• Basic Research •

Chronic photodamage in the chicken retina using 650-nm semiconductor laser

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Received: 2019-01-06 Accepted: 2019-05-05

Abstract

• AIM: To investigate the occurrence of chronic photodamage in the cone-based retina, following long-term exposure to a 650-nm semiconductor laser (power: 2 mW).

• METHODS: Chickens fed for 1mo under natural light after hatching were irradiated with 650-nm laser light at different times each day. Fifteen animals were included in each group. Group A was a control group, irradiated with natural light during the entire study. Group B was irradiated with laser for 3 min/d. Group C was irradiated with laser for 6 min/d. Group D was irradiated with laser for 30 min/d. The duration of the light experiment was 6mo. We obtained data at 1, 3, and 6mo, including measuring the retinal thickness *in vivo* using optical coherence tomography, hematoxylin and eosin staining, TUNEL assay, apoptosis staining, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and Western blotting to detect changes in L/M opsins and rhodopsin.

• RESULTS: At 1mo, the MDA content in Group D was higher than that observed in Group A (*P*=0.019). At 3mo the MDA content in Groups C and D was higher than that reported in Group A (*P*=0.026, 0.003). At 6mo, the MDA content in Groups B, C, and D was higher than that observed in Group A (*P*=0.038, 0.032, 0.000, respectively). There was no difference in SOD activity, and L/M opsin and rhodopsin content between the groups at 1 and 3mo. The SOD activity in group D was significantly decreased at 6mo (*P*=0.000), as was the content of rhodopsin. There was no significant reduction observed in retinal thickness, abnormal cell arrangement, and positive staining of TUNEL in the groups during the 6-month study period.

• CONCLUSION: Irradiation using a 650 nm semiconductor laser (power: 2 mW) for 6min per day over 6mo do not cause photodamage. Similarly, a 3-month exposure of 30min per day do not cause damage. However, irradiation for 6mo resulted in a significant increase in the content of free radicals and a decrease in the content of rhodopsin in the retina, suggesting the presence of photodamage.

• KEYWORDS: light/adverse effects; oxidative stress; red light; apoptosis; chicken retina

DOI:10.18240/ijo.2019.06.09

Citation: Wang YP, Liang ZY, Chen S, Yang WC, Kong JH. Chronic photodamage in the chicken retina using 650-nm semiconductor laser. *Int J Ophthalmol* 2019;12(6):936-942

INTRODUCTION

Red light plays roles in anti-inflammation and biological regulation of mitochondrial electron chain transfer in biological tissues[1-4]. In recent years, its use in clinical practice has gradually increased, promoting wound repair and tissue cell regeneration, improving blood circulation, treating peripheral nerve injury and chronic pain, and reducing damage to the retina, optic nerve, and other fields[5-10]. The clinically useful bands mostly range between 630 and 700 nm, and both light-emitting diodes and lasers exert similar therapeutic effects[11-12]. Laser is a special kind of light characterized by high brightness, monochromaticity, and good directionality. Its use is associated with advantages for the treatment of local lesions, such as in the macular area of the human eye. Studies have shown that exposure to low laser illumination does not cause obvious photodamage in the human eye in the short term[7,11]. However, studies investigating the safety of long-term exposure to laser illumination are currently lacking. In this study, the chicken retina was regularly irradiated using a 2 mW semiconductor laser for different periods of time to assess the effects of long-term exposure on the retina.

MATERIALS AND METHODS

Ethical Approval The study was approved by the Tianjin Medical University Medical Ethics Committee and complied with the Declaration of Helsinki and ARVO Statement. The procedures followed were in accordance with institutional guidelines.

Experimental Animals and Grouping Sixty Leghorn male chicken were raised under natural light after hatching for 1mo. They were randomly divided into four groups (15 animals per group). Exposures to laser per day included 0-min irradiation [Group A, (natural light, control)], 3-min irradiation (Group B), 6-min irradiation (Group C), and 30-min irradiation (Group D). The rest of the time, the chickens were exposed to natural light. Five experimental animals were randomly selected from each group for to observed the indicators at 1, 3, and 6mo.

Assessment of the Chicken Retina Using Optical Coherence Tomography At each detection time point, the heads of all experimental animals were fixed , and through the center of the pupil for optical coherence tomography (OCT) scanning of both eyes (Heidelberg, Germany). The scanning mode selected the ring of the nerve fiber layer thickness measurement mode of the machine. The obtained images were analyzed by using the ImageJ software (V1.8.0 National Institutes of Health). We adjusted the pictures to 8-bit, measured the pixel scale at the bottom of the left side of the measurement images, and selected the measurement area of the retina layer in the images.

Histological Observation After the animals were sacrificed, the left eye of the chicken was selected for tissue fixation, and the eye was cut along the equator of the eyeball on an ice table. After peeling, the retina was removed from the adherent vitreous and pigmented membrane and fixed with 4% paraformaldehyde for 24h. Subsequently, the retinal sections were dehydrated, treated with the clearing agent xylene, and embedded in paraffin. The slice thickness was 3 μm, and three slices were obtained from each sample. The sections were subjected to conventional hematoxylin and eosin (HE) staining and observed under a light microscope. Using TUNEL apoptosis detection kit to detect apoptosis (Beyotime, Shanghai, China), The sections were dewaxed three times using xylene, removal of xylene using gradient alcohol, dilution in distilled water for 2min, digestion with proteinase K for 15min at 37℃, rinsing with Tris buffer solution (TBS), then the tissues were incubated with a test solution containing fluorescein isothiocyanate fluorescent labeling (FITC) solution at 37℃ for 2h in a dark box, and then washed with TBS. Subsequently, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Olympus, Japan).

Determination of Malondialdehyde Content and Superoxide Dismutase Activity The right eyes of the chickens were selected to produce retinal homogenates. The posterior pole retina was placed on the ice platform and ultrasonically pulverized to produce a 10% retinal homogenate. The samples were centrifuged at 3500 r/min for 15min and the supernatants were collected for experimental use.

Malondialdehyde Content Determination The 0.1 mL of the 10% retinal homogenate was added to the malondialdehyde (MDA) detection reagent (Beyotime, Shanghai, China) and the mixture was placed in a 95℃ water bath for 30min. Subsequently, the mixture was centrifuged at 3500 r/min for 10min and the supernatant was collected. The concentration was calculated at a wavelength of 532 nm using a microplate reader.

Determination of Superoxide Dismutase Activity Diluted some 10% of the retina homogenate to 1% using TBS, 40 μL of the 1% of the retina homogenate was collected for use, and the detection reagent (Beyotime, Shanghai, China) was added to the mixture. The mixture was placed in a 37℃ water bath for 40min, and the developer (nitro-blue tetrazolium) was added for 10min. The concentration was calculated at a wavelength of 550 nm using a microplate reader.

The protein concentration was determined using the bicinchoninic acid (BCA) method. Briefly, 50 μL of 1% retinal homogenate were placed in the well plate, the BCA working solution (Beyotime, Shanghai, China) was added, and the mixture was placed at 37℃ for 30min. The protein concentration was calculated at a wavelength of 562 nm.

For the determination of MDA content and superoxide dismutase (SOD) activity, the MDA and SOD assay units were expressed as nmol/mg protein and nU/mg protein, respectively, based on the protein concentration.

L/M-opsin and Rhodopsin Determination Retinal homogenate (10%, 1 mL) was centrifuged at 10 000 r/min for 10min. The sample volume was calculated according to the protein concentration (25 μg protein), and an equal amount of loading buffer was added to the mixture. The mixture was placed in a 100℃ water bath for 5min, and 20 μL of the sample solution was used for electrophoresis. Of note, the voltage was first adjusted to 80 V. When the bromophenol blue indicator left the staking gel, the voltage was adjusted to 120 V. The reaction was terminated when the bromophenol blue reached the end of the separation gel. Transfer membrane electrophoresis was performed at 100 mA for 40min. After completion of the transfer, the polyvinylidene fluoride (PVDF) membrane was incubated in 5% skim milk for 4h, washed thrice with TBST, and incubated with primary antibody at 4℃ for 10h. The primary antibodies were L/M opsin rabbit polyclonal antibody (Biolab. Beijing, China) and rhodopsin mouse polyclonal antibody (Abcam, Cambridge, UK). The α-tubulin protein (Affinity, Changzhou, China) was used as an internal reference.

Subsequently, the membrane was washed thrice with TBST and incubated with secondary antibody at 37℃ for 2h. Photochemical development was used to obtain the pictures and the ImageJ software was used for image analysis.

Table 1 Retinal layer area in each group according to optical coherence tomography mean±SD

Month	Group A	Group B	Group C	Group D	*F*	*P*
1mo	17.67±1.49	17.63±1.47	18.08±1.23	18.48±1.77	1.066	0.371
3mo	22.4±1.34	21.95±1.19	21.65±1.45	21.66±0.83	0.850	0.476
6mo	22.58±1.17	22.68±0.70	22.38±0.73	21.94±0.50	0.817	0.503

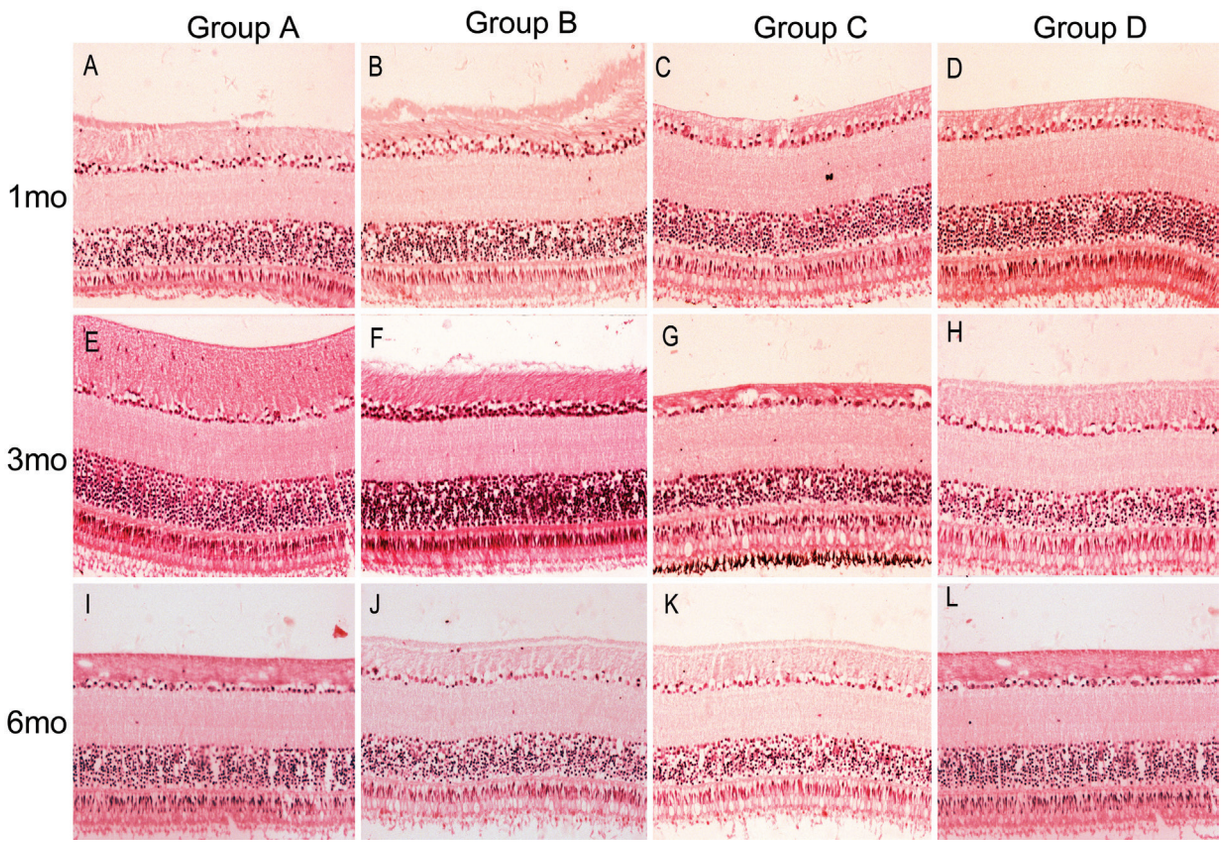

Figure 1 HE staining sections of retinas in each group The cells in the retina were arranged neatly and no obvious pathological changes were observed in each group.

RESULTS

Optical Coherence Tomography Retinal Thickness In the living state, the OCT images of the posterior retinas of the chickens were obtained using a circular scan mode. The obtained images were analyzed using the ImageJ software and the area of the retinal image was measured for component comparison. At 1, 3, and 6mo, the variance analysis of each group was $F1=1.066$ ($P=0.371$), $F2=0.850$ ($P=0.476$), and $F3=0.817$ ($P=0.503$), respectively, and the difference was not statistically significant. The mean area of the retina in each group is shown in Table 1.

Histological Observation

Hematoxylin and eosin staining Under light microscopy, the retinal structure in all groups was clear at all time points, and the inner and outer sections of the photoreceptor were neatly arranged. There was no obvious retinal cell structure disorder or fragmentation observed (Figure 1).

TUNEL staining All sections were visualized through fluorescence microscopy after staining with the TUNEL reagent and DAPI counterstaining. There was no obvious positive staining observed in the retinal nuclei of all groups at all time points. The green fluorescence observed in the picture was the autofluorescence of opsin (Figure 2).

Determination of Malondialdehyde Content and Superoxide Dismutase Activity The MDA content and SOD activity in the retinal homogenate of each group are shown in Tables 2 and 3, respectively. According to variance analysis using Dunnett *t*-test. At 1mo, the MDA content in each group was significantly different ($F=3.652$, $P=0.035$). However, there was no significant difference observed in Groups B and C compared with Group A ($P=0.105$ and $P=0.057$, respectively). Notably, in Group D the MDA content was significantly different versus Group A ($P=0.019$). At 3mo, there was significant difference in MDA content between the three groups ($F=5.784$, $P=0.007$). There was no significant difference between Group B and Group A ($P=0.091$). There was significant difference in Groups C and D compared with Group A ($P=0.026$ and $P=0.003$, respectively). At 6mo,

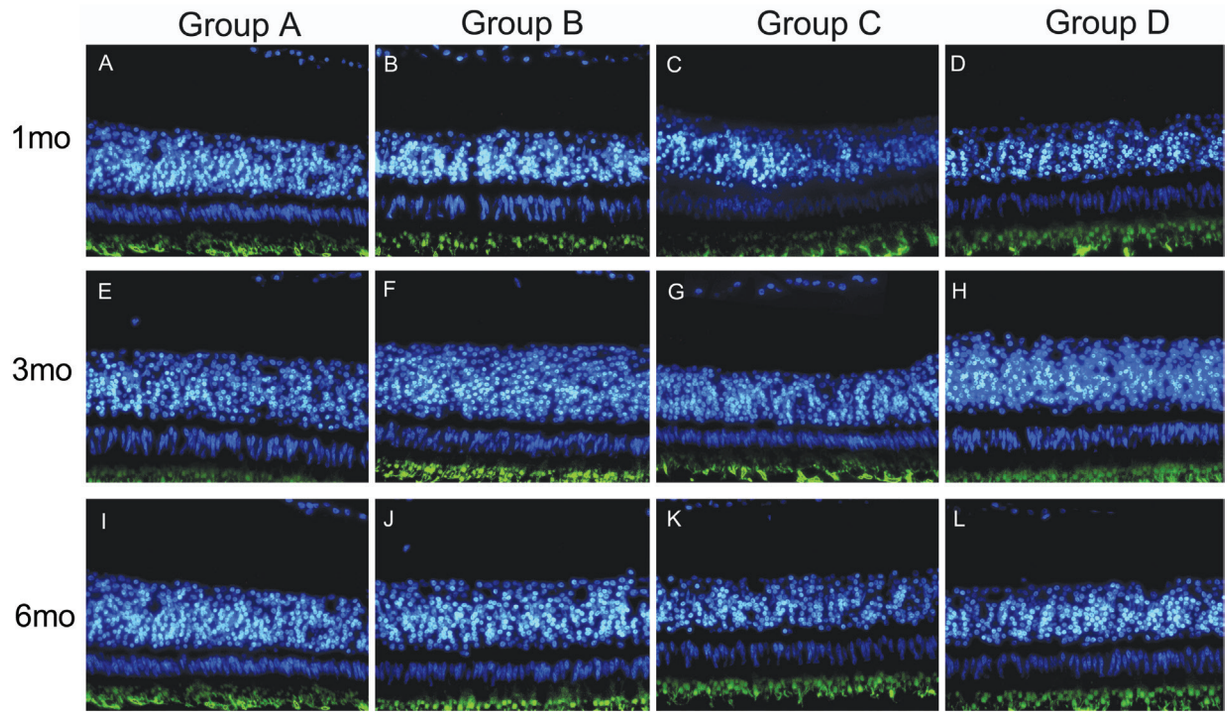

Figure 2 TUNEL immunofluorescence staining The fluorescent images were stained by TUNEL FITC and DAPI. No obvious positive staining was observed in the nucleus of each group. The green fluorescence below was the autofluorescence of opsin.

Table 2 MDA content mean±SD, nmol/mg protein

Month	Group A	Group B	Group C	Group D
1mo	77.12±2.86	82.26±3.78	83.03±3.81	84.32±4.19
3mo	79.81±2.58	83.77±2.72	84.89±2.01	86.83±3.49
6mo	75.18±3.78	85.23±2.00	85.52±3.41	109.32±10.24

Table 3 SOD activity mean±SD, nU/mg protein

Month	Group A	Group B	Group C	Group D
1mo	155.93±5.37	154.96±4.08	153.01±7.09	158.96±4.32
3mo	157.89±4.86	160.21±6.46	159.54±3.27	161.44±3.04
6mo	160.57±3.13	163.59±4.42	163.45±3.32	140.20±5.99

the MDA content in each group was significantly different according to variance analysis (F=31.153, P=0.000). There was significant difference in Groups B, C, and D compared with Group A (P=0.038, P=0.032, and P=0.000, respectively). At 1mo, variance analysis did not reveal significant differences in SOD activity between the groups (F=1.076, P=0.387). At 3mo, variance analysis did not show significant differences in SOD activity between the groups (F=0.513, P=0.679). At 6mo, there was significant difference in SOD activity according to variance analysis (F=33.215, P=0.000). There was no significant difference in Groups B and C compared with Group A (P=0.574 and P=0.607, respectively). There was significant difference between Group D and Group A (P=0.000).

Detection of L/M-opsin and Rhodopsin *via* Western Blotting At 1mo, there was no significant difference in the relative expression of L/M opsin and rhodopsin in all groups [F=0.165 (P=0.919), F=0.611 (P=0.617), respectively]. Similarly, at 3mo, there was no significant difference [F=1.523 (P=0.247), F=1.365 (P=0.289), respectively]. At 6mo, there was no significant difference in the relative expression of L/M opsin between the groups (F=0.333, P=0.802), whereas that of rhodopsin was significantly different (F=7.840, P=0.002). There was no significant difference in Groups B and C compared with Group A (P=0.726 and P=0.559, respectively). In addition, the expression level in Group D was different versus that observed in Group A (P=0.001; Figure 3).

DISCUSSION

In this study, the chicken retina was selected as the experimental subject, considering that it is mainly composed of cone cells, accounting for approximately 80% of retinal photoreceptor cells. Chicken cone cells can be divided into many kinds according to the absorption wavelength of the optic protein. The absorption peaks of long-, middle-, and short-wavelength cone cells are 571, 508, and 455 nm, respectively. Moreover, the absorption peak of rhodopsin in rod cells is 503 nm, which is similar to that reported in human cone-rod cells[13-14]. Moreover, the chicken retina and human macular area do not contain blood vessels. These similarities render the chicken an ideal animal model to simulate the human macular area. Studies investigating eye tissues have shown that the wavelength of red light bioregulation ranges between 630 and 700 nm, while the energy density of a single irradiation ranges between 0.1 and 8 J/cm^2[2,11,15-17]. In this study, three experimental groups were irradiated using a 2 mW laser for 3, 6, and 30min. The corresponding energy densities were 0.459, 0.917, and 4.586 J/cm^2, respectively.

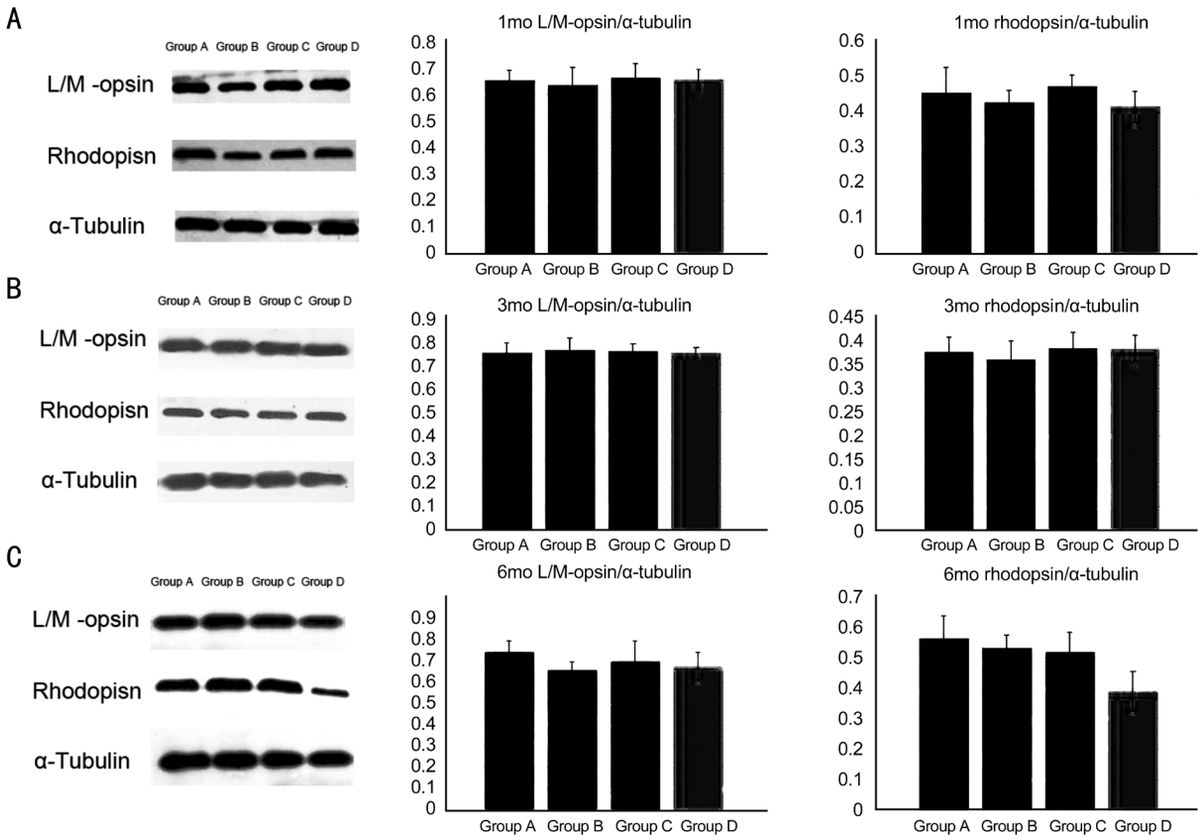

Figure 3 Western blot of L/M-opsion and rhodopsin A, B, and C are the Western results of 1, 3 and 6mo, respectively. There was no significant difference in the relative expression of L/M opsin and rhodopsin in all groups at 1 and 3mo. Whereas the rhodopsin was significantly different (F=7.840, P=0.002) at 6mo. The expression level in Group D was different versus that observed in Group A (P=0.001).

The occurrence of light damage is related to the power of the light, the irradiation time, and the wavelength of the light. Some studies have shown that irradiation with white light at 270 mW/cm^2 (instantaneous power density)[18] can cause light damage. The power of the light used in eye bioregulation is far below this level. However, this does not mean that low-power red light is safe, especially in the case of long-term exposure. It is thought that the excessive production of free radicals is closely related to the development of chronic light injury. The MDA content and SOD activity are commonly used indicators, reflecting the balance of free radicals in cells. The MDA content reflects the intracellular free radical content, while the SOD activity reflects the ability to scavenge free radicals in cells. Normally, the production and removal of intracellular free radicals are in a dynamic balance. The disruption of this balance by external factors, leads to the accumulation of free radicals. Excessive binding of free radicals to lipid membranes, proteins, and bases in nucleic acids in cells affects the normal biological functions of cells and induces apoptosis. There are many reasons for the observed increase in the levels of free radicals caused by chronic light injury, including dysfunction of organelles, excessive photodegradation of lipofuscin, accumulation of intermediate products in visual circulation, *etc*. Studies have shown that rod cells are more prone to light damage than cone cells. This observation may be related to the lower rate-limiting enzyme activity responsible for visual circulation in rod cells versus cone cells. Thus, when rod and cone cells receive the same amount of light, both retinal dimers and intermediates (which are similar to free radicals that disrupt normal cellular function) are more likely to accumulate in rod cells[19-21]. Early retinal photodamage is mainly manifested in cone-rod cells and their outer disc ganglia, including DNA damage and chromosome concentration, optin degradation, abnormal calcium metabolism, and peroxidation of polyunsaturated fatty acids in extraphotoreceptor segments. As an important protein in extracellular segment of cone-rod cells, the content of opsin reflects early injury[22].

In this study, at 1mo, the MDA content and SOD activity in Group D were higher than those reported in Group A. There was no significant difference in SOD activity among groups, which indicated that the generation of free radicals was increased by receiving additional laser irradiation. However, there is no excessive consumption of SOD, the increase of free radicals may only occur temporarily, and the body maintains the balance of free radical scavenging without obvious damage. At 3mo, the MDA content in Groups C and D was higher than that observed in Group A. In addition, there was no significant difference observed in SOD activity between the groups. At 6mo, the MDA content in Groups B, C, and D was higher than that observed in Group A, and the difference

was statistically significant. The MDA content in Group D was significantly higher than that reported in Group A (MDA content: 109.32±10.24 nmol/mg protein). Compared with Group A, there was no significant difference in SOD activity in Groups B and C; however, SOD activity decreased in Group D with statistically significant difference. The results showed that the balance of free radical scavenging was disrupted after 6mo of exposure to laser irradiation, and the accumulation of free radicals suggested the presence of light damage. In our study, we found that the free radicals produced by 650 nm laser irradiation in chicken retina progressively accumulated with time. At 1mo, the MDA content was increased in the 30-min irradiation group. At 3mo, the MDA content was increased in the 6-min and 30-min irradiation groups. At 6mo, the MDA content in each laser irradiation group was higher than that observed in Group A. Although there was no significant decrease in SOD activity after 3-min and 6-min irradiation, damage similar to that reported in the 30-min irradiation group may occur after prolonged irradiation time. At 1, 3, and 6mo, there was no statistically significant difference in L/M-opsin content in all groups. At 1 and 3mo, there was no statistically significant difference in rhodopsin content between the groups. However, at 6mo, the rhodopsin content in Group D was statistically significantly lower than that reported in Group A. This suggests that cone cells did not suffer from obvious damage to the outer disc ganglion during the 6mo of irradiation. In contrast, rod cells treated with 30min of irradiation per day for 6mo showed some damage to the outer disc ganglion and degradation of visual pigments. In this study, there was no significant decrease in retinal thickness, abnormal cell arrangement, and positive apoptotic staining. It may be inferred that chronic light injury does not reach the apoptotic level. However, it may also be hypothesized that cone cells are the main retinal cells in chickens, and the number of rod cells is too few to express in tissue sections and imaging examination at the initial stage of light injury.

In this study, it was found that light damage in rod cells appeared after 6mo of irradiation with a 650-nm laser (power: 2 mW) per day for 30min. Although there was no obvious damage noted in the 3-min and 6-min groups, the possible chronic accumulation of free radicals may lead to damage after prolonged exposure and observation. Furthermore, the sensitivity of the currently available detection methods may not be sufficient to recognize minute defects of light damage. Further studies investigating chronic light damage and the safety of red light photobiomodulation in the eye are required to support the present findings.

ACKNOWLEDGEMENTS

Conflicts of Interest: Wang YP, None; **Liang ZY,** None; **Chen S,** None; **Yang WC,** None; **Kong JH,** None.

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