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Effects of blue light spectra on retinal stress and damage in goldfish (*Carassius auratus*)

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**Abstract** There have been a number of studies on the negative effects of blue light exposure in various species; however, little information is available on the impacts of blue light intensity and duration on fish. We investigated the effects of blue light spectra on stress in the retinas of goldfish, using a blue (460 nm) light-emitting diode (LED) at three intensities (0.5, 1.0, and 1.5 W/m²). The experiment was conducted for 4 weeks, and sampling was performed at intervals of 1 week. We measured changes in the expression of cortisol, and the concentrations of hydrogen peroxide (H₂O₂), melanin-concentrating hormone receptor (MCH-R), and caspase-3 in the retinas of goldfish. In addition, we measured histological changes in the retina. We used a transferase dUTP nick end labeling (TUNEL) assay to evaluate the apoptotic response to blue LED spectra. Levels of cortisol, H₂O₂, MCH-R, and caspase-3 increased with exposure time and light intensity. Histological analysis revealed that the thickness of melanin granules increased with exposure time and light intensity. The progressive TUNEL assay revealed many apoptotic cells after exposure to blue LED light, increasing with exposure time and light intensity. Irradiation with blue light for longer than 1 week induced increased retinal stress and may induce apoptosis in the retinas of goldfish, even at a low intensity.

**Keywords** Blue light · *Carassius auratus* · Cortisol · Retinal damage · Stress

**Introduction**

For humans, the visible spectrum is 380 to 800 nm but for fish the visible spectrum is 277 to 737 nm, which includes UV wavelengths (Thibos et al. 1992; Palacios et al. 1998). Thus, goldfish retinas absorb lights of various wavelengths and have a wider range of sensitivity than humans. Light is transmitted to the brain through photoreceptors in the retina (Fischer et al. 2013) and induces behavioral and physiological responses in fish (Papoutsoglou et al. 2000; Owen et al. 2010). High light intensity can induce retinal and internal body stress and inhibit the immune response in fish (Migaud et al. 2007).

Fish retinas have photoreceptor cells that recognize visible light (277–780 nm) (Kuse et al. 2014) and chromophores (Youssef et al. 2011). The photoreceptors, or light-accepting cells, consist of rod and cone cells. The chromophores of the cone cells include short-wavelength opsin (S-opsin), medium-wavelength opsin (M-opsin), and long-wavelength opsin (L-opsin), and the chromophore of the rod cell is called rhodopsin (Sung and Chuang 2010; Youssef et al. 2011). Blue light (400–495 nm), which is a short wavelength of the visible light spectrum, has high enough energy to penetrate tissues, unlike some other wavelengths (Kuse et al. 2014). Therefore, damage from exposure to blue light is possible. In rats, damage to the intraocular...
photoreceptor has been caused by blue light but not by green light (Grimm et al. 2000).

When the retina is excessively exposed to light, cell regeneration proceeds very rapidly by photoreactivation (Grimm et al. 2000). This process involves S-opsin agglutination due to the strong light energy, resulting in denaturation of proteins in cone cells. M-opsin aggregation, which occurs along with S-opsin aggregation, is easily mitigated by proteasome degradation, but S-opsin aggregation is not easily mitigated (Zhang et al. 2011). Therefore, the strong energy of blue light induces the production of reactive oxygen species (ROS), which damage the retinal mitochondria (Godley et al. 2005).

Although there are many similarities between the retinas of fish and humans, there are also some differences (Wagner 1990; Kusmic and Gualtieri 2000). First, fish do not have eyelids and cannot control the size of the iris to protect the retina from intense light. Instead, fish control the distribution of melanin granules or the regeneration of photoreceptors in the retina (Allen and Hallows 1997; Allison et al. 2006). In other words, in fish exposed to light, melanin granules migrate within the inter-digits of the retinal pigment epithelium (RPE) cells to protect the outer segments of the photoreceptors from excessive light (Allen and Hallows 1997). Second, unlike other vertebrates, fish can regenerate their retina after retinal cell death (Wu et al. 2001; Cheng et al. 2006).

In fish, melanin-concentrating hormone (MCH) induces melanin to form granules (Rance and Baker 1979; Eberle 1988). This hormone acts on various physiological functions such as hypothalamus-pituitary-interrenal (HPI) axis regulation and stress response (Baker 1994; Gilchriest et al. 1999). Caspase-3 is widely used as a key indicator of apoptosis. This enzyme plays a central role in apoptosis, which is caused by various biochemical and morphological changes such as DNA damage and inflammation (Kerr et al. 1972; Hacker 2000). Wu et al. (2002) reported that exposure to blue light, as well as strong intensities of light, catalyzes apoptosis by promoting caspase-3 activity in photoreceptor cells.

Cortisol is a general stress indicator in various organisms. In fish, cortisol is used to measure the degree of stress in the body (Wendelaar 1997; Fevolden et al. 2002). In general, stress causes the production of ROS such as hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), hydroxyl radicals (OH$^-$), and singlet oxygen (1O$_2$) (Roch 1999). Excess ROS attack cells and impair cell functions (Kim and Phyllis 1998; Pandey et al. 2003) and can be used as stress indicators.

Recent studies have examined the relationships between various light sources and stress in fish (Volpato and Barreto 2001; Shin et al. 2011; Choi et al. 2012). Light-emitting diodes (LEDs) have advantages over other light sources in studies, due to their low power consumption, long life, and high efficiency. LEDs can emit light within a specific wavelength range with easily adjustable sensitivity (Migaud et al. 2007). They can be effectively applied to lighting systems used in the fish culture industry because they meet the needs of certain species that are sensitive to light. Thus, LEDs are appropriate for use in light-related research (Villamizar et al. 2009; Shin et al. 2011).

We investigated the effect of blue light on retinal stress and damage in goldfish, using blue (peak at 460 nm) LEDs at three intensities (0.5, 1.0, and 1.5 W/m$^2$), and using a white fluorescent bulb as a control. We measured changes in cortisol and H$_2$O$_2$ levels and in the height of melanin granules in the retina using histology. In addition, we measured changes in the expression of MCH-R and caspase-3. We also evaluated the apoptotic response of blue LED spectra using a terminal transferase dUTP nick end labeling (TUNEL) assay.

**Materials and methods**

**Experimental fish**

Common goldfish (Carassius auratus) (body length 7.4 ± 0.8 cm; mass 13.4 ± 3.0 g) were purchased from a commercial aquarium (Busan, Korea) and maintained in four 300-L circulation filter tanks prior to laboratory experiments. Four experimental conditions were applied to two tanks each, with 25 fish per tank. The fish were reared with automatic temperature regulation (JS-WBP-170RP; Johnsam Co., Bucheon, Korea), with the water temperature maintained at 22 °C, and were allowed to acclimate to the environmental conditions for 2 weeks prior to the beginning of the experiment.

Experimental groups were exposed to a blue (460 nm) LED (Daesin LED Co., Kyunggi, Korea) at various intensities, in addition to a white fluorescent bulb, while the control group was exposed to the white bulb only. The experiment was conducted for 4 weeks, and sampling was performed at intervals of 1 week. The photoperiod was 12:12-h light:dark (lights on at 07:00 h...
and lights off at 19:00 h). The LEDs were placed 40 cm above the surface of water. The irradiance at the surface of the water in the tank with external light interception was maintained at approximately 0.5, 1.0, or 1.5 W/m² (Fig. 1). The fish received commercial feed daily until the day prior to sampling. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec® ASD, CO, USA). After exposure to the light treatments, fish were anesthetized with 200 mg/L tricaine-methane-sulfonate (MS-222, Sigma, USA) to minimize stress prior to blood collection. Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000×g, 10 min) and stored at −80 °C until analysis. Fish were euthanized by spinal transaction (sampling at 11:00 h), at 1-week sampling intervals over 4 weeks, and the retinal tissue was collected under dim red light.

Real-time quantitative PCR

Total RNA was extracted from the retina using the TRI reagent® (Molecular Research Center, Inc., OH, 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 PCR (qPCR). qPCR was conducted to determine the relative expression levels of MCH-R, caspase-3, and β-actin mRNA using cDNA reverse-transcribed from the total RNA extracted from the retina. The primers used for qPCR are shown in Table 1. 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 protocol was 95 °C for 5 min, followed by 50 cycles at 95 °C for 20 s and 55 °C for 20 s.

H₂O₂ concentration

The retinal tissues were homogenized in 1× PBS. The homogenates were centrifuged at 5000×g for 5 min at 4 °C. The supernatant was removed, and the remaining pellet was used for the analysis. H₂O₂ concentrations were measured according to the modified method of Nouroozzadeh et al. (1994), using a PeroxiDetect™ kit (Sigma). Absorbance was read at 560 nm and the concentration of H₂O₂ was interpolated from a standard curve. The concentrations are expressed as nanomole per milliliter.

Table 1 Primers used for QPCR amplification

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer DNA sequences</th>
</tr>
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<tbody>
<tr>
<td>MCH-R (AB472590)</td>
<td>Forward 5′-GAC GAA TTC AAG GCG AAG AG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TCC ATA AAT GCT TGG CAT GA-3′</td>
</tr>
<tr>
<td>Caspase-3 (KT962123)</td>
<td>Forward 5′-GCT CCA CAG AAG TAT CGG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GTT ACA ATG ACC AGA CAG TTG-3′</td>
</tr>
<tr>
<td>β-Actin (AB039726)</td>
<td>Forward 5′-TTC CAG CCA TTC CTA T-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TAC CTC CAG ACA GCA CAG-3′</td>
</tr>
</tbody>
</table>

Fig. 1 Spectral profiles of the blue light-emitting diode (LED) light (460 nm) at intensities of 0.5, 1.0, and 1.5 W/m². The dotted line shows the spectral profile of the white fluorescent bulb (control group)
Retinal histology

To analyze the retinas exposed to LEDs for 4 weeks, four retinas from each experimental group were fixed in Bouin’s solution and subjected to histological examination. The samples were dehydrated in increasing concentrations of ethanol solution, clarified in xylene, and embedded in paraffin. Sections (5 μm thick) were selected and stained with hematoxylin and eosin for observation under a light microscope (Eclipse Ci, Nikon, Japan), and the images were captured using a digital camera (Eclipse Ci, Nikon, Japan).

Possible changes in retinal morphology were quantified by measuring the migration distance of melanin granules within the RPE. For variable factors, measurements were made using image analysis software (Image Pro Plus, v.4.5, Media Cybernetics, Inc., USA).

Caspase-3 concentration

The retinal tissues were homogenized in 1× PBS and the resulting suspension was subjected to two freeze-thaw cycles to further rupture the cell membranes. After that, the homogenates were centrifuged at 1500×g for 15 min. The supernatants were collected carefully and assays were used for the analysis. The caspase-3 concentration was determined using an immunoassay technique using Fish Caspase 3 (CASP3) ELISA kit (MBS012786, Mybiosource Inc., USA).

Cortisol concentration

The retina tissues were homogenized in 1× PBS. The homogenates were centrifuged at 5000×g for 5 min at 4 °C. The supernatant was removed, and the remaining pellet was used for the analysis. Cortisol concentration was determined using the immunoassay technique using Fish Cortisol ELISA kit (CSB-E08487f, Cusabio Biotech Co., LTD, China).

TUNEL assay

To evaluate the apoptotic response to red and green LED spectra, we performed a TUNEL assay using a commercially available in situ cell death detection kit for fluorescence (11 684 795 910, Roche, Switzerland). To prevent apoptotic cells from slipping off the slides, the slides were coated with polylysine. The retinas of fish that had been reared for 4 weeks under red and green LED spectra were washed and fixed with 4% buffered paraformaldehyde and permeabilized with freshly prepared 0.1% Triton X-100 in 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 treated as described above. Cells that demonstrated green fluorescence were apoptotic.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A one-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).

Results

Cortisol concentration in retina

The cortisol concentration of fish exposed to blue LED light was significantly higher than that of fish in the control group. Fish exposed to blue LED light for 4 weeks had the highest cortisol of all the light groups. The blue LED group that received the longest exposure and highest light intensity (1.5 W/m² intensity for 4 weeks) showed particularly high cortisol levels (0.86 ± 0.04 ng/mL) (Fig. 2a).

H₂O₂ concentration in retina

The H₂O₂ concentration was significantly higher in fish that received blue LED light than in the control group. With increasing exposure time and light intensity, the concentration in the blue LED light group increased. The concentration was highest in the group that received 1.5 W/m² for 4 weeks (6.59 ± 0.33 nmol peroxide/mL) (Fig. 2b).
Retinal histology

To investigate retinal morphology, we performed histological studies of the central region of the retina from fish that received 1.5 W/m² of blue LED light at 4 weeks and fish from the control group, as shown in Fig. 3. We also measured the thickness of the photoreceptor layer. There were no significant differences in the thickness of the photoreceptor layers between the experimental groups. However, the height of the melanin granules in relation to the photoreceptor layer was significantly higher in all the blue LED light groups than in the control group (Fig. 3e, f).

Changes in MCH-R mRNA expression levels

The expression of MCH-R mRNA was significantly higher in the blue LED group and was correlated with exposure time and light intensity. After 4 weeks of exposure, MCH-R mRNA expression levels in the groups that received 0.5, 1.0, and 1.5 W/m² of blue light were approximately 1.64-fold, 1.73-fold, and 1.75-fold higher, respectively, than those in the control group (Fig. 4).

Changes in caspase-3 mRNA expression levels and concentration

The expression of caspase-3 mRNA was significantly higher in the blue LED group and was correlated with exposure time and light intensity (Fig. 5a). Caspase-3 mRNA expression levels in the groups that received 0.5, 1.0, and 1.5 W/m² of blue light were approximately 1.37-fold, 1.42-fold, and 1.43-fold higher, respectively, than those in the control group.

The concentration was significantly higher in groups that received blue light than in the control and was highest in the group that received 1.5 W/m² of blue LED light (25.72 ± 1.3 pmol/L).

TUNEL assay

There were significant visible differences in cells labeled by the TUNEL assay between the groups (Fig. 6); there were more apoptotic cells in groups that had been exposed to blue LED light than in the control group. Fish that received the highest intensity of light (1.5 W/m²) had more apoptotic cells than fish from other groups (Fig. 6).
Discussion

We investigated the effect of irradiation with blue light on stress, retinal damage, and apoptosis in the retinas of goldfish. We also analyzed the evidence of stress in the body as a whole. Plasma cortisol concentrations were significantly higher in fish exposed to blue LED light than those in the control group and were correlated with duration of exposure and light intensity.

The results of the present study support those of a previous study by Migaud et al. (2007), who demonstrated higher plasma cortisol levels in Atlantic salmon (*Salmo salar*) exposed to blue wavelength light for a certain period. In addition, as the intensity of light...
increased, the concentration of cortisol increased. In the present study, the cortisol concentration was significantly higher in the group exposed to blue LED light than in the control group. Evidently, blue wavelength light causes stress in fish and the amount of stress is associated with the intensity of light.

The plasma concentration of H$_2$O$_2$, a type of ROS induced by stress, was also significantly higher in the groups that received blue LED light than in the control group and was correlated with duration of exposure and light intensity. These results are similar to those of Gaillard et al. (1995), who showed that intense blue light induced production of ROS in the retina, which ultimately destroyed the cell membranes of retinal cells and reduced retinal function. Godley et al. (2005) also reported that the increasing concentration of ROS in RPE cells induced by exposure to blue light was closely associated with mitochondria possessing blue-sensitive chromophores. Photoreceptor cells in the retina contain S-opsin, a protein that absorbs light with short wavelengths, such as blue light. Thus, exposure to blue light activates S-opsin aggregation and cone cell degeneration, which leads to oxidative stress. Blue light appears to induce oxidative stress in goldfish by promoting the production of H$_2$O$_2$.

We also observed differences in retinal damage according to the intensity of the wavelength. Melanin granules in the retina thicken when stimulated by light and the melanin granules were thicker in the blue LED group than in the control group, and thickest of all in the blue LED group that was exposed for 4 weeks.

Migaud et al. (2007) reported that when Atlantic salmon (Salmo salar) were exposed to blue light, the layer of melanin granules was thicker than at night time, when they were not exposed to light. Bogerts (1981) also reported that in humans under stress, MCH was secreted from the retina and melanin pigment was formed as a byproduct, forming a melanin granule layer. Therefore, in the present study, we conclude that goldfish formed thick melanin granules to protect their retinas from blue light. This conclusion is supported by the differences we observed in the expression level of MCH-R mRNA in the retina, indicating that the degree of melanin aggregation in goldfish retina was correlated
with duration of light exposure and light intensity. The expression of MCH-R mRNA increased significantly as blue light exposure and intensity increased.

MCH is known to induce melanin aggregation and regulate the stress response in fish through the HPI axis (Gilham and Baker 1985). According to Green and Baker (1991), repeated administration of saline to rainbow trout promoted higher MCH levels in plasma, which stimulated the HPI axis and promoted stress-related hormone secretion. As a result, the secretion of MCH increased due to stress. The expression of MCH-R in the retina also increased due to increased MCH.

To confirm the effect of blue light irradiation on apoptosis in fish retinal cells, we analyzed goldfish retinal tissue for caspase-3 activity and mRNA expression. Both the expression of caspase-3 and its concentration significantly increased with the duration of blue light exposure and light intensity. These results are similar to those of Wu et al. (2002), who showed that, in rats, the expression of caspase-3 mRNA in the retina increased after irradiation with blue LED light. Sparrow and Cai (2001) also reported that the caspase-3 concentration in human RPE cells was higher after exposure to blue light.

In the present study, as in previous studies, the mRNA expression and concentration of caspase-3 were higher in the group that received blue LED than in the control group. The blue LED wavelength appears to directly increase the concentration of caspase-3 by promoting the production of ROS in the retina and the adverse effects on the retina increase as the intensity of the blue LED wavelength increases.

Exposure to blue light is thought to induce cell death by amplifying stress in goldfish. Jaadane et al. (2015) reported that when photoreceptor cells in rats were exposed to strong light stress, a TUNEL assay indicated high numbers and distribution of TUNEL-positive cells. According to Vihtelic and Hyde (2000), after zebrafish...
(Danio rerio) were denied light for 7 days and were then suddenly exposed to strong light (8000 lx), the death (apoptosis) of rod and cone cells occurred. Our results support these findings, suggesting that photoreceptor cells undergo apoptosis when light stress exceeds their ability to recover.

In conclusion, blue wavelength light promotes the production of ROS in the retina of goldfish and induces stress in the fish, leading to retinal damage (apoptosis). Many aquariums use blue light due to the visual esthetics of aquariums lit with this wavelength. Shin et al. (2011) reported that irradiation with blue light for a short time (less than 28 h) has a positive effect on physiological function (growth, maturation, etc.) in fish. However, irradiation with blue light for longer than 1 week appears to have a negative influence on fish retinas, even at a low intensity (0.5 W/m²). In addition, in the case of fish, which are capable of regenerating retinal cells, chronic exposure to blue light raises the possibility that excessive induction of cell death may lead to slow or no cell regeneration.

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