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More light components and less light damage on rats' eyes: evidence for the photobiomodulation and spectral opponency

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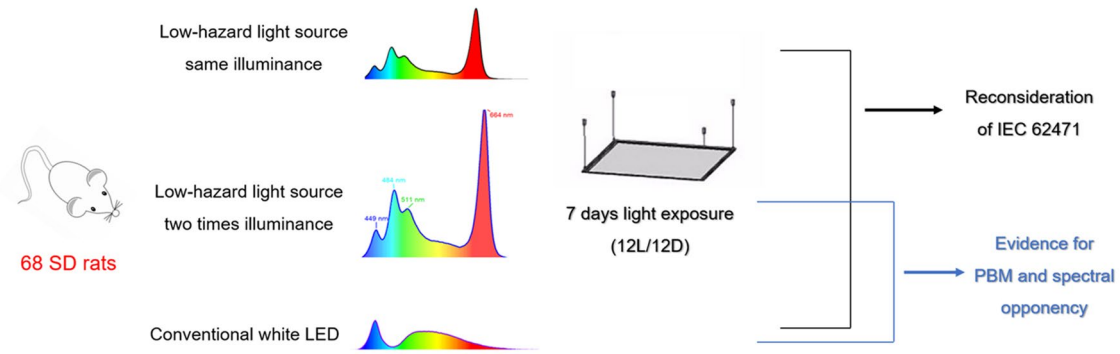
Abstract

The blue-light hazard (BLH) has raised concerns with the increasing applications of white light-emitting diodes (LEDs). Many researchers believed that the shorter wavelength or more light components generally resulted in more severe retinal damage. In this study, based on the conventional phosphor-coated white LED, we added azure (484 nm), cyan (511 nm), and red (664 nm) light to fabricate the low-hazard light source. The low-hazard light sources and conventional white LED illuminated 68 Sprague–Dawley (SD) rats for 7 days. Before and after light exposure, we measured the retinal function, thickness of retinal layers, and fundus photographs. The expression levels of autophagy-related proteins and the activities of oxidation-related biochemical indicators were also measured to investigate the mechanisms of damaging or protecting the retina. With the same correlated color temperature (CCT), the low-hazard light source results in significantly less damage on the retinal function and photoreceptors, even if it has two times illuminance and blue-light hazard-weighted irradiance (E_B) than conventional white LED. The results illustrated that E_B proposed by IEC 62471 could not exactly evaluate the light damage on rats' retinas. We also figured out that more light components could result in less light damage, which provided evidence for the photobiomodulation (PBM) and spectral opponency on light damage.

Graphical abstract



Keywords Blue-light hazard · Light-emitting-diode · Photobiomodulation · Spectral opponency

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1 Introduction

Light-emitting diode (LED) has become the most widespread artificial light source in recent years because of its high efficiency, high brightness, small size, long

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lifetime, tunable spectrum, and fast response time [1, 2]. It is common to fabricate phosphor-coated blue LED or red–green–blue (RGB) LED to obtain white light. Therefore, the white LED usually has more blue-light components than natural daylight and incandescent lamps [3]. Referring to the photobiological safety standard proposed by the International Electrotechnical Commission (IEC), the monochromatic light in the range of 435 to 440 nm has the most serious blue-light hazard (BLH) on the retina [4]. Overexposure to blue light promotes the production of reactive oxygen species (ROS), then damages the retinal cells by oxidative stress and inflammatory response [5–9]. Exposure to blue-light or blue-enriched white LED also results in the loss of photoreceptors, lipid peroxidation, as well as the autophagy and apoptosis of retinal cells [5, 7, 10, 11].

The biological experiments evaluating the light damage are primarily conducted on retinal cells or animals [12–14]. Most studies fix the illuminance and exposure duration of white light and investigate the damage on rats' retinas or retinal cells [15–17]. Filtering or removing the short-wavelength light in white light effectively decreases the light damage [13, 14, 17–19]. However, the chromaticity coordinates of the filtered white light would deviate from the Planckian locus, which is negative for the color preference and color fidelity [20–22]. Less blue light in the white light also causes circadian disruption, increases the risk of depression, and decreases the alertness and work efficiency [23, 24]. Moreover, according to IEC 62471, it seems unavoidable to change the chromaticity coordinates of light when fabricating low-hazard white light [25]. Another routine to reduce the BLH is photobiomodulation (PBM). Exposure to red or infrared light for several minutes is helpful to activate the metabolism of mitochondria, reduce the oxidative stress of retinal cells, and enhance the retinal function [5, 26–30]. PBM protects the retina or promotes the recovery from light-induced damage. In most cases, rats or retinal cells are only exposed to red or infrared light before or after light exposure, but few reports use red or infrared light as the additional component in the general illumination.

The conventional white LED is believed to damage the photoreceptors and decrease the retinal function after illuminating Sprague–Dawley (SD) rats at hundreds of lx for several days [15, 17, 31]. In this study, referring to PBM, the red light of 664 nm is added to the conventional white light to lower BLH. Another azure (484 nm) and cyan (511 nm) LEDs are also added to maintain the chromaticity coordinates of the mixed white light. Deducing from IEC 62471, the E_B of low-hazard light source would approximate the conventional white LED if they present the same illuminance and chromaticity coordinates [25]. Moreover, the spectral opponency indicates the additional light components would decrease the effect caused by conventional white LED. Though there are spectral opponency effects on the light-induced melatonin suppression [32–34], the IEC 62471 ignores the potential spectral opponency on light damage. Therefore, it is significant to evaluate the BLH exactly after concerning the PBM and spectral opponency on light damage.

In this study, we used 6-week-old SD rats to investigate the light-induced damage on retinas. All the rats were exposed to different light sources for 7 days with 12-h light/12-h dark (12L/12D) cyclic routine. The light sources included a conventional white LED, two low-hazard light sources, and a dark control group. The illuminance on rats is approximately 200–400 lx, approximating the occupational household illuminance levels. We measured the retinal function, thickness of retinal layers, and fundus photographs of rats before and after light exposure. We used western blots to determine the expression levels of autophagy-related proteins, including Beclin-1, autophagy mark light chain 3 (LC3), mitogen-activated protein kinase (MAPK), P62, and protein kinase R-like endoplasmic reticulum kinase (PERK). These proteins are associated with the autophagy process on the retina. PERK participates in the process that oxidative stress induces the production of ROS, and then modulates the autophagy of photoreceptors [35]. P62 is involved in both oxidative stress and autophagy. It is synthesized during oxidative stress and degraded by autophagy [35]. Beclin-1 is an important indicator in the apoptosis pathway marking the initiation of autophagy [36]. The activation of MAPK is related to the regulation of oxidative stress, which results in the ROS-induced apoptosis by modulating the pro- and anti-apoptotic proteins of mitochondria [37]. The increasing expression levels of LC3 indicate the autophagosome formation or the increase of autophagy. To investigate the oxidative stress on rats' retinas, we analyzed some oxidation-related biochemical indicators after light exposure, including hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide dismutase (SOD), and total anti-oxidant capacity (TAC). H_2O_2 is associated with oxidative stress and induces cellular injury [38]. Increasing MDA concentration generally implies the lipid peroxidation of mitochondrial membranes [6]. In contrast, SOD, an enzymatic anti-oxidant, would protect retinal cells against oxidative stress by eliminating ROS [6]. The concentration of TAC also reflects the capacity of reducing oxidative damage on cells [39]. As a result, with the additional light components, the low-hazard light sources decrease the oxidative stress-related autophagy and effectively protect the retina from light-induced damage. The mechanisms of protecting retina and alleviating light damage were also discussed.

2 Methods

2.1 Animals and ethics statement

The albino rat is a suitable model for investigating light-induced retinal damage [40]; hence, we selected albino SD rats (male, 6 weeks) in vivo in the experiments. All the experimental rats are male because they have better physical health than female rats, and the estrous cycle or menstrual period of female rats may affect the performances. They lived in the transparent cages individually, and the size of cage is 30 cm × 20 cm × 20 cm. All rats were obtained from the Peking University Animal Center and raised in the animal room in Peking University People's Hospital. All the animal experiments were performed in accordance with Animal in Ophthalmic and Vision Research (ARVO) statements for the Use of Animals in Ophthalmology and Vision Research and the Institutional Animal Care and Use Committee of Peking University. All the experimental procedures were approved by the Animal Care and Use Committee of Peking University People's Hospital. After experiments, we euthanized the rats by CO_2 inhalation [41].

2.2 Main parameters of light source

The IEC 62471 standard describes the photobiological safety of the light source and proposes BLH weighted irradiance (E_B) to evaluate the light damage [4]:

$$E_B = \sum_{380}^{780} S(\lambda)B(\lambda)\Delta\lambda \tag{1}$$

where $S(\lambda)$ is the spectral power distribution (SPD) of light source and $B(\lambda)$ is the BLH spectral weighting function [4]. The CIE TN 002:2014 proposes blue-light hazard efficiency of radiation (BLHER), which is defined as the quotient of blue-light hazard-weighted radiant flux to the corresponding radiant power [42]:

$$\mathrm{BLHER} = \frac{\sum_{380}^{780} S(\lambda)B(\lambda)\Delta\lambda}{\sum_{380}^{780} S(\lambda)\Delta\lambda} \tag{2}$$

The illuminance, E_V, is defined as

$$E_V = K_m \sum_{380}^{780} S(\lambda)V(\lambda)\Delta\lambda \tag{3}$$

where K_m is the maximal luminous flux and equals 683 lm/W, and $V(\lambda)$ is the photopic vision function. The quotient of E_B to E_V, which means the BLH per luminous flux, can be deduced as [25]

$$\frac{E_B}{E_V} = \frac{\sum_{380}^{780} S(\lambda)B(\lambda)\Delta\lambda}{K_m \sum_{380}^{780} S(\lambda)V(\lambda)\Delta\lambda} = \frac{\sum_{380}^{780} S(\lambda)B(\lambda)\Delta\lambda}{\sum_{380}^{780} S(\lambda)\bar{z}(\lambda)\Delta\lambda} \cdot \frac{1-x-y}{y} \cdot \frac{1}{K_m} \tag{4}$$

where x and y represent chromaticity coordinates, and $\bar{z}(\lambda)$ is the color matching function. The curves of $B(\lambda)$ and normalized $\bar{z}(\lambda)$ almost overlap, especially at the wavelength of more than 440 nm, so $\sum_{380}^{780} S(\lambda)B(\lambda)\Delta\lambda / \sum_{380}^{780} S(\lambda)\bar{z}(\lambda)\Delta\lambda$ is almost constant for different light sources. It means that the chromaticity coordinates of light mainly influence the BLH under the same illuminance, and the correlated color temperature (CCT) can be approximately calculated by the chromaticity coordinates [43]:

$$\begin{cases} A = \frac{x-0.329}{y-0.187} \\ CCT = 669A^4 - 779A^3 + 3660A^2 - 7047A + 5652 \end{cases} \tag{5}$$

2.3 Conventional white LED and low-hazard light sources

The light sources in this study were fabricated by Shenzhen Jinghong Lighting Company. The conventional white LED is consisted of blue LED and yttrium aluminum garnet (YAG) phosphor. The peak wavelengths of blue LED and phosphor are 449 and 534 nm, respectively. The CCT is about 5600 K, which is common in general illumination. The SPD of the conventional white LED is shown in Fig. 1a, and its chromaticity coordinates are shown in Fig. 1b.

The low-hazard light source is based on the conventional white LED, combined with additional light components. The additional light components contain red, azure, and cyan LEDs. The red light has been confirmed to alleviate oxidative stress and protect the retina from light-induced damage [26–28]. The peak wavelength of red light is designed to be 670 nm, referring to PBM. However, only adding red light would change the chromaticity coordinates and decrease the CCT of white light. The changes of chromaticity coordinates and CCT would affect human color perception of light source, and we aimed to design the low-hazard light sources without changing the color perception of humans. So, another azure and cyan LEDs are added to keep the same chromaticity coordinates as the conventional white LED. The peak wavelength of azure LED is designed to be more than 480 nm because the $B(\lambda)$ at 480 nm was less than 0.45, indicating a low BLH efficiency [4]. With additional light of three LEDs, the low-hazard light source presents the same chromaticity coordinates and CCT as the conventional white LED. As shown in Fig. 1b, the chromaticity coordinates of the three additional LEDs form a triangular area in the CIE 1931 color space, and the Planckian locus is in the triangular area, indicating the three additional LEDs can match the white light of different CCTs.

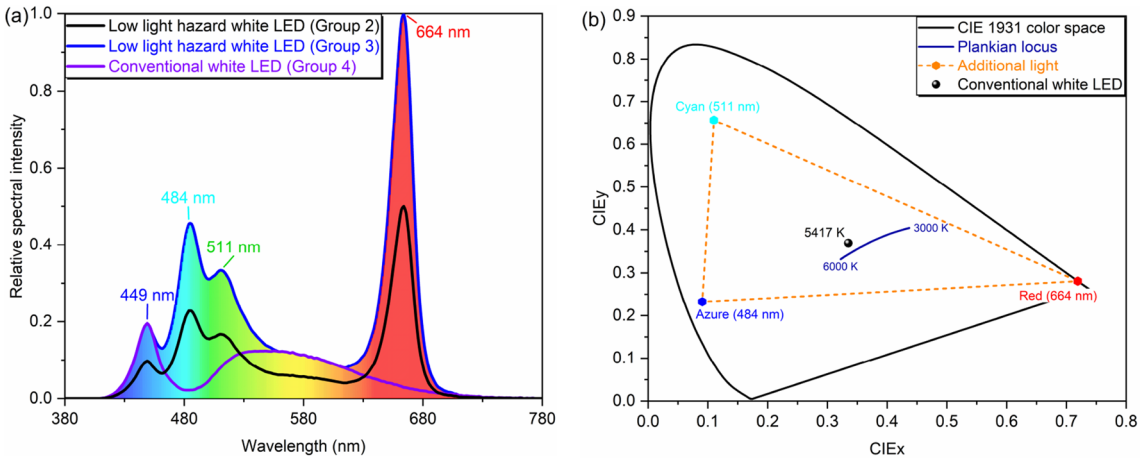

Fig. 1 **a** The SPDs of the low-hazard (Groups 2 and 3) and conventional (Group 4) white LEDs. The maximal peak spectral intensity of light source in Group 3 is set as the unit. The peak wavelengths of white and additional LEDs are 449, 484, 511, and 664 nm. **b** The chromaticity coordinates of conventional white, azure, cyan, and red LEDs, combined with Planckian locus in the CIE 1931 color space

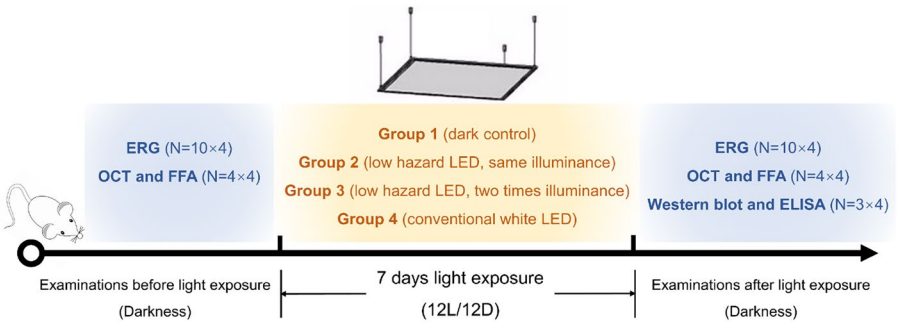

Fig. 2 Schematic diagram of the experimental procedure

The rats are divided into four groups exposed to different light sources after the dark adaptation. We design a blank control group (Group 1) to reflect the naturally retinal changes without light exposure [15, 17]. The rats in control group are reared humanely in the dark environment, to investigate the effects of environmental factors and the growth of rats on the retina. With three additional LEDs on the conventional white LED, Groups 2 and 3 are low-hazard light environments with different illuminances. Rats in Group 4 are illuminated by the conventional white LED, and the illuminance is in the range of 200–220 lx. The light sources in Groups 2–4 are designed to have similar relative SPDs, chromaticity coordinates, and CCTs. The CCTs of white light are around 5600 K, which is common in the illumination. With the additional light components, the low-hazard light source in Group 3 presents higher illuminance of 400–440 lx. The light source in Group 2 has the similar relative SPD as Group 3 and its illuminance decreases a half, so the illuminance of Group 2 is also about 200–220 lx. Figure 1a shows the typical SPDs and peak wavelengths of Groups 2–4.

In the experiment, we measured the light parameters on the first and last day of the 7-day light exposure. The measurements were on the bottom of the transparent cages by the spectrometer (PLA-20, Everfine, China). The measured light parameters contain chromaticity coordinates, CCT, and illuminance. In addition, the E_B and BLHER of light source can be calculated according to the SPD and illuminance.

2.4 Experimental procedure

Figure 2 shows the experimental procedure. After obtaining the experimental rats, there was more than 24-h dark adaptation before examinations and light exposure, to eliminate the effects of previous rearing environment. Then, all 68 rats were randomly divided into 4 groups, each with 17 rats. For each group, we took retinal examinations on 14 rats before light exposure. 10 rats in one group were conducted electroretinography (ERG) to evaluate electrophysiological

function. The other four rats in one group took optical coherence tomography (OCT) for the thickness of retinal layers and fluorescein fundus angiography (FFA) for fundus photograph. After these examinations, all rats illuminated for 7 days with 12L/12D cyclic routine, and rats in different groups were exposed to different light sources. The light sources of four groups are illustrated in Sect. 2.3. After 7-day light exposure, all rats also had 24-h dark adaptation, then we took retinal examinations after light exposure. ERG, OCT, and FFA were conducted in vivo, so these examinations were conducted on the same 14 rats before and after light exposure. The other three rats in each group, which did not take any examinations before light exposure, were killed and extracted their eyeballs for western blot analysis and enzyme-linked immunosorbent assay (ELISA). Western blot analyzed autophagy-related proteins and ELISA measured oxidation-related biochemical indicators.

2.5 Electroretinography (ERG)

An ERG recording system (RETIport, Poland Consult, Germany) was used to detect the retinal electrophysiological response to the light stimulation. ERG responses reflect the pathological change and function between photoreceptors and ganglion cells. The ERG responses contain a-wave and b-wave, and we concentrated on their amplitudes. The amplitude of a-wave reflects the pathological change of the photoreceptors, and the amplitude of b-wave mainly reflects the conduction of the visual impulse by the bipolar cells [44]. The ERG recordings were taken before and after 7-day light exposure. They were conducted on 40 rats (10 rats in each group) and took 80 times. The rats experienced the dark adaptation for 24 h before ERG recordings and all the procedures were conducted in a dark room under dim red light (> 650 nm, < 10 lx). In the preparation, rats were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) under dim red light. Then, we used eyedrops to dilate the rats' pupils, and the eyedrop contained 0.5% tropicamide (Alcon Laboratories, Inc). After anesthetization and mydriasis, we placed one stainless steel subdermal needle electrode at the tail as the ground electrode and another two electrodes under the eyelids as the reference electrodes. The recording gold electrodes were placed directly onto the cornea, combined with 0.1% sodium hyaluronate eyedrops (Santen Pharmaceutical). The ERG recordings were under dark-adapted, light-adapted, and light-adapted flicker conditions, and the electrophysiological responses were recorded simultaneously from both eyes. Under dark-adapted condition, rats were stimulated by white light of 0.001, 0.003, 0.3, and 3.0 cd s m^{-2}. After that, rats were in light adaptation (25 cd s m^{-2}) for 10 min. Then, rats were under light-adapted conditions and were stimulated by white light of 1.0, 3.0, and 10.0 cd s m^{-2}. Finally, the responses to 30 Hz flicker stimulation were recorded at 3.0 cd s m^{-2}. Each recording was the average response to three times stimulation at the above illuminance levels.

2.6 Optical coherence tomography (OCT)

The analysis of the OCT images can figure out the thickness of retinal layers, and then reflect the loss of different retinal cells after light exposure. Comparing the OCT images before and after light exposure, we evaluate the light damage on the retinal cells. Before and after the 7-day light exposure, we took OCT images on the 16 rats (four rats in each group). The OCT images were recorded with an OCT Scan Head on a Micron IV Fundus Camera. The camera was equipped with a mouse objective lens (Phoenix Research Labs, Pleasanton, CA, USA). Images were recorded from the eyes through pupils dilated with 0.5% tropicamide (Alcon Laboratories, Inc). 0.1% sodium hyaluronate eyedrops (Santen Pharmaceutical) were applied on the cornea to prevent dehydration. The images were quantitatively analyzed using the "In Sight" software. The software can automatically detect the boundaries of retinal layers and calculate the thickness of layers. The thickness of retinal layers of each eye was assessed, including retinal pigment epithelium (RPE), inner/outer segments of photoreceptors (IS/OS), external limiting membrane (ELM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), ganglion cell layer (GCL) and inner plexiform layer (IPL), and inner limiting membrane (ILM) and nerve fiber layer (NFL).

2.7 The fundus photograph and fluorescein fundus angiography (FFA)

The fundus photograph and FFA on the fundus photograph were taken on the same rats as the OCT. The fundus photographs of the retina demonstrate whether damage occurs on the retina after light exposure. Micron IV Retinal Imaging Microscope was used to take photographs on the ocular fundus of rats. Before FFA, rats were anesthetized with an intramuscular injection of 50 mg/kg of ketamine and 5 mg/kg of xylazine. Then, 0.2% carbomer was applied to the cornea to prevent desiccation and to keep the corneal surface optically smooth.

2.8 Western blot analysis

The retinal tissues were extracted to analyze protein expressions after light exposure. Previous studies confirmed that the light damage on retina is associated with the oxidative stress and the autophagy of retinal cells [45, 46]. Therefore, we analyzed the autophagy-related proteins, including Beclin-1, LC3, MAPK, P62,

and PERK. After light exposure, we killed three rats in each group, then enucleated the eyeballs and isolated the retina tissues for western blot analysis. Two eyeballs of each rat were analyzed as independent samples. The retina issues were washed three times with ice-cold phosphate-buffered saline (PBS) (4 °C, pH 7.4) for 5 min and prepared using a protein extraction kit and protease inhibitor kit (Pierce, USA). After centrifugation, the supernatant was collected, and the protein content of each lysate was measured with a bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's instructions. Equal amounts of protein (40 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a 0.22 μm polyvinylidene fluoride membrane (Millipore, USA). The primary antibodies used to probe the membranes included anti-PERK (1:1000, Cell Signaling Technology, USA), anti-p38 mitogen-activated protein kinases (p38 MAPK) (1:1000, Cell Signaling Technology, USA), anti-phosphorylated p38 MAPK (1:1000, Cell Signaling Technology), anti-P62 (1:2000, MBL International, USA), anti-light chain 3 (LC3) (1:1000, Cell Signaling Technology), anti-Beclin-1 (1:2000, Abcam), and anti-β-actin (1:2000, Cell Signaling Technology). The membranes were washed and incubated with peroxidase-conjugated secondary antibody (1:5000, Boster, USA) for 1 h at room temperature. The proteins were visualized with enhanced chemiluminescence western blot detection reagents (Millipore). The density of each band was analyzed with ImageJ software (NIH, USA), and relative changes in protein expression were calculated by the comparison with controls. We repeated each experiment at least three times.

2.9 Measurement of oxidation-related biochemical indicators

The light-induced damage on retina is associated with the ROS production and oxidative stress. Therefore, the activities of some typical oxidants and anti-oxidants were examined to evaluate the oxidative stress of rats under different light sources. After euthanizing rats, we immediately dissected the retina issues. The retina issues were placed on ice and homogenized in the assay kit to detect the concentration of biochemical indicators. Then, we centrifuged them at 8000*g* for 10 min (4 °C) and took the supernatant on ice for testing. H_2O_2 and MDA were examined to detect the oxidative capacity of retinal cells after light exposure. The expression level of H_2O_2 was detected with hydrogen peroxide assay kit (Solarbio, Beijing, China), and the expression level of MDA was detected with malondialdehyde assay kit (Solarbio, Beijing, China). We also used SOD and TAC to detect the anti-oxidative capacity of retinal cells after light exposure. The expression levels of SOD and TAC were detected with total superoxide dismutase assay kit and total anti-oxidant capacity assay kit, respectively (Solarbio, Beijing, China). Then, the absorbance of samples was detected by Microplate Reader (SpectraMax190, Molecular Devices, USA).

2.10 Statistical analysis

The ERG recordings and OCT images were taken on the rats in vivo, so they were conducted on the same rats before and after 7-day light exposure. For ERG and OCT results, paired sample t-test was adopted to evaluate the difference before and after light exposure. The change score of rat's retina after light exposure was calculated as

$$\text{Change score} = \frac{X_{\text{after}} - X_{\text{before}}}{X_{\text{before}}} \times 100\% \tag{6}$$

where X_{before} and X_{after} are the amplitudes of ERG responses and thickness of retinal layers before and after light exposure, respectively. As for comparing different groups, Shapiro–Wilk tests were used to evaluate normality assumption. Then, Kruskal–Wallis tests were adopted on the variables with nonparametric distribution, while one-way analysis of variance (ANOVA) was adopted on the variables with parametric distribution, followed by Bonferroni's test. As for the western blot and ELISA, there were only results after light exposure for the four groups, so we used one-way ANOVA and Bonferroni's test to evaluate the significant differences between groups. $p < 0.05$ is regarded as the significant difference in all the statistical tests. $p < 0.01$ and $p < 0.001$ are also marked with different symbols.

3 Results

3.1 Parameters of the experimental light sources

The light source parameters were measured two times on the first and last day during the 7-day light exposure. The parameters contain chromaticity coordinates, CCT, illuminance, E_B, and BLHER, as shown in Table 1. Group 1 is the dark control group and rats are reared in the dark environment, so the light parameters of Group 1 are not measured and recorded. It is shown in Table 1 that the light parameters generally maintain stable during the 7-day light exposure. The illuminance changes less than 10% and the fluctuation of CCT is less than 161 K. The illuminance and CCT decrease little in most cases. The chromaticity coordinates, E_B, and BLHER also have no obvious change. Generally, all the light sources in three groups have similar CCT and chromaticity coordinates, as we designed. The low-hazard light sources in Groups 2 and 3 have much less BLHER than conventional white LED (Group 4). Moreover, the illuminance and E_B of Group 3 are about two times

Table 1 The light source parameters on the first and last day during the 7-day light exposure

	Group 2		Group 3		Group 4	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
Illuminance (lx)	226.0	222.5	445.8	438.6	228.1	220.4
CCT (K)	5626	5787	5698	5609	5612	5546
Chromaticity coordinates (CIEx, CIEy)	(0.329, 0.360)	(0.325, 0.359)	(0.328, 0.356)	(0.330, 0.356)	(0.330, 0.361)	(0.331, 0.364)
$E_B(W/m^2)$	0.151	0.171	0.306	0.302	0.157	0.155
BLHER	0.145	0.155	0.139	0.147	0.235	0.231

than Group 4, while the illuminance and E_B of Group 2 are similar to Group 4.

3.2 Amplitudes of a- and b-waves in ERG

According to the standard of International Society for Clinical Electrophysiology of Vision (ISCEV), we concentrated on the amplitudes of a- and b-waves when rats were under dark-adaptation condition and stimulated by the white light of 0.3 cd s m^{-2} [44]. The right and left eyes of one rat are regarded as two samples, so there are 20 samples in each group.

Figure 3a and c presents the amplitudes of a- and b-waves for the rats in four groups. Paired sample t-test is used to compare the amplitudes before and after the 7-day light exposure. After 7-day light exposure, the amplitudes of a-wave in Groups 1 and 2 decrease a little, while the difference is not significant ($DF = 19$, $t = 0.459$, $p = 0.652$ for Group 1 and $DF = 19$, $t = 1.558$, $p = 0.136$ for Group 2). The changes of b-wave in Groups 1 and 2 are also insignificant ($DF = 19$, $t = -0.209$, $p = 0.834$ for Group 1 and $DF = 19$, $t = 1.018$, $p = 0.322$ for Group 2). In contrast, the amplitudes of a-wave in Groups 3 and 4 significantly decrease after 7-day light exposure ($DF = 19$, $t = 6.273$, $p = 0.000$ for Group 3 and $DF = 19$, $t = 11.658$, $p = 0.000$ for Group 4). Similarly, the amplitudes of b-wave in Groups 3 and 4 present significant decreases ($DF = 19$, $t = 7.120$, $p = 0.000$ for Group 3 and $DF = 19$, $t = 10.042$, $p = 0.000$ for Group 4).

Figure 3b and d shows the change scores of the amplitudes of a- and b-waves. According to Shapiro–Wilk tests, the change scores of a- and b-waves are nonparametric variables, and Kruskal–Wallis test indicates that they are significantly different between four groups ($DF = 3$, $p = 0.000$). As for the pairwise comparisons, we concentrated on the comparisons with Group 4 (conventional white LED) and Group 1 (dark control), as shown by the blue and black symbols at the bottom of Fig. 3b and d. Compared with Group 1, the changes of b-wave in Group 2 have no significant difference ($p = 0.331$ for a-wave and $p = 0.527$ for b-wave), while Groups 3 and 4 decrease significantly ($p < 0.01$ for both a- and b-waves in Groups 3 and 4). In addition, the change scores of a- and b-waves in Group 2 are significantly higher than Group 4 ($p < 0.001$), and the change scores of a- and b-waves in Group 3 are also significantly higher than Group 4 ($p = 0.003$ for a-wave and $p = 0.012$ for b-wave).

3.3 Thickness of the retinal layers

Figure 4A shows the typical OCT images of rats in four groups before and after light exposure. According to the boundary of retinal lines, we obtained the thickness of retinal layers of four groups before and after light exposure, including RPE, IS/OS, ELM, ONL, OPL, INL, GCL and IPL, ILM and NFL. We also measured the total thickness of retina. The change scores on the thickness of retinal layers reflect the loss of retinal cells after light exposure [47]. Each group has four rats and eight samples of eyes, except Group 2. The left eye of one rat in Group 2 fails to obtain an effective OCT image.

Figure 4b and c, respectively, presents the change scores of superior and inferior retinal layers after light exposure. Figure 4b also presents the change scores of the total thickness of retina. According to Shapiro–Wilk tests, the change scores of retinal layer thickness are not nonparametric variables ($p = 0.003$). Kruskal–Wallis tests are used to compare the difference of change scores between four groups. Generally, the change scores of the thickness of total retina, IS/OS, ELM, and ONL significantly differ. Their p values are 0.000, 0.000, 0.036, and 0.000, respectively, while other layers have no significant differences between different groups. Then, we move to the layers with significant differences. There is no significant difference between Groups 1 and 2 ($p > 0.05$) in all the layers after pairwise comparisons on the change scores, while Groups 3 and 4 present significant decrease on the thickness of IS/OS, ELM, ONL, and total retina than Group 1 ($p < 0.05$). Compared with Group 4, Group 1 has significantly more change scores on the thickness of these three layers and total retina ($p < 0.01$). The thickness of Group 2 also presents

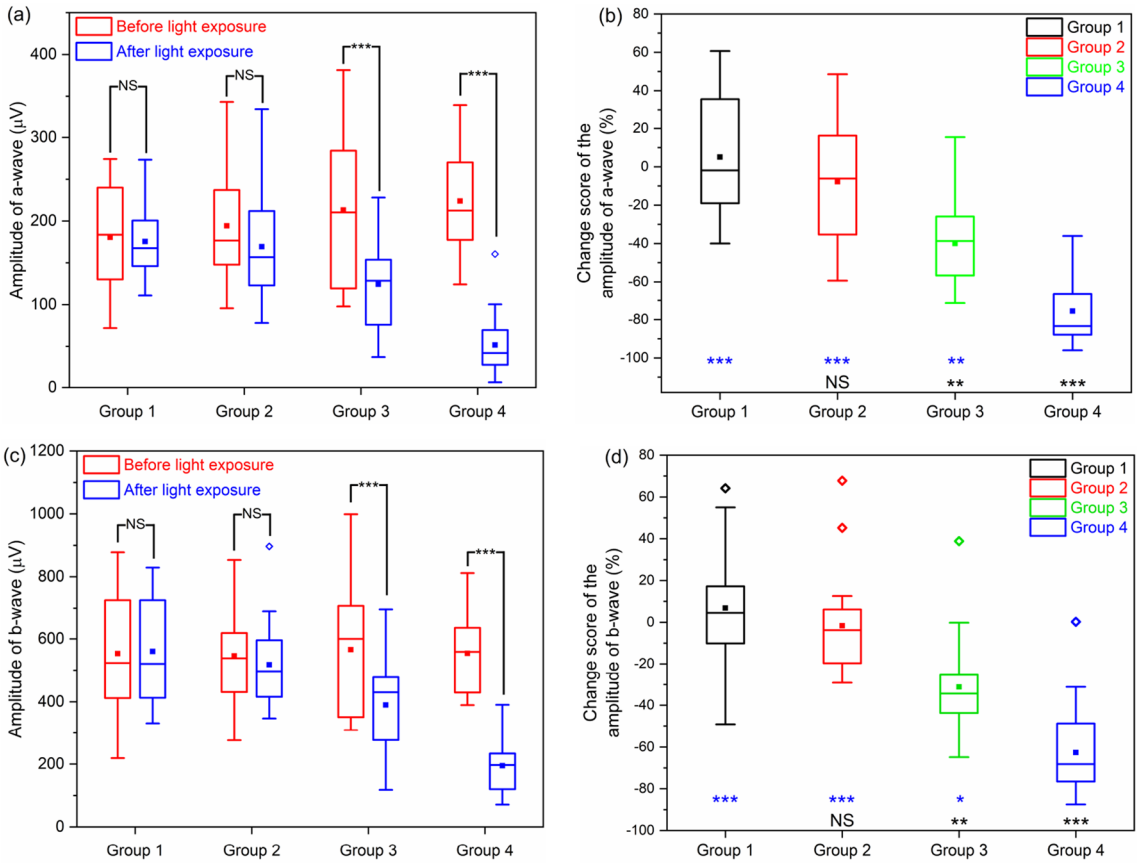

Fig. 3 The ERG responses under dark-adaptation condition with white light of 0.3 cd s m^{-2}. **a** The amplitudes of a-wave before and after light exposure. **b** The change scores of amplitudes of a-wave after light exposure for four groups. **c** The amplitudes of b-wave before and after light exposure. **d** The change scores of amplitudes of b-wave after light exposure for four groups. The blue and black symbols at the bottom represent the comparison with Group 4 and Group 1, respectively. $***p \le 0.001$, $**p \le 0.01$, $*p \le 0.05$, NS, $p > 0.05$. The bottom and top of the box are the first and third quartiles, and the band and dot inside the box are the second quartile (median) and mean value, respectively. The single points outside the box represent the outliers which are defined as the data outside quartile + 1.5 × interquartile range. The ends of whiskers represent the minimum and maximum of all data except for outliers

significantly less change than Group 4 for the layers of IS/OS, ONL, and total retina. In contrast, the thickness of ELM has no significant difference between Groups 2 and 4. As for the comparison between Groups 3 and 4, there is no significant difference in most layers, while Group 3 has significantly higher change scores on the thickness of ONL than Group 4 ($p < 0.05$).

3.4 Retinal fundus photograph and FFA

As shown in Fig. 5, the fundus examinations reveal no retinal pathological changes after 7-day light exposure. There is no evidence of retinal atrophy, vascular leakage, or neovascularization [48, 49]. The results indicate that low-hazard light sources and conventional white LED at 200–400 lx hardly cause severe damage on retinal blood vessels.

3.5 Expression of autophagy-related proteins

Western blot was used to determine the expression levels of PERK, P62, Beclin-1, MAPK, and LC3 in the four groups after light exposure, as shown in Fig. 6a. The expression level of β-actin was also determined as the reference. The expression levels of autophagy-related proteins were calculated by comparing the optical densities of their bands with the β-actin band. As shown in Fig. 6b, rats in Group 1 express the least autophagy-related proteins, while rats in Group 4 have the most autophagy-related protein expression levels. In addition, the expression level in Group 3 is higher than Group 2 for the five autophagy-related proteins. After Shapiro–Wilk tests, the expression levels of these proteins are normal distributions ($p = 0.471$), and they present significant differences between groups by ANOVA ($p < 0.05$).

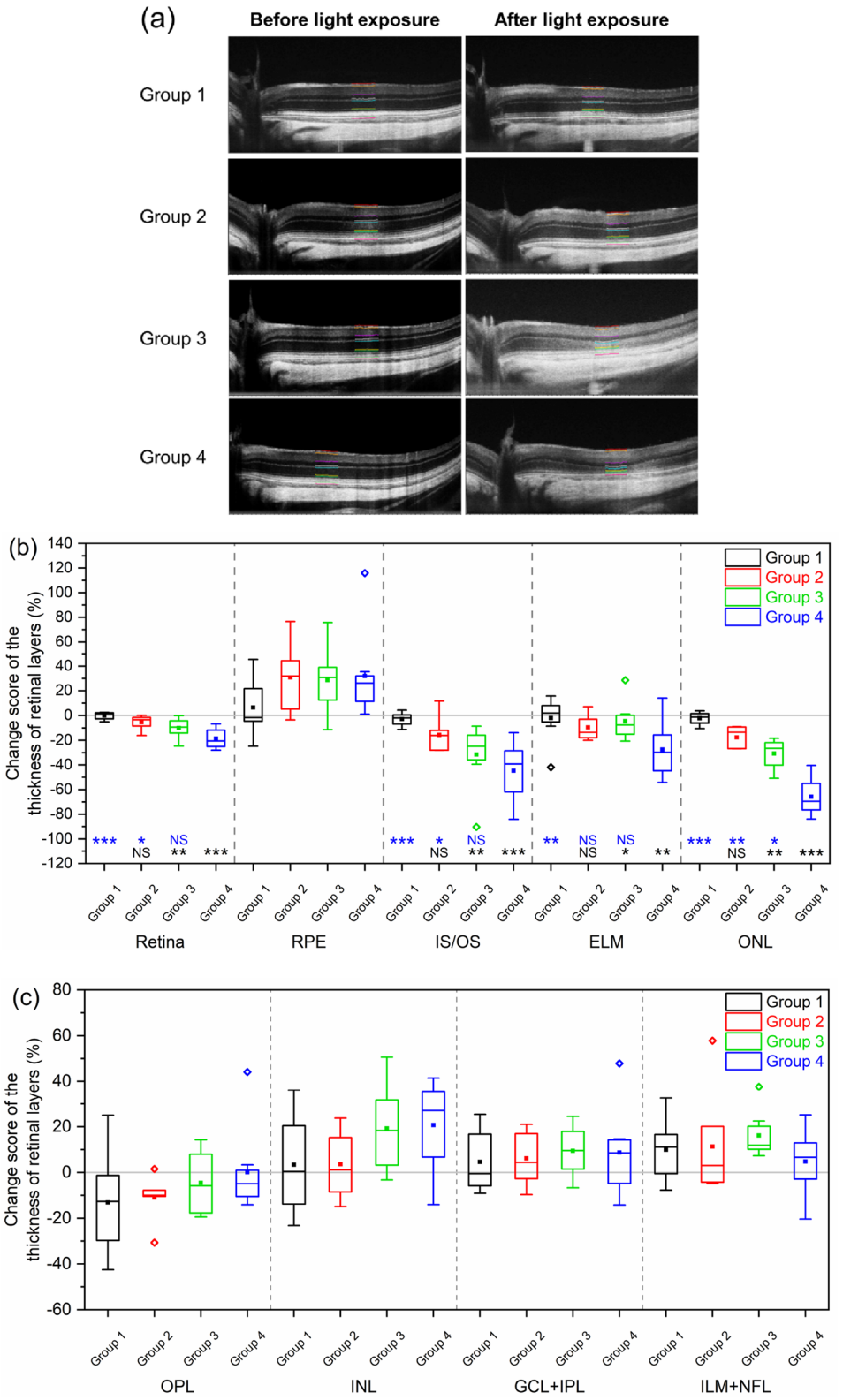

Fig. 4 **a** The OCT images of rats in four groups before and after light exposure. The colorful lines represent the boundaries of different retinal layers. **b**, **c** The change scores on the thickness of retinal layers after light exposure. **b** Change scores on the thickness of total and superior retina, including RPE, IS/OS, ELM, and ONL. **c** Change scores on the thickness of inferior retina, including OPL, INL, GCL + IPL, and ILM + NFL. The blue and black symbols at the bottom represent the comparison with Group 4 and Group 1, respectively. ***$p \le 0.001$, **$p \le 0.01$, *$p \le 0.05$, NS, $p > 0.05$. The bottom and top of the box are the first and third quartiles, and the band and dot inside the box are the second quartile (median) and mean value, respectively. The single points outside the box represent the outliers which are defined as the data outside quartile + 1.5 × interquartile range. The ends of whiskers represent the minimum and maximum of all data except for outliers

Then, Bonferroni's tests are adopted to evaluate the differences between the two groups, especially the comparison with Groups 1 and 4. For all the autophagy-related proteins, there is a significant difference between Groups 1 and 4 ($p < 0.05$), while there is no significant difference between Groups 3 and 4 ($p > 0.05$). Meanwhile, rats in Group 2 express significantly fewer autophagy-related proteins than Group 4, including PERK, P62, MAPK,

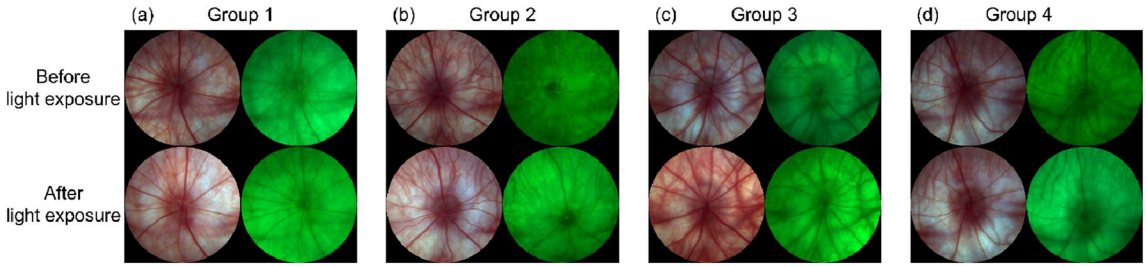

Fig. 5 The typical retinal fundus photograph and FFA of rats in **a** Group 1, **b** Group 2, **c** Group 3, and **d** Group 4 before and after light exposure. In each figure, the left image is the retinal fundus photograph, and the right image is the FFA

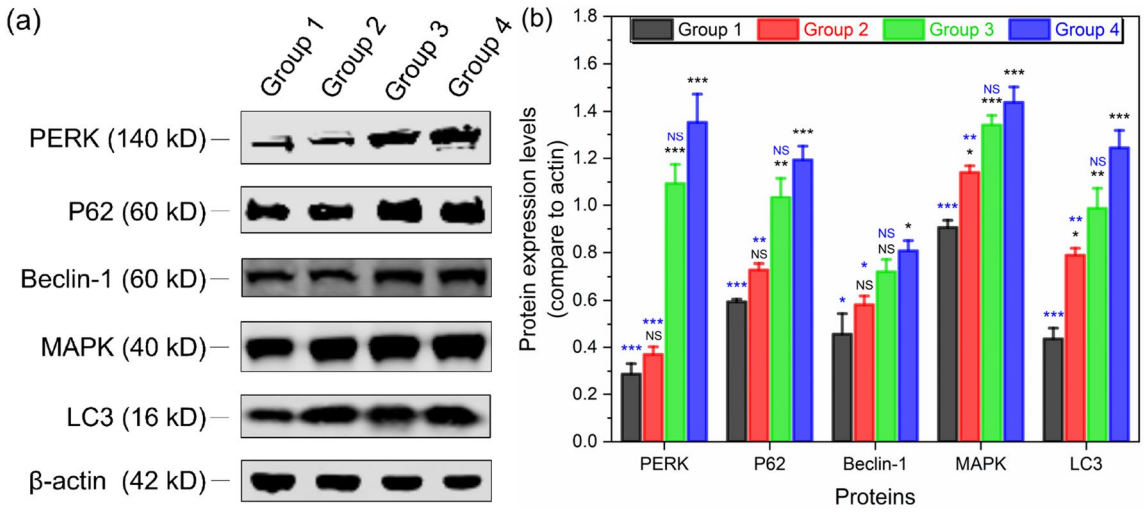

Fig. 6 **a** The representative western blot results of rats in four groups after 7-day light exposure. **b** The expression levels of proteins (Mean ± SEM) after 7-day light exposure, including PERK, P62, Beclin-1, MAPK, and LC3. The blue and black symbols above the error bars represent the comparison with Group 4 and Group 1, respectively. ***$p \le 0.001$, **$p \le 0.01$, *$p \le 0.05$, NS, $p > 0.05$

LC3, and Beclin-1. In addition, the expression levels of PERK, P62, and Beclin-1 between Groups 1 and 2 have no significant difference ($p > 0.05$), while MAPK ($p = 0.032$) and LC3 ($p = 0.027$) show significant differences. As for the comparison between Groups 1 and 3, rats in Group 3 express significantly more proteins than Group 1 in most cases ($p < 0.01$), while there is no significant difference on Beclin-1 ($p = 0.075$).

3.6 Oxidative stress

ELISA was conducted to evaluate the concentration of oxidants (H_2O_2, MDA) and anti-oxidants (SOD, TAC) in the rats in four groups after 7-day light exposure. As shown in Fig. 7, the rats in Group 4 have the most oxidants and anti-oxidants concentrations, while the rats in Group 1 are the least. The oxidants and anti-oxidants concentrations in Group 3 are less than Group 4, and rats in Group 2 are also less than Group 3. In most cases, oxidative stress leads to the increase of oxidants and the decrease of anti-oxidants [50]. However, in this study, the concentrations of oxidants on rats' retinal issues have a positive correlation with anti-oxidants. It can be explained that the initial step of oxidative process also increases anti-oxidant activity, due to the defense mechanism against oxidative damage [39].

According to Shapiro–Wilk tests, the concentration of these oxidants and anti-oxidants are normal distributions ($p = 0.221$), and ANOVA confirms the significant differences on the concentration of oxidants and anti-oxidants between the groups ($p < 0.001$). Then, Bonferroni's tests are used to evaluate the difference between the two groups. Generally, rats in Group 2 have significantly more oxidants and anti-oxidants than Group 1 ($p < 0.05$). In addition, oxidants and anti-oxidants in Groups 3 and 4 are significantly more than Group 1 ($p < 0.001$), while there is no significant difference between Groups 3 and 4 ($p > 0.05$). In addition, rats in Group 2 have significantly less MDA, SOD, and TAC concentration

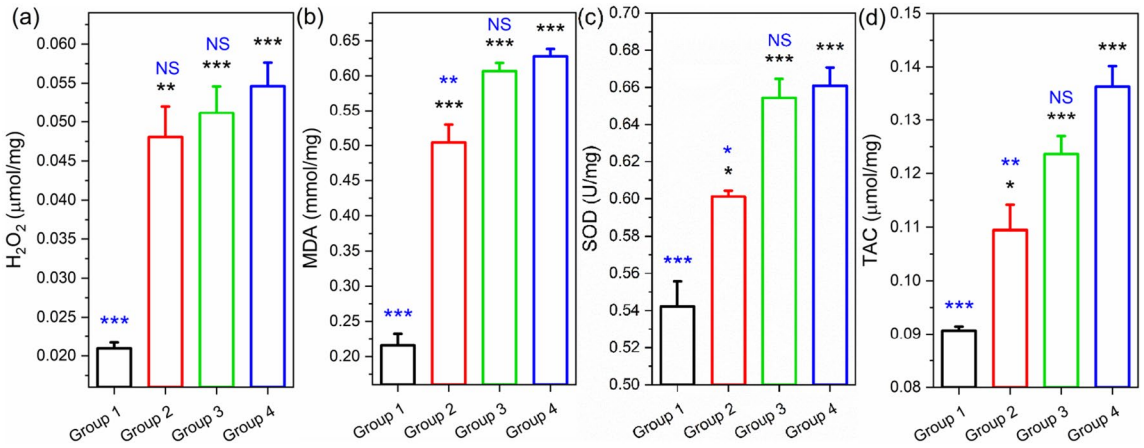

Fig. 7 The concentration of oxidants and anti-oxidants on the rats of four groups after 7-day light exposure, including **a** H_2O_2, **b** MDA, **c** SOD, and **d** TAC. The blue and black symbols above the error bars represent the comparison with Group 4 and Group 1, respectively. $^{***}p \le 0.001$, $^{**}p \le 0.01$, $^{*}p \le 0.05$, NS, $p > 0.05$

than Group 4 ($p < 0.05$), but no significant difference on the concentration of H_2O_2 ($p > 0.05$).

4 Discussion

The CCTs of the conventional and low-hazard white LEDs are approximately 5600 K during the 7-day light exposure. It is the common CCT in general light sources. The illuminance of Groups 2 and 4 is about 200 lx, and it increases to about 400 lx for Group 3 because of the additional light components. The illuminance of about 200–400 lx is also the common occupational illuminance [51]. In brief, we concentrate on the light damage caused by the white LEDs in the common lighting application. Previous studies found that the effects of light exposure on SD rats are similar to humans, especially the light damage on retina [5, 52], so SD rats are widely used as the model of chronic light damage [40]. ERG, OCT, and FFA are used to evaluate the light damage on retina comprehensively. Meanwhile, we also investigated the mechanisms of light damage and retinal protection. The expression levels of autophagy-related proteins are associated with autophagy, apoptosis, and loss of retinal cells. The concentration of some typical oxidants and anti-oxidants is associated with the oxidative stress of retina.

4.1 The light damage of conventional white LED

The conventional white LED illuminated the rats in Group 4 for 7 days. Compared with rats in Group 1 (dark control group), the amplitudes of a- and b-waves decrease significantly, indicating the significant loss of retinal function. Meanwhile, based on the OCT images, the thickness of most superior retinal layers in Group 4 also decreases, including IS/OS, ELM, and ONL. As shown in Fig. 4b, the thickness of RPE increases a little after light exposure. Previous studies reported the increase of apoptosis and the decrease of cell viability on RPE cells after light exposure [10, 53], but the results in this study are quite different. They indicate that the moderate illuminance of about 200 lx in Group 4 is not enough to decrease the thickness of RPE layer, and the growth of retina in the 7 days may contribute to the little increase of RPE layer. The results illustrate that the conventional white LED damages the structure of some retinal layers after 7-day light exposure. The significant decrease of ONL thickness indicates the loss of photoreceptors on retina, which would affect the responses of photoreceptors and bipolar cells. These results conform to the attenuated amplitudes of a- and b-waves.

Though the normal fundus photograph illustrates that conventional white LED with moderate illuminance causes no retinal pathological changes, the ERG and OCT results indicate the significant changes on retinal function and photoreceptors. The conventional white LED has a higher blue-light component and BLHER, and the short-wavelength blue light has high BLH efficiency [3, 4]. BLHER reflects the blue-light component in the spectrum after being weighted by $B(\lambda)$. As the reference, the BLHER of typical daylight is 0.17 at 5600 K. According to Table 1, the BLHER of conventional white LED in Group 4 reaches 0.23, which is about 35% higher than the typical daylight [3]. We also found that rats in Group 4 have significantly higher expression of autophagy-related proteins, combined with more oxidants and anti-oxidants than Group 1. The light-induced damage on retina is usually attributed to the production of ROS and oxidative stress, which leads to the autophagy of retinal cells [5, 12, 52]. The loss of retinal

cells, especially photoreceptors, would affect the retinal function [44]. Previous studies confirmed that the white light at thousands of lx would damage the retina [16, 19, 47], while the general illumination of white LED is about hundreds of lx. This study indicated that the white LED at hundreds of lx also has potential risks on retina. Though SD rats' retina is easier to be damaged than humans because of the lack of pigments, the potential retinal toxicity caused by common white LED should raise concern in the wide application.

4.2 The light sources with similar E_B result in different damage on retina

The illuminance of Group 2 is about 200 lx, which is similar to Group 4 of conventional white LED. The BLHER of the low-hazard light source in Group 2 is about 0.15, which is about 35% less than conventional white LED, and also less than typical daylight at 5600 K. The additional red, azure, and cyan LEDs decrease the relative proportion of blue light in the spectrum, then result in a lower BLHER. We have deduced that the quotient of E_B to illuminance primarily depends on the chromaticity coordinates [25], and the light sources in Groups 2 and 4 present similar chromaticity coordinates and illuminance, so the two light sources would have similar E_B, as shown in Table 1.

After 7-day light exposure, the retinal function of rats in Group 2 has no significant change, while rats in Group 4 are damaged significantly, according to ERG results. Meanwhile, the thickness of ONL, which is mainly consisted of photoreceptors, also has a similar change after light exposure. The rats in Group 2 present no significant change on the thickness of ONL, while rats under conventional white LED suffer from the significant decrease of ONL layer. The previous study also found that after light exposure and light damage, the retinal function can recover, but the damage on the ONL can hardly recover after several days [17]. As for the autophagy-related proteins, oxidants, and anti-oxidants, rats in Group 4 express more PERK, P62, MAPK, and LC3 than rats in Group 2. They also have more MDA, SOD, and TAC than Group 2. These results indicate that rats in Group 4 suffer from more serious oxidative stress and autophagy of cells. To some extent, rats in Group 2 also suffer from oxidative stress and autophagy, according to the increase of MAPK, LC3, and oxidants and anti-oxidants than the dark control group. However, the little increase of oxidative stress and autophagy in Group 2 hardly damages the retinal cells and affects the retinal function, while the significant increase of oxidative process in Group 4 damages the structure and function of retina.

Though the light sources in Groups 2 and 4 have similar CCT, E_B, and illuminance, the low light hazard light source results in much less damage on retina, especially the retinal function and thickness of photoreceptors. Filtering the blue light and decreasing the CCT of white light are the common methods to protect the retina [13, 14, 17–19]; however, these would result in the decrease of illuminance and deviation of chromaticity, which has negative effects on the color perception and non-visual effects [20–24]. Here, we provided the low-hazard light source to decrease the light damage and protect the retina without changing the CCT and illuminance of white light. It also illustrates that CCT and illuminance cannot fully reflect the potential risks of light damage on retina [47]. E_B, which is proposed by IEC 62471, is inversely proportional to the maximum permissible exposure duration ($t_{\max}$), and $t_{\max}$ is used to evaluate the risk groups of light sources [4, 54]. The low-hazard and conventional white LEDs in Groups 2 and 4 have similar E_B and $t_{\max}$, and they are expected to have similar risks for photobiological safety, according to IEC 62471. However, the low-hazard light source in Group 2 actually results in much less damage than conventional white light in Group 4. As a result, E_B and $t_{\max}$ cannot exactly reflect the light hazard and photobiological safety of the light source. Some evidence also indicated that E_B in IEC 62471 cannot exactly reflect the light-induced damage. The E_B mainly takes account of the light damage on RPE, while chronic exposure to light can also damage the photoreceptors [47]. We realize the main difference between the two light sources of Groups 2 and 4 is BLHER, which indicates BLHER might be more effective in evaluating the light damage than E_B in some cases. The comparison between Groups 2 and 4 also indicates the significance of the relative proportion of blue light on the light damage when the light presents similar and moderate illuminance. In addition, the spectral opponency and PBM on the light damage should also be concerned, which is discussed in the following section.

4.3 The additional light components decrease the damage on retina

The light source in Group 3 is fabricated by adding red, azure, and cyan LEDs to the conventional white LED in Group 4. As a result, the illuminance and E_B in Group 3 are two times of Group 4. Meanwhile, though the light sources in Groups 3 and 4 have the same blue-light intensity, the additional light decreases the relative proportion of blue light in the spectrum, so the BLHER in Group 3 decreases to 0.14. The chromaticity coordinates and CCT are almost same in Groups 3 and 4, as listed in Table 1. Both light sources in Groups 3 and 4 result in the loss of amplitude of b-wave and the thickness of superior retinal layers after 7-day light exposure, indicating the significant light damage on the retinal function and cells. However, the change scores in Group 3 on the amplitude of b-wave and thickness of

ONL are significantly less than those in Group 4, indicating the light source in Group 3 has less damage on retina.

The light sources in Groups 3 and 4 have the same spectral intensity of blue light, and the difference is that the light source in Group 4 has additional light components. Therefore, we believe the additional light components, consisting of red, azure, and cyan LEDs, effectively decrease the damage on the retinal function and photoreceptors. It provides the evidence of spectral opponency on the light damage that additional light components result in less light damage. Referring to PBM, the red light around 670 nm could increase the mitochondrial membrane potential of retinal cells, and then promotes the metabolism of mitochondria, which is helpful to block the mitochondrial permeabilization and attenuate the ROS-induced autophagy of photoreceptors [26, 28, 30]. Previous studies mostly used PBM before or after light exposure to benefit the retina [29, 55, 56]. The PBM after light-induced damage and other types of retinal damage could promote the recovery of retina [29]. PBM before light exposure is also effective to protect the retina from light-induced damage [55, 56]. However, few studies used PBM simultaneously with light exposure. In this study, we utilize the red light around 670 nm and add it directly to the general illumination. Considering that the addition of red light would change the chromaticity coordinates of white light, we also add another azure and cyan LEDs to maintain the chromaticity coordinates. As a result, the rats in Group 3 have significantly less damage on the retinal function and photoreceptors, indicating that exposure to additional light components effectively decreases the light-induced damage on retina. The red light around 670 nm is believed to alleviate the production of ROS and reduce the autophagy of photoreceptors [26, 28].

The expression levels of autophagy-related proteins in Group 3 are less than Group 4, and rats in Group 3 also have fewer oxidants and anti-oxidants than Group 4, but the difference is insignificant. These tendencies conform to the mechanism of PBM from red light, while the insignificant difference could not fully explain the significantly better performances of the retinal function and thickness of photoreceptors in Group 3. Besides the red light, the additional azure and cyan components may present spectral opponency on the light damage. Previous studies confirmed the spectral opponency on melatonin suppression [32–34, 57] that the additional green light of 525 nm effectively alleviated the melatonin suppression of blue light of 450 nm [33]. Though the spectral opponency has not been concerned on the light damage previously, to some extent, melatonin suppression is associated with light damage. Melatonin is a kind of anti-oxidants, which can reduce oxidative stress and autophagy [58, 59]. There are tens of pg/ml of melatonin in the plasma of SD rats during the daytime [60], and the additional light components could have spectral opponency on melatonin suppression. As a result, the additional light components might protect the retina via the spectral opponency on melatonin, other than the oxidants and anti-oxidants measured in this study.

There are still some limitations of this study. We only examined the light damage caused by the white light with moderate illuminance and common CCT, so the results might not be suitable in some extreme conditions with thousands of lx. We used SD rats to examine the photobiological safety because they are sensitive to the light damage and they have similar retinal structures as humans. Still, animal experiments are not enough to fully represent the actual effects on humans. Furthermore, we will concentrate on more direct evidence of the specific structures or matters, to investigate the mechanism of spectral opponency on light damage.

5 Conclusions

In summary, we fabricated the low-hazard light source by adding azure (484 nm), cyan (511 nm), and red (664 nm) light components into conventional phosphor-coated white LEDs. The low-hazard and conventional white LEDs illuminated SD rats for 7 days with moderate illuminance (200 to 400 lx) and common CCT (5600 K). The conventional white light at moderate illuminance significantly damages the functions and structures of rats' retinas. As for the low-hazard light source, it has no significant damage on the retina after 7-day light exposure at about 200 lx. The low-hazard light source also results in significantly less oxidative stress and cellular autophagy, then effectively decreases the light damage on retinal function and structures. The results indicate that the E_B for BLH proposed by IEC 62471 should be reconsidered. In addition, we figure out that proper additional light components can decrease the light damage on retina due to PBM and spectral opponency. Consequently, the spectral opponency on the light damage provides a new method to promote photobiological safety without changing the chromaticity coordinates of white light.

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Data availability Data are available upon request directed to the corresponding author.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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