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Effect of green light spectra on the reduction of retinal damage and stress in goldfish, *Carassius auratus*

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ABSTRACT

We investigated the effect of light spectra on retinal damage and stress in goldfish using green (530 nm) and red (620 nm) light emitting diodes (LEDs) at three intensities each (0.5, 1.0, and 1.5 W/m^2). We measured the change in the levels of plasma cortisol and H_2O_2 and expression and levels of caspase-3. The apoptotic response of green and red LED spectra was assessed using the terminal transferase dUTP nick end labeling (TUNEL) assay. Stress indicator (cortisol and H_2O_2) and apoptosis-related genes (caspase-3) decreased in green light, but increased in red light with higher light intensities over time. The TUNEL assay revealed that more apoptotic cells were detected in outer nuclear layers after exposure to red LED over time with the increase in light intensity, than the other spectra. These results indicate that green light efficiently reduces retinal damage and stress, whereas red light induces it. Therefore, red light-induced retina damage may induce apoptosis in goldfish retina.

1. Introduction

Fish retina absorbs light of different wavelengths. Light is transmitted to the brain through the photoreceptor in the retina, and affects behavioral and physiological responses and causes stress in fish [1–3]. When fish are stressed by the light environment, their immune function deteriorates [4], resulting in an adverse effect on growth and maturation [5], possibly affecting their survival. In fish, environmental stress can induce oxidative stress, and an increased generation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$) [6]. Also, when fish are exposed to stressful environments, such as light and thermal change, the hypothalamus-pituitary-interrenal (HPI) axis is activated, facilitating the release of adrenocorticotropic hormone (ACTH) that stimulates the synthesis and release of cortisol [7,8]. Increased cortisol suppressed the metabolism and decreased the fertility [9,10].

Although there are many similarities between fish and human retinas, there are some differences [11,12]. First, fish do not have eyelids, and cannot adjust the iris to protect the retina from high light intensity. To overcome this, fish control the melanin granules, or the expression of the photoreceptor cells [13,14]. Therefore, when fish are subjected to light stimulation, melanin granules wrap the photoreceptor to form a thick layer of retinal pigment epithelium (RPE) to protect the retina from light [13]. Second, fish retina can be renewed, even after the retinal cell death, unlike most vertebrates [15,16]. According to Vihtelic and Hyde [17], after adapting zebrafish, *Danio rerio,* to an environment without light for 7 days, then suddenly exposing the fish to a strong light of 8000 lux, rod and cone cell death (apoptosis) occurred. After 28 days of light exposure, the regeneration of cells was promoted through the differentiation of rod and cone cells.

During apoptosis, cysteine dependent aspartate-specific proteinases (caspases) function as intercellular proenzymes. Apoptosis-related proteolysis is largely achieved by a gene family of caspases [18]. Caspase-3 plays a fundamental role during apoptosis, including DNA damage, and inflammation resulting from biochemical and morphological changes [19,20]. Wu et al. [21] showed that illumination of 0.64 W/m^2 or more light on rat retinal cells, sharply increased caspase-3 mRNA expression in photoreceptor cells, promoting apoptosis.

Recent studies have shown the relationship between various light wavelengths and stress in fish [22–24]. In particular, among various light sources, light emitting diodes (LEDs) have the dual advantage of emitting light within a specific wavelength range with

Abbreviations: LEDs, light emitting diodes; TUNEL, terminal transferase dUTP nick end labeling.

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easily adjustable sensitivity. Thus, LEDs are effective for such studies [25]. Although the effects of light on fish stress and its physiological changes have been studies [22,26], there are limited studies on the sensitivity of fish retina to the wavelength of light. LEDs can be efficiently applied to lighting systems, and are effective in the fish farming industry, because the environmental sensitivity of certain species can be controlled [23,27].

In the present study, we investigated the effect of light spectra on retinal damage and stress in goldfish, *Carassius auratus*, using green (peak at 530 nm) and red (peak at 620 nm) LEDs at three intensities each (0.5, 1.0, and 1.5 W/m^2), and a white fluorescent bulb as a control. We measured the changes in the levels of cortisol, H_2O_2, and caspase-3. We also evaluated the apoptotic response of green and red LED spectra using the terminal transferase dUTP nick end labeling (TUNEL) assay.

2. Materials and methods

2.1. Experimental fish

For each experiment, common immature goldfish (total length, 6.1 ± 0.5 cm; mass, 12.5 ± 0.4 g) were purchased from a commercial aquarium (Busan, Korea) and maintained in seven 300-L circulation filter tanks prior to experiments in the laboratory. The experimental conditions were performed in duplicate with 25 fish per tank. The water temperature was maintained at 22 ± 1 °C using an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Buchoen, Korea). The goldfish were acclimated to the experimental conditions for 24 h.

The light control group was exposed to light from a white fluorescent bulb (27 W, wavelength range 350–650 nm); the light intensity at the water surface was approximately 0.96 W/m^2. The experimental groups were exposed to green (peak at 530 nm) and red (peak at 620 nm) LEDs (Daesin LED Co. Kyunggi, Korea) were placed 40 cm above the surface of water; the light intensity at the water surface was approximately 0.5, 1.0, and 1.5 W/m^2 (Fig. 1). The fish in the control and experimental groups were exposed to a 12-h light:12-h dark photoperiod (lights on at 07:00 h and lights off at 19:00 h). The fish were reared under these conditions with a daily feeding of commercial feed until the day prior to sampling. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, CO, USA). The fish were anesthetized with 200 mg/L 2-phenoxyethanol (Sigma, St. Louis, MO, USA) to minimize stress by spinal transaction weekly at 11:00 a.m. for 4 weeks prior to blood and tissue (retina and pituitary) collection. Blood was collected rapidly from the caudal vein using a 1 mL syringe coated with heparin. Plasma samples were separated from blood samples by centrifugation (4 °C, 10 000*g*, 10 min) and stored at −80 °C until analysis. The tissue samples were removed from the fish, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. Quantitative PCR (qPCR)

Total RNA was extracted from the retina and pituitary using the TRI reagent® (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed in a total volume of 20 μL, using an oligo-d(T)$_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting cDNA was stored at 4 °C for use in quantitative (q) PCR. qPCR was conducted to determine the relative expression levels of caspase-3 and β-actin mRNA using cDNA reverse-transcribed from the total RNA extracted from the retina. The primers used for qPCR are designed with reference to the known sequences of the goldfish (GenBank accession numbers: caspase-3, KT962123; β-actin, AB039726): caspase-3 forward (5′-GCT CCA CAG AAG TAT CGG-3′) and reverse (5′-GTT ACA ATG ACC AGA CAG TTG-3′) primers; β-actin forward (5′-TTC CAG CCA TCC TTC CTA T-3′) and reverse (5′-TAC CTC CAG ACA GCA CAG-3′) primers. PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR program was as follows: 95 °C for 5 min, followed by 50 cycles of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with β-actin, and all data are expressed relative to the corresponding β-actin threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using the 2−ΔΔCt method: [$\Delta\Delta Ct = 2^{-}(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.3. Caspase-3 concentration

The retina tissues were homogenized in 1 × PBS. The resulting suspension was subjected to two freeze-thaw cycles to further break the cell membranes. After that, homogenates were centrifuged at 1500*g* for 15 min. The supernatant were used for the

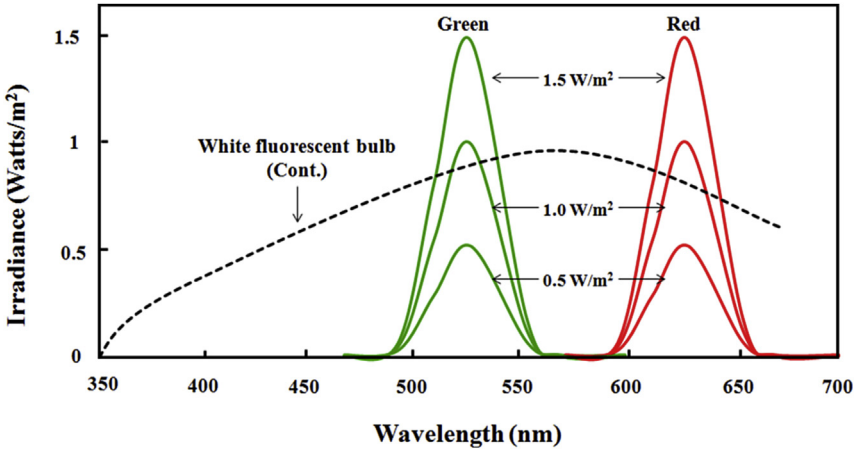

Fig. 1. Spectral profiles of the green (530 nm) and red (620 nm) LEDs with light intensities of 0.5, 1.0, and 1.5 W/m^2. The dotted line shows the spectral profile of white fluorescent bulbs (control). Reprinted from Shin et al. [23], with permission from *Comparative Biochemistry and Physiology Part A*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

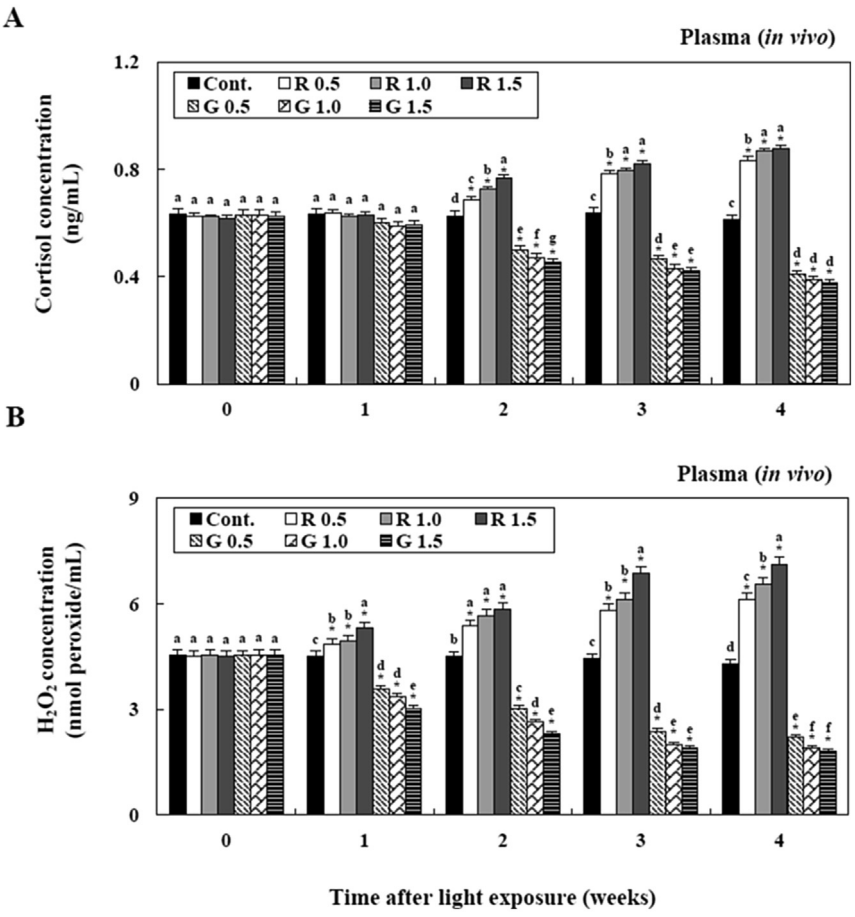

Fig. 2. Changes in the concentration of plasma cortisol (A) and H_2O_2 (B) under red (R) and green (G) LEDs, its irradiance was approximately 0.5, 1.0, and 1.5 W/m^2, and a white fluorescent bulbs (control), as measured by an enzyme-linked immunoassay. Values with different characters are significantly difference exposed to the different LEDs spectra in fish within the same time ($P < 0.05$). The asterisks (*) indicates significant differences between times within the same LEDs spectra ($P < 0.05$). All values are means ± SE ($n = 5$).

analyses. An immunoassay technique was used to determine the caspase-3 concentration using Fish Caspase 3 (CASP3) ELISA kit (MBS012786, Mybiosource Inc., USA). The caspase-3 concentration of retina was analyzed using a microplate reader.

2.4. TUNEL assay

To evaluate the apoptotic response of retina tissue after exposed to red and green LED spectra, we performed the TUNEL assay using the commercially available *In Situ* Cell Death Detection Kit, Fluorescein (TUNEL technology, Cat. No. 11 684 795 910; Roche, Switzerland). Slides were coated with polylysine to ensure the adherence of apoptotic cells. After rearing for 4 weeks under red and green LED spectra, the retina were washed and fixed with 4% buffered paraformaldehyde and permeabilized with freshly prepared 0.1% Triton X-100, 0.1% sodium citrate solution. These retinas were then incubated with the TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. The slides were washed three times with PBS, and the incorporated biotin-dUTP was detected under a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon, Japan). For the paraffin-embedded tissue sections, slides were dewaxed and fixed according to standard protocols and then were treated as described above. The green fluorescent cells indicated apoptotic cells.

2.5. Plasma parameter analysis

Plasma was collected using heparin as an anticoagulant, and centrifuged at 1000*g* for 15 min. The supernatant was used for the analyses. The cortisol levels were determined by an immunoassay technique using a Fish cortisol ELISA kit (CSB-E08487f, Cusabio biotech Co., LTD., China).

Plasma H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. [28] and a PeroxiDetect™ kit (Sigma). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve. The concentrations are expressed as nmole peroxide/mL.

2.6. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A two-way ANOVA followed by Tukey's post-hoc test was used to compare differences in the data ($P < 0.05$). The values are expressed as the means ± standard error (SE).

3. Results

3.1. Plasma cortisol and H_2O_2 concentration

The plasma cortisol concentration in the red LED groups was significantly higher than that in the control and green LED groups

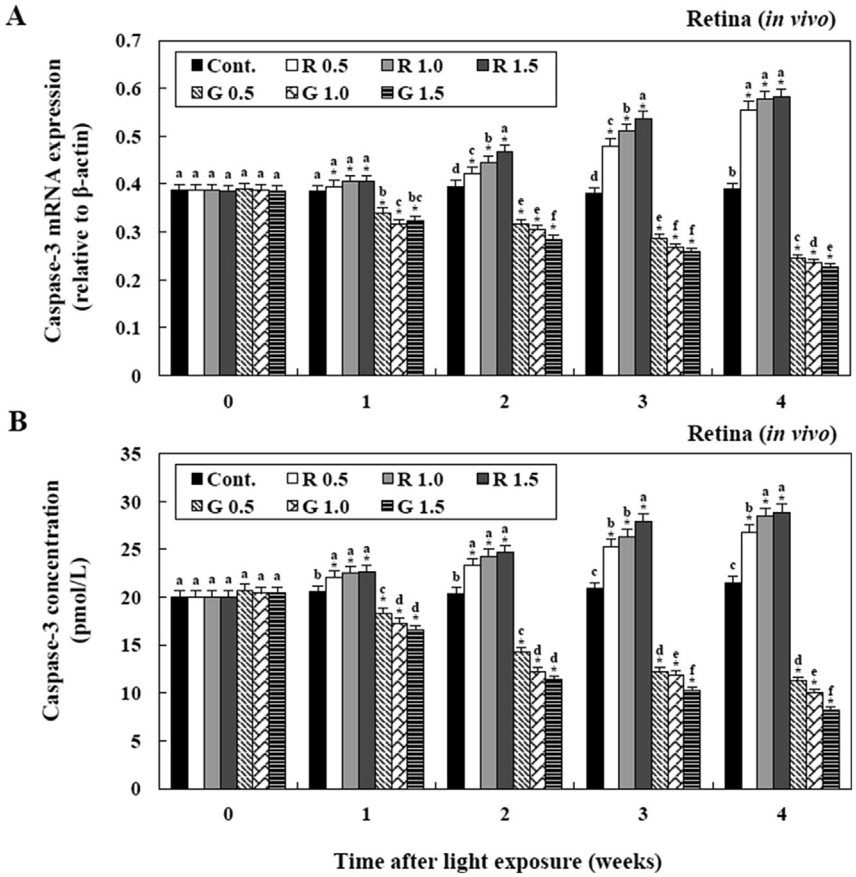

Fig. 3. Changes in the expression levels of caspase-3 mRNA (A) and caspase-3 concentration (B) in the retina under red (R) and green (G) LEDs, its irradiance was approximately 0.5, 1.0, and 1.5 W/m^2, and a white fluorescent bulbs (control), as measured by quantitative PCR. Values with different characters are significantly difference exposed to the different LEDs spectra in fish within the same time ($P < 0.05$). The asterisks (*) indicates significant differences between times within the same LEDs spectra ($P < 0.05$). All values are means ± SE ($n = 5$).

(Fig. 2A). Green LED groups at 4 week had lower cortisol concentration than the other light groups. In particular, with increased time and light intensity, the cortisol concentration in the green LED groups (1.5 W/m^2 intensity at 4 week; 0.38 ± 0.02 ng/mL) decreased, while it increased in the red LED groups (1.5 W/m^2 intensity at 4 week; 0.88 ± 0.04 ng/mL).

The Plasma H_2O_2 concentration of red LED groups was significantly higher than controls and green LED groups (Fig. 2B). 1.5 W/m^2 intensity in red LED group at 4 weeks are the highest than other light groups. In particular, as time passes and as intensity of light increases, the concentration in red LED group (1.5 W/m^2 intensity at 4 weeks; 7.12 ± 0.2 nmole peroxide/mL) was increased, while in green LED group (1.5 W/m^2 intensity at 4 weeks; 1.80 ± 0.2 nmole peroxide/mL) was decreased.

3.2. Changes in caspase-3 mRNA expression levels and concentration (in vivo and in vitro)

The expression of caspase-3 mRNA using cDNA extracted from the goldfish retina significantly increased in the red LED group with increasing time and light intensity (Fig. 3A). At 4 week, caspase-3 mRNA expression levels in the green LED group significantly decreased with increasing time and light intensity by 0.62-, 0.60-, 0.58-fold for the 0.5, 1.0, and 1.5 W/m^2 intensity groups, respectively than those in the control group, while caspase-3 mRNA expression levels of 0.5, 1.0, and 1.5 W/m^2 intensity groups in the red LED group increased by approximately 1.42-, 1.48-, and 1.49-fold, respectively, than those in the control group (Fig. 3A). The caspase-3 concentration in red LED groups was significantly higher than that in the control and green LED groups. Green LED groups at 4 week had the highest caspase-3 concentration than the other light groups. In particular, with increasing time and light intensity, the caspase-3 concentration in the red LED group (1.5 W/m^2 intensity at 4 week; 28.84 ± 1.4 pmol/L) increased, while in the green LED group (1.5 W/m^2 intensity at 4 week; 8.23 ± 0.5 pmol/L), it decreased (Fig. 3B).

3.3. TUNEL assay

There were visible significant differences in cells labeled by the TUNEL assay between control (white), red, and green LEDs (Fig. 4). The apoptotic cells decreased in number after exposure to green LED than those in the control. In contrast, many apoptotic cells were detected after exposure to red LED than in the other groups.

4. Discussion

This study investigated how irradiation of specific wavelengths regulates stress and apoptosis in retina of goldfish. We irradiated them with various light wavelengths in their experimental tanks (using a white fluorescent light bulb, 27 W; green LED, 530 nm; red LED, 630 nm). We subsequently analyzed the changes in the oxidative stress. Furthermore, we measured change of plasma caspase-3 levels, in order to investigate how particular wavelengths

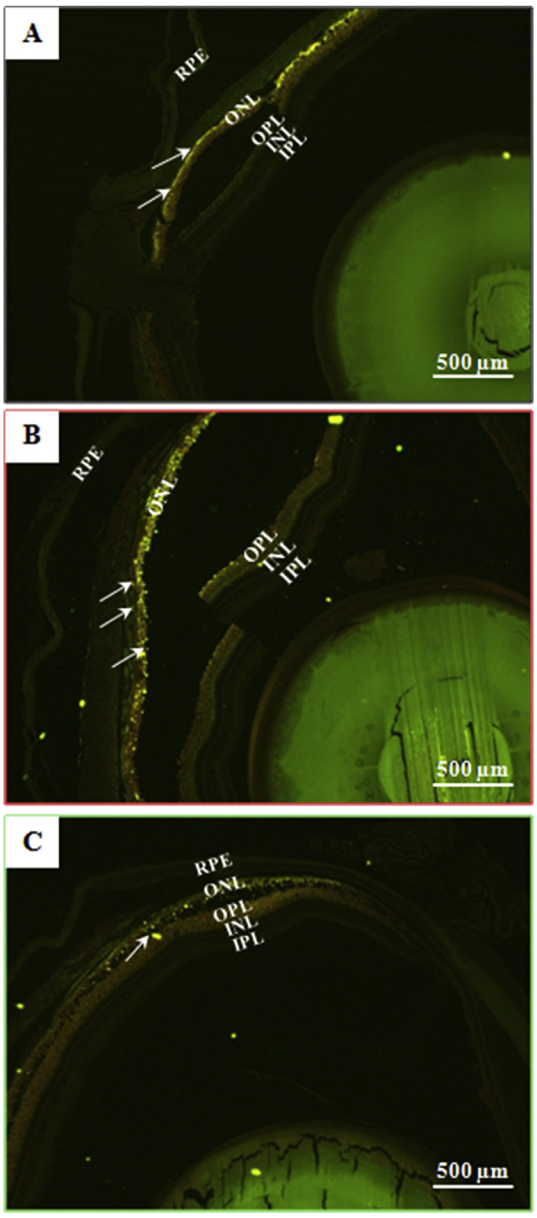

Fig. 4. TUNEL detection of retina cell apoptosis of goldfish under different lighting conditions for 4 week using a white fluorescent bulb (control, A), red (B), and green (C) LEDs at 1.5 W/m^2 light intensity. Cells were stained with acridine orange and then imaged using a fluorescence microscope. Green fluorescent cells and white arrows indicate apoptotic cells. Scale bar = 500 μm. Thickness in the peripheral retina for nuclear (ONL; outer nuclear layers and INL; inner nuclear layers), synaptic layers (OPL; outer plexiform layer and IPL; inner plexiform layer), and RPE (retinal pigment epithelium). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

influenced apoptosis at relatively various light wavelengths.

In the present study, cortisol concentrations decreased in the green LED group, which suggested that green spectra suppress stress in fish. In contrast, cortisol and H_2O_2 concentrations increased in the red LED group, which suggested that red spectra induce stress in fish. The results of the present study are in agreement with a previous study by Mariana et al. [29], which demonstrated that when human retinas were illuminated using red wavelengths for a certain period, plasma cortisol increased. Van der Salm et al. [26] demonstrated that cortisol levels of red porgy, *Pagrus pagrus,* in rearing aquaria with red background considerably increased than those with white backgrounds. Volpato and Barreto [22] demonstrated that the cortisol levels of Nile tilapia, *Oreochromis niloticus* in the green LED group were significantly lower than those in the other groups. In the present study, similar results were obtained for the green LED groups, whereas in the red LED groups, the cortisol levels were significantly higher than those in the control. This suggested that green spectra reduced stress, whereas red spectra induced stress in fish retina. In addition, higher light intensities induced more stress.

When comparing the different light groups, plasma H_2O_2 concentrations in the fish irradiated with green light were lower than the control and red groups, whereas the opposite was true for the goldfish receiving only red light. The latter treatment also showed an increase in oxidative stress at higher light intensities (i.e., 1.5 vs.1.0 vs. 0.5 W/m^2). These results are similar to those of Choi et al. [24], showed that starvation caused oxidative stress in cinnamon clownfish (*Amphiprion melanopus*), with groups that were exposed to green and blue (LED) light having significantly decreased levels of H_2O_2. In contrast, cinnamon clownfish exposed to red (LED) light displayed significantly increased H_2O_2 levels in their plasma. In sum, the results of our study are similar to those of previous studies that green LED light can play a role in reducing this temperature-induced oxidative stress and ROS generation.

To confirm this reducing effect of green light irradiation in apoptosis on the fish retina, we analyzed the goldfish retina tissue for their caspase-3 activity and mRNA expression. Thus, in the present study, green spectra suppressed oxygen free radical (H_2O_2) and reduced the levels of caspase-3, suggesting that green spectra reduce stress in fish retina in contrast to red spectra. The results of caspase-3 are in agreement with those of a previous study by Kuse et al. [30], which demonstrated that when murine cone photoreceptor-derived cells (661 W) were exposed to green LED, antioxidant genes and caspase-3 expression decreased. Kanan et al. [31] indicated that stress caused by light promoted the production of free radicals, thereby increasing the caspase levels. Therefore, increased caspase-3 levels induced disease in the retina or promoted the progress of apoptosis. Therefore, we consider that our findings, like other studies, show that green light irradiation has similar effects as antioxidant treatments, and can play an important role in reducing apoptosis. Furthermore, it showed that irradiation with green light can reduce the oxidative stress, and hence inhibit caspase-3 activity.

Furthermore, the TUNEL assays we performed showed that the groups held at red LED clearly had increased apoptosis rates compared with the groups held at green and control. The TUNEL assay results of the present study are in agreement with those in a previous study by Kuse et al. [30], which demonstrated that when murine cone photoreceptor-derived cells (661 W) were exposed to white, blue or green LEDs, the number TUNEL-positive cells were lower in the green LED group than in the other spectra. Jaadane et al. [32] showed that when the photoreceptor cells of the rat were exposed to strong light as a stress environment, the TUNEL assay indicated that high generated and distribution of TUNEL-positive cells. This is beyond the levels that photoreceptor cells may recover by themselves, indicating that apoptosis is induced by stress. In the present study, the results indicate that cell death was suppressed with reduced stress from green spectra in goldfish. According to previous studies by Wu et al. [15] and Cheng et al. [16], fish can regenerate the retinal cells, and it is likely that the green spectra play an effective role in the regeneration of goldfish retina.

In conclusion, 1) Green spectra reduce caspase production and apoptosis, leading to decreased damage of retina. 2) Fewer TUNEL-positive cells in the green LED groups indicate that the retina of goldfish recognize green spectra as a stable environment. It is possible that green spectra effectively enhance the regeneration of

retina cells. Therefore, further studies are required to elucidate retina cell-death mechanisms in relation to different light spectra.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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