNEL+ cells

TUNEL was used to quantify photoreceptor apoptosis during bright light exposure, and performed on retinal cryosections using a protocol published previously (Rutar et al., 2010). For negative control experiments, the terminal deoxynucleotidyl transferase (TdT) enzyme was omitted. A retinal histological section containing the optic nerve head (ON) was taken to compare all regions of the retina in the superior and inferior regions. In each region, the number of TUNEL+ cells was quantified in increments of 500 μm along the full length of the retina, starting at the optic nerve (ON) head and extending toward the periphery in the superior and inferior regions. In addition, the average number of TUNEL+ cells was calculated for the superior and inferior regions of each retina. To investigate the extent of cell death, the process of quantification was performed on two retinal sections, and calculated as the average for each animal. The values for the dim-reared control and each light damage group (LD 1 day, 3, 5 and 7 days) were compared by one-way ANOVA followed by Tukey's multiple comparison post-test (TUNEL counts across time points) and two-way ANOVA (TUNEL counts for eccentricity and superior-inferior analysis) followed by Sidak's multiple comparison post-test, with a n = 6 per experimental group.

2.5 ONL thickness measurement

Retinal cryosections were stained with the DNA-specific dye bisbenzimide (Calbiochem, US, 1:1000 dilution of a 10 mg/ml stock) to visualise the cellular layers. For each

section, digitised images of the entire retina were captured with a NIKON A1 Confocal Microscope (Nikon, USA) at 10x magnification. Thickness of the outer nuclear layer (ONL) for the dim-reared control and each light damage group was measured at increments of 600 μm along the full length of a retinal cryosection containing the optic nerve (ON) head. ONL thickness was calculated as the ratio of ONL thickness to the distance between the outer and inner limiting membrane (OLM-ILM). The total ONL ratio from each retina was the average of two retinal sections at the comparable locations. The procedure was repeated for six animals (n=6), and calculated as an average. Significance was determined using one-way ANOVA (ONL thickness ratio across time points) followed by Tukey's multiple comparison post-test and two-way ANOVA (eccentricity and superior-inferior analysis) followed by Sidak's multiple comparison post-test.

2.6 Immunohistochemistry

Adjacent sections to those used for TUNEL assay were selected for immunohistochemistry. Details of antibodies used are displayed in Table 1. Sections were incubated in 10% normal goat serum (Sigma Aldrich, Australia) for 1 hr at room temperature (RT), followed by overnight incubation in primary antibody at 4°C. Antigen retrieval was performed only for antibody IBA1 and F4/80. With this antibody, sections were incubated in Revealit-Ag Antigen Recovery Solution (ImmunoSolutions, QLD, Australia) for 1 hr at 37°C before the overnight incubation of the antibody. The sections were then washed in 0.1M PBS, and incubated with appropriate secondary antibody-AlexaFluor 488 or 594 for 4 hrs at room temperature. For F4/80 and 8OHG, biotinylated secondary antibody was used for 2 hrs incubation followed by 1.5 hrs incubation of streptavidin-AlexaFluor 488 or 594 conjugates at room temperature. For 4-HNE, goat anti-rabbit polyclonal secondary antibody AlexaFluor 488 was used. Sections were then stained with bisbenzimide to identify cellular layers, and

coverslipped with Aqua-Poly/Mount (Polysciences, PA, USA). To control for non-specific binding the primary antibody was omitted from some sections. Visualisation of immunofluorescence and image acquisition was performed using the Nikon A1 Confocal Microscope.

2.7 Quantification of IBA1+ microglia

The number of IBA1+ cells was counted at 500 μm intervals across the retinal cryosections for both the superior and inferior regions (n=6 per experimental group). The average IBA1+ cell counts for the superior and inferior regions was analysed with two-way ANOVA followed by Sidak's multiple comparison. The total IBA1+ cells from each retina was the average of two retinal sections and analysed with one-way ANOVA followed by Tukey's multiple comparison post-test.

2.7 Quantitative Real-time Polymerase Chain Reaction

Following purification of RNA, cDNA was synthesized using Tetro cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's protocol. A 20 μl reaction mixture was used in conjunction with 1 ug RNA, 500 ng Oligo dT primer and 200U reverse transcriptase. Gene expression was measured using mouse specific TaqMan probes (ThermoScientific, MA, USA), as shown in Table 2. The TaqMan probes, cDNA and TaqMan Gene Expression Mastermix (ThermoScientific), were plated in a 384-well transparent plate. The amplification of each sample was performed in technical duplicates, carried out using a QuantStudio 12K Flex RT-PCR machine (ThermoScientific). Relative fold change was expressed as a percentage change compared to the dim-reared control and normalised to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Significance for each gene was determined using one-way ANOVA with Tukey's multiple comparison post-

test (n=6 per experimental group). For each analysis, differences with a $p < 0.05$ were considered statistically significant.

2.8 Measurement of retinal function using electroretinography (ERG)

Full-field scotopic ERGs were performed to assess the retinal function of dim-reared control and 7 days light damaged animals. Briefly, mice were dark adapted overnight, anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) and the pupils dilated with a single drop of 1% atropine sulfate (8.3 mg of atropine). A single- or twin-flash paradigm was used to elicit mixed (rod and cone) or isolated cone responses, respectively. Flash stimuli for mixed responses were provided by an LED-based system (FS-250A Enhanced Ganzfeld, Photometric Solutions International, Melbourne), over a stimulus intensity range of 6.3 log $cd \cdot s \cdot m^{-2}$ (range -4.4 - 1.9 log $cd \cdot s \cdot m^{-2}$). Interstimulus interval was increased from 2 s for the lowest intensities to 5 min for the highest intensities to allow complete recovery of the b-wave between stimuli. Isolated cone responses were obtained at 1.6 log $cd \cdot s \cdot m^{-2}$ following a rod-saturating stimulus of 1.9 log $cd \cdot s \cdot m^{-2}$ given 400 ms before the test stimulus. This short interval after a rod-saturating flash does not allow recovery of rod function, thereby revealing cone-only responses. The a-wave amplitude was measured from the baseline to the trough of the a-wave response and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Data are expressed as the mean wave amplitude ± SEM (μV). Two-way ANOVA, with Tukey's multiple comparisons Post-hoc test, was performed to compare the responses from control and light damaged mice over the flash stimulus range. The a-wave and b-wave data were fitted with a Naka-Rushton equation [$R/R_{max} = I/(I + K)$] using the Solver function in Excel (Microsoft Version 2013) to determine R_{max}

(maximum amplitude) and K (semisaturation constant) from the response amplitude (R) and the flash intensity (I) over the range of -4.4 to 1.9 log cd·s·m^{-2}. Statistics were performed using Prism (GraphPad Software V5; GraphPad Software, Inc., La Jolla, CA, USA) and either a 2-way ANOVA for mixed a-wave and b-wave and students t-test for cone b-wave. For each analysis, differences with a $p < 0.05$ were considered statistically significant.

3 RESULTS

3.1 Emergence of focal outer retinal atrophy and photoreceptor death following light damage

The effect of light exposure on photoreceptor cell death was investigated using TUNEL assay (Figure 2). No apoptotic cell bodies were found in the ONL following 1 day light exposure (Figure 2B). At 3 days' light exposure a number of TUNEL+ cell nuclei were detected in the ONL (Figure 2C) with increasing numbers detected at 5 days (Figure 2D) and 7 days' light exposure (Figure 2E). Both dim-reared control (Figure 2A) and negative control retinas (Figure 2F) showed no positive cell labelling in any retinal layers. Superior-inferior regional analysis showed that TUNEL+ nuclei were localised in the superior region at 5 and 7 days ($p < 0.05$, Figure 3A). Their distribution in the superior region was focal, showing a significant increase in the number of TUNEL+ cell nuclei from 0.5 mm eccentricity from the optic nerve and peaking at around 1 mm ($p < 0.05$, Figure 3B). In addition, the number of TUNEL+ cells was detected up to 2.0 mm in the superior retina ($p < 0.05$, Figure 3B), whereas in the inferior retina there was less photoreceptor cell death distributed over a less extensive region (0.5-1.5 mm).

The cumulative effects of photoreceptor cell death were examined by measuring ONL thickness over the light damage timecourse (Figure 4A-E). At 1 and 3 days' light exposure, there was no significant change in the ONL thickness (Figure 4A), but reduced ONL thickness was detected at 5 days, with a further reduction at 7 days. At both timepoints the ONL was significantly thinner than the dim-reared control ($p < 0.05$, Figure 4A-C). Regional analysis

demonstrated that ONL thickness in superior retina was significantly thinner than in inferior retina, after both 5 and 7 days exposure ($p < 0.05$, Figure 4D). At 5 days' light exposure the ONL was thinnest at 0.6 mm eccentricity from the ON head and by 7 days, the area of ONL thinning was larger, extending up to 1.8 mm from the ON head ($p < 0.05$, Figure 4E). In inferior retina, there was no significant change in ONL thickness across the eccentricity points.

3.2 Measurements of retinal function using electroretinography

The functional consequences of bright light exposure on the pigmented mouse retina were assessed using flash ERG (Figure 5). Representative traces of scotopic control and 7 day light damaged animals are show in Figure 5A. There was a clear change of retinal function in response to 7 day light damaged animals. Experimental light damaged mice showed a significant reduction in the a-wave response at flash intensities greater than -0.4 log cd.s.m^{-2} ($p < 0.0001$, Figure 5B) and of the b-wave response at flash intensities greater than -1.1 log cd.s.m^{-2} ($p < 0.0001$, Figure 5C). With the maximum stimulation intensity (1.9 log cd.s.m-^{2}), the amplitudes of both a- and b-waves in light damaged mice were significantly reduced to 22.6% (R_{max} Control 721 μV; light damage 163 μV) and 24.3% (R_{max} Control 1604 μV; light damage 389 μV) of the values in controls, respectively. The semisaturation constant of the a-wave was 0.002 log cd.s.m^{-2} for control and 0.39 log cd.s.m-^{2} for light damage, and b-wave was -0.61 log cd.s.m^{-2} for control and -1.13 log cd.s.m-^{2} for light damage. Using a twin-flash paradigm, we isolated the cone-derived waveform and found that cone-derived b-waves were significantly ($p < 0.05$) suppressed by light exposure to 30% of control values at 1.6 log cd.s.m^{-2} (Figure 6D).

3.3 Immunoreactivity for the stress marker GFAP during light exposure

Faint immunoreactivity of GFAP was detected in the ganglion cell layer (GCL) of the dim-reared control retina. At 1 day of light exposure, we observed a strong increase in GFAP

immunoreactivity in the processes of astrocytes and Müller cells. (Figure 6B, arrows). Stronger GFAP expression was seen at 3 days, and continued until more processes were labelled GFAP+ at 5 and 7 days. (Figure 6C-E, arrows).

3.4 Mobilisation of IBA1+ and F4/80+ microglial cells to the outer retina over the course of light damage

Recruitment of microglia/macrophages to the outer retina following light exposure was assessed using IBA1 immunolabelling (Figure 7A-H). In dim-reared controls, IBA1+ cells had a ramified morphology, and were localised to the inner and outer plexiform layers (Figure 7A). After 1 and 3 days' light exposure, the IBA1+ cells remained concentrated in the same retinal layers as the dim-reared control with no IBA1+ cells detected in the outer retina (Figure 7B-D). By 5 days, a substantial incursion of amoeboid IBA1+ cells into the photoreceptor layer and subretinal space was detected (Figure 7E-F, arrows). Amoeboid IBA1+ cells persisted in the outer retina throughout the light exposure timecourse (Figure 7G, arrows). Double labelling with antibody F4/80 confirmed the identity of IBA1+ cells as retinal microglia/macrophage (Figure 7I-K, arrows). Quantification of IBA1+ cells in outer retina is shown in Figure 7. The number of IBA1+ cells present in the outer retina was significantly increased at 5 days of light exposure, compared to dim-reared control and remained significantly different from control levels at 7 days ($p < 0.05$, Figure 7).

Comparison of IBA1+ cell numbers in superior and inferior retina shows that at 5 days' exposure the total numbers of IBA+ cells in superior retina were significantly higher than in inferior retina ($p < 0.05$, Figure 8A). Comparison of the distribution of recruited cells indicates that, compared to controls, the number of IBA1+ cells were substantially higher in superior retina between 0.5mm and 1.5 mm eccentricity, at 3, 5 and 7 days ($p < 0.05$, Figure 8B). In

inferior retina the numbers of recruited IBA1+ cells were significantly different from controls between 0.5 and 1.0 mm, at 5 and 7 days' light exposure ($p < 0.05$, Figure 8B).

3.5 Changes in immunoreactivity for 4-HNE and 8-OHG oxidative stress markers during light exposure

Oxidative stress following light exposure was assessed using antibodies to 4-HNE and 8-OHG immunolabelling (Figure 9 A-O). The control retina shows faint immunoreactivity to 4-HNE in inner/outer segments (IS/OS) of photoreceptors (Figure 9A), which is markedly increased at 1 and 3 days of light exposure when strong labelling both IS/OS and photoreceptor cell nuclei is detected (Figure 9B-C, F-G). Increased 4-HNE immunolabelling is detected in photoreceptor segments at both 5 and 7 days (Figure 9 D-E, H-I). Immunoreactivity for 8-OHG was intense in the GCL following a peak at 1 day light exposure and gradually returning to below baseline levels by 7 days (Figure 9J-O). Whilst the majority of the 8-OHG staining was seen in the GCL, light-exposed retinas for 1 day and 3 days showed some evidence of 8-OHG immunoreactivity in the photoreceptor layer that was not seen at other time points.

3.6 Gene expression changes in inflammation and oxidative stress- related markers over the light damage time course

Marked increases in chemokines (*Ccl2*, *Ccl3* and *Ccl12*) mRNA expression were detected after 1 day of light exposure, and persisted throughout the light exposure period. Peak expression of each of these chemokines was detected after 1 day light exposure ($p < 0.05$, Figure 10A-C).

A significant differential expression in complement component 3 (*C3*), compared to dim-reared controls, was also detected over the protracted time course ($p < 0.05$, Figure 10D), with expression peaking at 5 days ($p < 0.05$).

In addition, we detected significant differential expression of heme oxygenase 1 (*Hmox-1*) over the protracted time course ($p < 0.05$, Figure 10E). *Hmox-1* was substantially elevated upon 1 day of light exposure, followed by a decrease in the expression level at 3 days and a steady increase up to 7 days.

4 DISCUSSION

Despite the known resistance of C57BL/6J mice to light damage due to their pigmentation and possessing the *Rpe65* Leu450Met polymorphism, we demonstrate the efficiently of an LED-based model in inducing chronic outer-retinal degeneration in these mice over a 7 day period. In this new model we characterised outer-retinal degeneration from both histological and functional perspectives, which coincides with an exuberant induction of oxidative stress and inflammation. The changes we describe are comparable with those elicited in albino rat models, and likewise feature a focal lesion of degeneration and inflammation in superior retina (LaVail et al., 1987; Marc et al., 2008; Rohrer et al., 2011; Rutar et al., 2010).

Our new model was able to efficiently replicate the gross morphological changes that are a hallmark of light damage, including photoreceptor and RPE degeneration, atrophy of the adjoining choriocapillaris, and the focal predominance of these features in the superior retina (Hao et al., 2002; LaVail et al., 1987; Marc et al., 2008; Rapp et al., 1985; Rutar et al., 2010). A key difference in our new model, however, is that it necessitated a light stimulus that is up to 100-fold greater in intensity and around 2-7 times longer in duration than previous light-damage paradigms in albino rodents. While such parameters could usually risk inducing heat

stress and its confounding side-effects on retinal damage (Organisciak et al., 1995; Rinkoff et al., 1986), our use of high-efficiency LED technology and carefully-placed fans allowed us to safely maintain this intensity without any elevation in ambient temperature within the light boxes over the period of exposure.

Our findings are also a significant advancement on previous models using pigmented rodents, as these utilised incandescent or fluorescent light damage methodologies and thus yielded relatively small amounts of cell death (Hayes and Balkema, 1993; LaVail et al., 1987; Wenzel et al., 2001). Moreover, previous models in pigmented animals have mainly relied on acute stimuli ranging from only minutes-to-hours (Lansel et al., 1998; Marco-Gomariz et al., 2006) whereas our new model offers the advantage of inducing chronic retinal damage over a multi-day period. This protracted time course allows for enhanced temporal separation of the features of light-induced degeneration, and better scope for its use in studying pathogenic mechanisms of human conditions in which degeneration is progressive rather an acute (such as AMD).

We further describe the emergence of indicators of oxidative stress and inflammation in our new model, which have been not well-characterised in other pigmented light damage paradigms. Oxidative stress was evidenced by robust increases in immunoreactivity for the oxidative damage by-products 4-HNE and 8-OHG, and up-regulation of the oxidative stress response gene *Hmox-1*, which preceded the progressive onset of photoreceptor death. Inflammation was indicated by robust recruitment of both IBA1+ and F4/80+ microglia/macrophages to the ONL and subretinal space, coincident with increases in leukocyte-guiding chemokines *Ccl2, Ccl3,* and *Ccl12,* as well as the central complement component *C3*. The relatively late peak in C3 expression (5 days) coincides with peak microglial / macrophage numbers in the retina, suggesting that these cells have a significant influence on C3 accumulation in the retina, as described in the albino rat model (Rutar et al

2011a). Our findings compare favourably with the spatiotemporal emergence of these similar oxidative stress (Imai et al., 2010; Organisciak et al., 1999; Rutar et al., 2012a; Tanito et al., 2005) and inflammatory response (Chen et al., 2004; Rohrer et al., 2007; Rutar et al., 2015; Rutar et al., 2011a) observed in light-damage studies conducted with albino rodents, and thus support the use of our new model as an extension of these previous paradigms.

5 Conclusions

Models of light-induced retinal degeneration have been used extensively to study the roles of inflammation and oxidative stress, particularly in relation to human retinal diseases including AMD where such pathways are heavily implicated (Anderson et al., 2010; Hollyfield, 2010; Hollyfield et al., 2008; Karlstetter et al., 2010). While existing models using albino mice and rats have furthered our understanding of these factors, ongoing investigations using these paradigms are hindered by the inherent albinism. Our new model of chronic photo-oxidative degeneration in C57BL/6J mice overcomes a number of hurdles in existing models, including greater semblance to normal retinal physiology, chronic degeneration elicited via a sustained stimulus, and easier access to gene technology such as knockouts where the C57BL/6J strain is the predominant genetic background. Consequently, our new model has substantial value in furthering our understanding of the causative factors of photo-oxidative damage, as well as that of human dystrophies such as AMD.

Acknowledgments

Supported by the financial contributions of the National Health and Research Council (NHMRC – Grant 1049990, Dr. K. Valter and Prof. J Provis) and the Ophthalmic Research Institute of Australia (ORIA, Dr M. Rutar, Dr R. Natoli, & Dr T. Sandercoe - New Investigator

Grant 2014). We would also like to acknowledge the contributions of Joshua A. Chu-Tan and Nilisha Fernando for assisting with the electroretinograms and recording of temperature/light levels in the photo-oxidative experiments.

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Figure Legends

Figure 1. The light damage paradigm used for C57BL/6J mice. (A) Shows the light damage paradigm with animals either being exposed to 100k lux constant light or 5 lux cyclic light (12 hrs, light/dark) over a time course of 7 days. Each animal was administered a single drop of atropine in each eye twice daily in order to dilate the pupils. B-D shows the design of the light boxes including the array of boxes (B), the front view with hole for the water bottle (C) and the rear view with venting for reducing heat (D). The LEDs produce a spectrum of light that is similar to that of daylight (E). Data logging over a 7 day period showing stable recordings for both temperature (F, degrees Celsius (°C)) and light (G, kilolux (klx)).

Figure 2. Localisation and quantification of light-induced photoreceptor cell death using TUNEL immunolabelling on retinal cryosections. From 3 days there was a significant increase in cell death compared to controls (one-way ANOVA, * denotes $p < 0.05$ $n = 6$ per group). Cell death began to increase at 3 days in the ONL, continued at 5 days and was maximal at 7 days (* denotes $p < 0.05$, $n = 6$ per group). Representative images were captured for all time points including dim-reared control (A-F). TUNEL+ photoreceptor nuclei (red) appeared at 3 days and increased at 5 days and 7 days. Their

distribution was sparse but focal at the ONL at 3 days, and became dense at 5 days and 7 days (arrows). Negative control confirms that the TUNEL labelling was specific and not due to background staining. Scale bar: 100 μm. INL, inner nuclear layer, ONL, outer nuclear layer, GCL, ganglion cell layer.

Figure 3. Distribution of photoreceptor cell death in the ONL following light exposure. (A) The total TUNEL+ counts across the superior and inferior region of the retina showed that labelling was concentrated in the superior retina, with increasing severity at 5 days and 7 days (* denotes $p < 0.05$, n = 6 per group). (B) TUNEL+ counts across the whole length of retina demonstrated a focal localisation of TUNEL+ nuclei at 0.5 mm-2.0 mm from ON head in the superior retina at 3 days, 5 days and 7 days. Inferior retinal TUNEL was only significantly different from control at 0.5 mm-1.5 mm. There was no change in the photoreceptor cell death at 1 day of light exposure (* denotes $p < 0.05$, n =6 per group).

Figure 4. Effect of prolonged light exposure on the thickness of outer nuclear layer. (A) The ONL thickness ratio was significantly lower at 5 days and 7 days compared to the dim reared control (* denotes $p < 0.05$, n = 6 per group). (B-C) Representative retinal images showed a lesion site at 7 days of light exposure with a much thinner ONL thickness, compared to the retina of dim-reared control (white circle). (D) Superior-inferior regional analysis of ONL:Retina thickness ratio showed a substantially reduced ONL thickness in the superior region than the inferior at 5 and 7 days of light exposure (* denotes $p < 0.05$, n = 6 per group). (E) Eccentricity analysis of ONL:Retina thickness ratio across the length of retina demonstrated a significant reduction of ONL thickness occurred at 5 days, exclusively at 0.6mm superior to the ON head. The reduced ONL thickness persisted up to 1.8mm at 7 days indicating a superior specific l[illegible]sive cell death ($p < 0.05$, n =6 per group).

Figure 5. Full field scotopic flash electroretinogram recordings of control and 7 days light damaged animals. (A) Representative stimulus intensity-response traces of control (dim-reared) and 7 days light damaged animals recorded over a range of 6.3 log units ($cd.s.m^{-2}$). Mean intensity-response characteristics show reduced a-wave and b-wave amplitudes, which were quantified and fitted to a Naka-Rushton curve (B-C) across the intensity series. Both a-wave and b-wave amplitudes for the 7 day light damages animals were significantly different from dim-reared controls ($p<0.0001$, two-way ANOVA, n =3 per group). (D) Cone b-wave amplitudes in 7 days light damaged animals at a flash intensity of 1.6 log $cd.s.m^{-2}$ were significantly reduced compared to dim-reared control levels ($p<0.01$, students t-test, n =3 per group).

Figure 6. GFAP expression following continuous light exposure. Sections immunolabelled with GFAP (green, A-E), and with secondary antibody only negative c[illegible]F). (A) Normal GFAP expression in the superior retina from a dim-reared animal. B: Immunoreactivity for GFAP was evident in Müller cell processes (arrows). (C) GFAP immunoreactivity continued to increase. (D-E) Intense GFAP immunoreactivity featured heavily across the entire retina. Scale bar: 100μm (A-F).

Figure 7. IBA1-positive cells in light-exposed retina. (A) In normal retinas, scattered IBA1 positive ramified microglial cells were located in the outer plexiform layer and inner plexiform layer and ganglion cell layer, but not in the outer nuclear layer (ONL) or the subretinal space. (B-G) Infiltration of IBA1-positive microglial cells in the ONL was observed at 1 day, 3 days, 5 days and 7 days of light damage. Microglial cells began to migrate to the ONL at 3 days, with an elongated structure (arrow, D). By 5 days, these cells accumulated in the ONL with an amoeboid morphology (arrows, F), but decrease at 7 days of light damage with some still remaining in the subretinal space. (H) Negative control confirmed IBA1-positive staining was specific. (I-J) Co-localisation of IBA1 positive microglial cells

with F4/80 using double labelled immunohistochemistry. The scale bar represents 100 μm in all images, except F and D (F and D scale bar 50 μm). The histogram was the quantitative analysis of IBA-positive microglial cells in the ONL, showing a significant increase at 5 days and 7 days of light exposure. (* denotes $p < 0.05$, $n = 6$ per group)

Figure 8. Regional analysis of microglial cells in light-exposed retinas. (A) Superior-inferior regional analysis shows that the superior region of the ONL had more microglial cells than the inferior region (* denotes $p < 0.05$, $n = 6$ per group). (B) In the superior retina, localisation of microglial cells was significantly different from dim-reared control in a region from 0.5 mm to 1.5 mm at all time points, except for 1 day. The inferior retina was only significantly different from the dim-reared control at 0.5 mm to 1.0 mm at 5 and 7 days ($p < 0.05$, $n = 6$ per group).

Figure 9. Immunoreactivity of oxidative stress markers, 4-HNE and 8-OHG during light exposure periods. (A) Faint immunoreactivity of 4-HNE was seen in the IS/OS of dim-reared control retinas. (B, C) Intense 4-HNE immunoreactivity featured heavily in both the ONL and IS/OS upon 1 day of light exposure. 4-HNE immunoreactivity was found in the photoreceptor nuclei (arrows, B2). By 3 days, 4-HNE immunoreactivity was intense in the IS/OS, but reduced in the ONL (arrow). (D, E) Strong immunoreactivity of 4-HNE persisted and accumulated in the subretinal space at 5 and 7 days continuous light exposure. (F-I) Magnification of 4-HNE immunoreactivity in the subretinal region. (J) Control retinas had faint 8-OHG labelling in the GCL. (K, L) 8-OHG expression was intense at 1 day of light exposure in the GCL and persisted until 3 days. (M, N) 8-OHG immunoreactivity was less at 5 days and almost diminished in 7 days of light exposure. (O) Negative control confirmed positive staining seen at the GCL was not due to background. Scale bars: A-E, J-O: 100 μm, F-I: 50 μm.

Figure 10. Gene expression in light-exposed mouse retinas. Retinas of each light exposure period were extracted and analysed by RT-PCR for changes in expression of chemokines (*CCL3, CCL2, CCL12*), complement activation (*C3*) and oxidative stress (*Hmox-1*). (A-G) Expression trends for all genes were significantly different compared to dim-reared control ($p < 0.05$, $n = 6$ per group). (A-C) Chemokines, *CCL2, CCL3* and *CCL12* showed a peak at 1 day of light exposure. (E-F) C3 showed a peak at 5 days while *Hmox-1* showed an initial large increase at 1 day, decreasing at 3 days before gradually increasing up to 7 days ($p < 0.05$, $n = 6$ per group).