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Effects of different light wavelengths from LEDs on oxidative stress and apoptosis in olive flounder (*Paralichthys olivaceus*) at high water temperatures

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ABSTRACT

We investigated how different light spectra affect thermal stress in olive flounder (*Paralichthys olivaceus*), using light emitting diodes (LEDs; blue, 450 nm; green, 530 nm; red, 630 nm) at two intensities (0.3 and 0.5 W/m^2) at relatively high water temperatures (25 and 30 °C, compared to a control condition of 20 °C). We measured the expression and activity of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and the levels of plasma hydrogen peroxide (H_2O_2) and lipid peroxidation (LPO). Furthermore, the levels and mRNA expression of caspase-3 were measured, and terminal transferase dUTP nick end labeling (TUNEL) assays of liver and comet assays were performed. The expression and activity of antioxidant enzymes, as well as plasma H_2O_2 and LPO levels were significantly higher after exposure to high temperatures, and significantly lower after exposure to green and blue light. Caspase-3 levels and mRNA expression showed a similar pattern. The TUNEL assay showed that apoptosis markedly increased at higher water temperatures, compared with the 20 °C control. In contrast, green light irradiation decreased apoptosis rate. Furthermore, the comet assays showed that nuclear DNA damage was caused by thermal stress, and that green light irradiation played a role in partially preventing this damage. Overall, these results suggest that light with green and blue wavelengths can reduce both high temperature-induced oxidative stress and apoptosis, and that particularly green light is efficient for this. Therefore, green light can play a role in protecting in olive flounder from thermal stress damage.

1. Introduction

Water temperature is an important environmental factor for fish, as it affects physiological processes such as growth, reproduction, metabolism, and immune system functioning [1,2]. In fish, acute water temperature changes 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 ($^1O_2$) [3,4]. In addition, overproduction of ROS can increase the lipid hydroperoxide (LPO) levels, and negatively affect cell viability by causing cell membrane damage, DNA and proteins denaturation, and an acceleration of apoptosis [5,6].

Fish have various antioxidant systems to protect themselves from stress-induced ROS generation caused by acute environmental change. Generally, the antioxidant enzymes from these systems are activated to directly remove ROS [6]. Antioxidant enzymes involved in endogenous antioxidant mechanisms include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) [7,8]. The basic mechanisms of these antioxidant enzymes are as follows: SOD converts O_2^- into H_2O_2, and CAT and GPX convert the produced toxic H_2O_2 into water and molecular oxygen (O_2), thus eliminating the toxic effects [9,10]. These antioxidant enzymes are found virtually in all tissues of vertebrates, but show in general, high activity in the liver [11,12].

Although cells have numerous mechanisms to protect themselves against stress, enhanced stress caused by sudden changes in

Abbreviations: CAT, catalase; H_2O_2, hydrogen peroxide; LED, light-emitting diode; LPO, lipid hydroperoxide; SOD, superoxide dismutase; TUNEL, terminal transferase dUTP nick end labeling.

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the environment, such as acute water temperature changes, leads to disrupted cell signaling, extensive DNA damage, and cell apoptosis [13,14]. Apoptosis is regulated by the cysteine protease family caspases, and it is characterized by morphological events such as DNA fragmentation and cellular shrinkage [15]. Particularly, caspase-3 is known to play a central role in apoptosis, by influencing biochemical and morphological processes that respond to DNA damage and inflammation [16,17].

Light is known to affect fish physiological responses, including their growth and maturation [18,19]. Furthermore, light-induced changes, including a change in the photoperiod, can cause stress in fish [20,21]. Studies that investigated the influence of light on fish functioning recently showed that light-emitting diodes (LEDs) with specific wavelengths affect diverse physiological responses [22–24]. The wavelength of green light is particularly known for its influence in maintaining or regulating physiological homeostasis, and for alleviating stress in fish exposed to high water temperatures and toxic environments. In contrast, the wavelength of red light can cause stress in fish, and negatively affect fish physiological functioning [8,25]. Furthermore, the light emitted by LEDs can regulate homeostasis or the environmental sensitivity of different fish species, and control the generation of oxidative stress in fish. As a result, LED light may be an effective tool to use in the fish-farming industry [4,24,26].

In this study, we investigated how light with a particular wavelength controls oxidative stress and apoptosis in olive flounder (*Paralichthys olivaceus*) exposed to high water temperatures. For this, we exposed olive flounder to high water temperature (25 and 30 °C) and different wavelengths of light (white fluorescent bulb, 27 W; blue, 450 nm; green, 530 nm; red, 630 nm) and different light intensities (0.3 and 0.5 W/m^2). We measured the expression of mRNA, protein levels, the activity of SOD and CAT, as well as the changes in oxidative stress by measuring plasma H_2O_2 and LPO concentrations. Furthermore, we investigated the changes in caspase-3 mRNA expression and levels, in order to determine the effects of the different light wavelengths on apoptosis. Finally, we conducted terminal transferase dUTP nick end labeling (TUNEL) assays, as well as and comet assays, in order to determine how green light reduced the effects of DNA damage and apoptosis in the liver cells. Particular attention was given to green light, as this wavelength is known to affect ROS scavenging.

2. Materials and methods

2.1. Experimental fish and conditions

From a commercial aquarium (Jeju, Korea), we purchased olive flounders (*P. olivaceus*, length 11.5 ± 0.5 cm; mass 18.2 ± 0.8 g), and the fish subsequently acclimated in eight 300-L circulation filter tanks in the laboratory for two weeks. All fish were first exposed to 20 °C (the control temperature). The water temperature was subsequently increased from 20 °C to 30 °C, with daily increments of 1 °C, using an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Seoul, Korea). At 20, 25, and 30 °C, fish were selected from the tanks to form different experimental groups with five replicate fish per group. The experimental groups were exposed to either blue (peak at 450 nm), green (peak at 530 nm), or red (peak at 630 nm) light from LEDs (Daesin LED Co., Kyunggi, Korea), whereas the control group was irradiated with a white fluorescent bulb (27 W; GX24Q-3, PHILIPS, Amsterdam, Netherlands) (Fig. 1). The photoperiod for all treatments was a 12-h light (L): 12-h dark (D) cycle (lights on at 07:00 and lights off at 19:00). The light control group was exposed to light from a white fluorescent bulb (wavelength range 350–650 nm); placed 50 cm above the water surface and the light intensity at the water surface

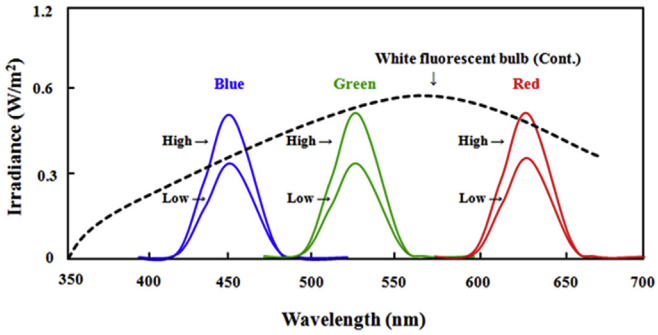

Fig. 1. Spectral profiles of light emitting diodes (LEDs; blue, 450 nm; green, 530 nm; red, 630 nm) and the white fluorescent bulbs (control) used in this study. For each LED light treatment, two different intensities were used (low, 0.3 W/m^2; high, 0.5 W/m^2). Reprinted from Shin et al. [23], with 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.)

was approximately 0.96 W/m^2. The LEDs were placed 50 cm above the water surface, and irradiance levels at the water surface was maintained at approximately 0.3 or 0.5 W/m^2. The fish were kept for 24 h under experimental conditions, with a daily serving of commercial feed until the day before the sampling. Spectral analyses of the white fluorescent bulb and LEDs were performed with a spectroradiometer (FieldSpec, ASD Inc., CO, USA). The fish were anaesthetized with 200 mg/L tricaine methanesulfonate (Sigma, USA) in order to minimize the stress before collection of blood and liver tissue samples. Blood was collected from the caudal vein with a 1-mL syringe coated with heparin. From the blood samples, plasma samples were derived through centrifugation (4 °C, 10,000× *g*, 5 min), and the plasma samples were subsequently stored at −80 °C until the analyses. For total RNA and protein extraction, liver tissue immediately frozen in liquid nitrogen, and stored at −80 °C. Furthermore, for experiment of cell culture and assay of comet and TUNEL, several liver tissues immediately used the each experiment, according to the manufacturer's instruction.

2.2. Total RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from each sample using TRI Reagent® (Molecular Research Center, Inc., USA) and was treated with DNase, according to the manufacturer's instruction. Subsequently, 2 μg of total RNA was reverse transcribed in a 20 μL reaction volume, using an oligo-(dT)$_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. The resulting cDNA was diluted, stored at 4 °C, and subsequently used for PCR and real-time qPCR analyses.

The qPCR analysis was conducted to determine the relative expression levels of caspase-3 and the antioxidant enzymes SOD and CAT, using the total RNA extracted from the olive flounder

Table 1
Primers used for QPCR amplification.

Genes (accession no.)	Primer	DNA sequences
SOD (EF681883)	Forward	5′-CGT TGG AGA CCT GGG GAA TGT G-3′
	Reverse	5′-ATC GTC AGC CTT CTC GTGGAT C-3′
CAT (GQ229479)	Forward	5′-CCA AAC TAC TAT CCC AAC AGC-3′
	Reverse	5′-CCA CAT CTG GAG ACA CCT T-3′
Caspase-3 (JQ394697)	Forward	5′-GCA AAT CGC TGG TGG GAA A-3′
	Reverse	5′-CAT CGT CTA CAC TGT CTG TTT CG-3′
β-actin (HQ386788)	Forward	5′- GGA CCT GTA TGC CAA CAC TG-3′
	Reverse	5′- TGA TCT CCT TCT GCA TCC TG -3′

livers. The qPCR primers pairs to span splice exon-exon junctions were designed using known olive flounder sequences (Table 1). We conducted the qPCR amplification using a Bio-Rad iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and the iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. As a control, the β-actin gene was also amplified for each sample, and all data were expressed as their difference with the corresponding calculated β-actin threshold cycle (Ct) levels. The Ct values of the PCR products formed the basis for all analyses. The Ct levels were defined as the PCR cycle in which the fluorescence signal crossed a threshold during the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) per sample and their internal control (β-actin) were calculated as follows: $[\Delta\Delta Ct = 2^{\hat{}} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})]$. After the PCRs were completed, the qPCR data from three replicate samples were analyzed using Bio-Rad to estimate the transcript copy numbers of each sample.

2.3. Western blot analyses

The total protein content from the olive flounder livers was extracted using a T-PER® tissue protein extraction reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), following the manufacturer's instructions. Subsequently, 30 μg of protein was loaded per lane into Mini-PROTEAN® TGX™ gels (Bio-Rad, Hercules, CA, USA), and a protein ladder (Bio-Rad, Hercules, CA, USA) was used as a reference. The samples were electrophoresed at 180 V; thereafter, the gels were immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) at 85 V for 3 min, using the Trans-Blot® Turbo™ transfer system. Subsequently, the membranes were blocked with 5% milk in Tris-buffered saline buffer (pH 7.4) for 45 min, after which they were washed in the in Tris-buffered saline buffer without milk. Membranes were incubated with SOD antibodies (1:2,000 dilution, NBP1-47443, Novus Biologicals, USA) and CAT antibodies (1:2,000 dilution, sc-58332, Santa Cruz Biotechnology, USA), and subsequently incubated with horseradish peroxidase conjugated anti-mouse IgG secondary antibodies (SOD and CAT dilution 1:2,000, Bio-Rad, Hercules, CA, USA) for 60 min. As the internal control, β-tubulin (dilution 1:5000, ab6046, Abcam, Cambridge, UK) was used. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA), and 30 s of exposure with a Molecular Imager® from ChemiDoc™ XRS + Systems (Bio-Rad, Hercules, CA, USA). The membrane images were scanned using a high-resolution scanner, and the band densities were estimated using Image Lab™ Software, version 3.0 (Bio-Rad, Hercules, CA, USA).

2.4. In vitro caspase-3 treated liver cells culture

Liver cells cultures were prepared using both enzymatic and mechanical procedures for olive founder kept at either green light (530 nm; 0.3 and 0.5 W/m^2) or a white fluorescent bulb. The photoperiod for these treatments was the same as mentioned above. The liver tissue was quickly removed from the fish, and placed in 3 mL ice-cold dispersion buffer (pH 7.4, Dulbecco's phosphate-buffered saline, without calcium chloride or magnesium chloride, containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone; Gibco-BRL, Rockville, MD, USA). The isolated liver tissue was then transferred to 6 mL fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma). The connective tissue and other impurities were removed, and the liver parts were cut into small pieces using scissors. The liver cells and the minced liver tissue were subsequently transferred to a flask, and incubated, with slow stirring, at room temperature for 10 min. The mixture of dispersed liver cells and tissue was filtered, and a culture medium (medium 199, Invitrogen, USA) was added. The cell suspension was centrifuged at 800× *g* for 10 min, and the cells were then resuspended into a fresh culture medium. The liver cells (1.2×10^6 cells 800 μL/well) were then placed in a 24-well tissue culture plate. The liver cells in the culture plate were treated with different levels of caspase-3 (0, 100, 200, and 300 ng/μL). The recombinant caspase-3 was kindly provided by Professor J. Lee (Jeju National University, Jeju, Korea; Elvitigala et al. [27]). We sampled the cells after 6, 12, 24, 36, and 48 h of the caspase-3 treatment. Changes in SOD and CAT mRNA expression in the cells were assessed as described above for the samples.

2.5. Plasma parameter analysis

From the blood, plasma samples were derived through centrifugation (4 °C, 10,000× *g*, for 5 min). H_2O_2 levels in the plasma were measured using a modified version of the methods described by 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. In addition, SOD, CAT, LPO, and caspase-3 levels were analyzed using an immunoassay from an ELISA kit (SOD, CSB-E15929fh; CAT, CSB-E15928fh; Cusabio Biotech, Hubei, China; and LPO, MBS013426; caspase-3, MBS012786; Mybiosource Inc., San Diego, USA).

2.6. Terminal transferase dUTP nick end labeling (TUNEL) assay

To evaluate the apoptotic response of the fish liver cells to green LED light, we performed the terminal deoxynucleotide transferase dUTP nick end labeling technique with a commercially available *in situ* cell death detection kit (11 684 795 910, Roche, Switzerland). To avoid apoptotic cells losing adherence to the slides, the slides were coated with polylysine. The fish liver tissue was washed and fixed with 4% buffered paraformaldehyde, and permeabilized with freshly prepared 0.1% Triton X-100, 0.1% sodium citrate solution. This liver tissue was then incubated with the TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. The slides were washed three times with phosphate buffered saline (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. The green fluorescent cells indicated they were apoptotic.

2.7. Comet assay

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells [29]. The liver cells (1×10^5 cells/mL) were examined using a CometAssay® Reagent kit for single cell gel electrophoresis assay (Trevigen Inc., Maryland, USA), according to the method described by Singh et al. [30], with some modifications. Cells were immobilized in an agarose gel on CometAssay® comet slides and immersed in a freshly prepared alkaline unwinding solution for 20 min. The slides were subsequently electrophoresed at 15 V for 30 min. The samples were stained with SYBR® Green (Trevigen Inc.), and kept for 30 min in the dark. They were subsequently read using a fluorescence microscope (excitation filter 465–495 nm; Eclipse C*i*, Nikon, Japan). At least 100 cells from each slide were assessed. For a quantitative analysis of the comet assays, we used comet assay IV image analysis software (version 4.3.2, Perceptive Instruments Ltd., UK) to analyze the comet tail lengths (distance of DNA migration from head), percentage of DNA in the tails (tail intensity/total intensity in tail),

and the tail moments (amount of DNA damage, product of tail length and percentage of DNA in tail).

3. Results

3.1. Changes in SOD and CAT expression and activity

The levels of mRNA and protein expression, as well as the activity of SOD and CAT, increased significantly with temperature rises in all experimental groups (Fig. 2). Furthermore, the fish that were irradiated with green and blue light had significantly lower (expression) levels than the groups that were irradiated with white fluorescent light, especially at higher light intensities (by approximately 1.2- to 2.1-fold). In contrast, the groups that were irradiated with red light had significantly higher levels of mRNA expression, as well as higher SOD and CAT activities, which increased with light intensity (by approximately 1.2- to 1.7-fold). In particular, the green light groups had significantly lower expression levels of mRNA and protein activity than the other LED treatment groups.

3.2. Change in concentration of H_2O_2 and LPO

The plasma H_2O_2 concentrations in all experimental groups increased significantly at higher temperatures (Fig. 3A). The concentrations of H_2O_2 in the green and blue light groups were significantly lower (~1.4-fold) than the control groups, and this difference increased with light intensity. However, the H_2O_2 concentrations significantly increased (~1.3-fold) in the red light irradiation treatments. The variations in the plasma LPO concentrations were similar to the results of the H_2O_2 concentrations (Fig. 3B). Furthermore, the plasma LPO concentrations in the 30 °C groups were significantly higher than in 20 °C groups. As with the H_2O_2, the plasma LPO concentrations in the green and blue light groups were significantly lower than in control groups.

3.3. Changes in mRNA expression and levels of caspase-3

The caspase-3 levels and mRNA expressions in the 30 °C groups were significantly higher than in the other temperature groups (~3.9-fold, Fig. 4). Furthermore, the caspase-3 levels and mRNA expression in green and blue light exposed fish cells were significantly lower than in control groups that were irradiated with white fluorescent light. In particular, the caspase-3 levels and mRNA expression in the green light groups were significantly lower than in fish cells from groups irradiated with blue or red LEDs. Furthermore, the caspase-3 levels and mRNA expression in the red light groups were higher under temperature stress.

3.4. Change in mRNA expression of SOD and CAT by caspase-3 (in vitro)

In the *in vitro* experiments that used liver cells culture, SOD and CAT mRNA expression in the caspase-3 treatment groups was significantly higher than untreated groups (Fig. 5). In the cells treated with 100 and 200 ng/μL caspase-3, and for all their post-treatment times, the groups irradiated with green light had

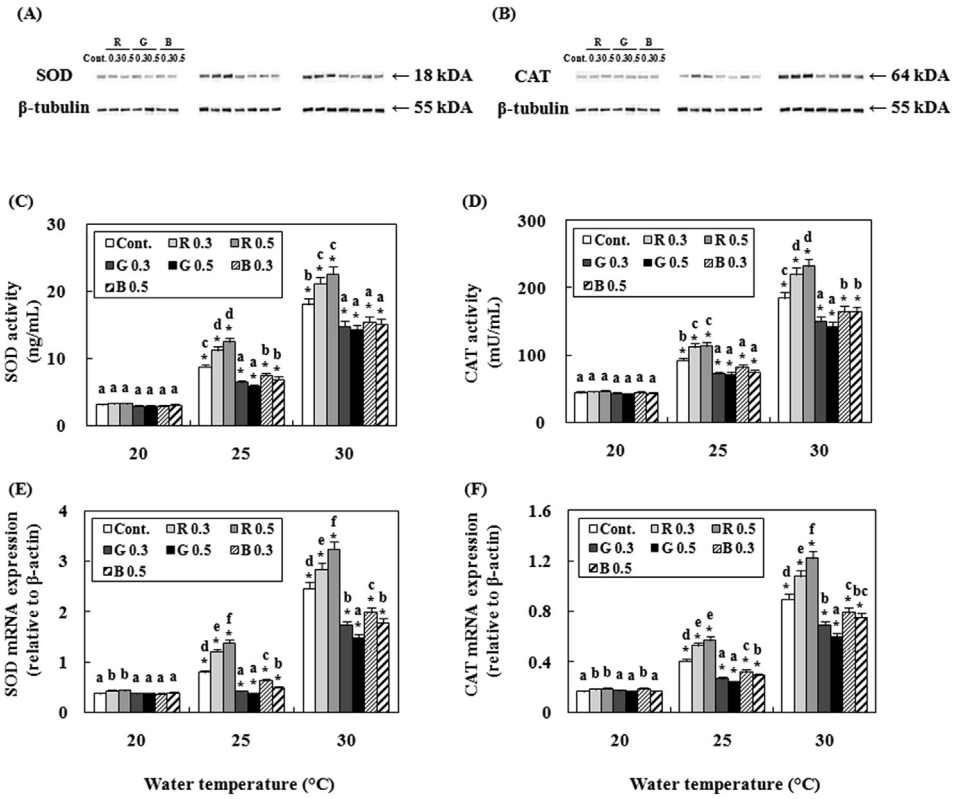

Fig. 2. SOD protein expression (A), activities (C), and relative mRNA expression (E), and CAT protein expression (B), activities (D), and relative mRNA expression (F). Results are shown for different thermal regimes, and groups exposed to different wavelengths of light (red (R), green (G), and blue (B) LEDs) at different irradiance intensities (0.3 and 0.5 W/m^2). or a white fluorescent bulb (Cont.). Results are based on an enzyme-linked immunoassay and quantitative real-time PCR (qPCR), for which 2 μg of total liver RNA was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels in each sample. A western blot was used to determine the expression of antioxidant enzymes [SOD (18 kDa) and CAT (64 kDa)] in the livers during the thermal changes, and β-tubulin (55 kDa) was used as an internal control. The lowercase letters with different characters denote significant differences between groups that received different wavelengths of light and the same temperature regime ($P < 0.05$). The asterisks (*) indicate significant differences between different temperature regimes per wavelength ($P < 0.05$). All values are represented as means ± SE ($n = 5$).

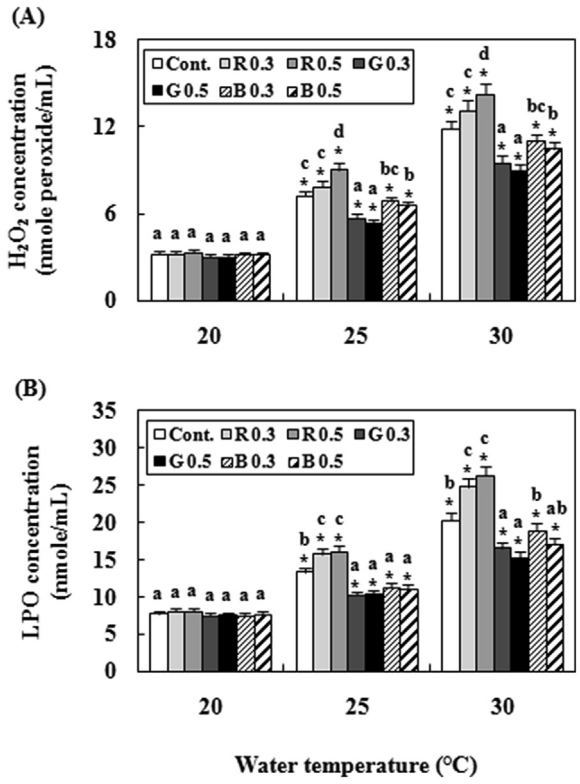

Fig. 3. Concentrations of plasma H_2O_2 (A) and LPO (B) at different thermal regimes and light conditions using red (R), green (G), and blue (B) LEDs, measured with a microplate reader. The lowercase letters with different characters denote significant differences between groups that received different wavelengths of light and the same temperature regime ($P < 0.05$). The asterisks (*) indicate significant differences between different temperature regimes per wavelength ($P < 0.05$). All values are represented as means ± SE ($n = 5$).

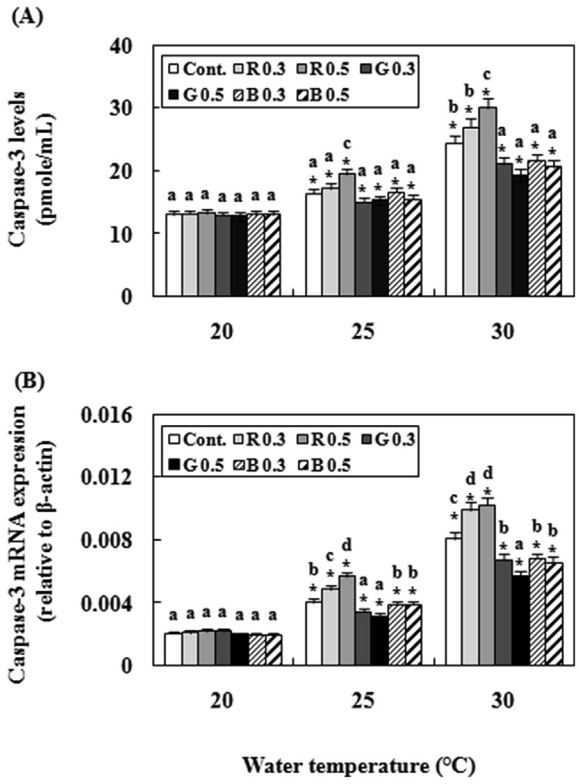

Fig. 4. Plasma caspase-3 levels (A), and caspase-3 mRNA expression levels (B) at different thermal regimes and light conditions using red (R), green (G), and blue (B) LEDs, measured with a microplate reader and quantitative real-time PCR (qPCR). Total liver RNA (2 μg) was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels per sample. The lowercase letters with different characters denote significant differences between groups that received different wavelengths of light and the same temperature regime ($P < 0.05$). The asterisks (*) indicate significant differences between different temperature regimes per wavelength ($P < 0.05$). All values are represented as means ± SE ($n = 5$).

significantly lower SOD and CAT mRNA expression levels than the control groups that received white fluorescent light. The treatments that received 300 ng/μL caspase-3 and were irradiated with green light significantly decreased their SOD and CAT mRNA expression levels at 36 h post-treatment and thereafter. No significant differences were observed between the groups receiving green light at different intensities.

3.5. TUNEL assay

We visually assessed the TUNEL assays of liver, which is an increasingly applied method to investigate active apoptosis (Fig. 6). The assays showed that the liver cells from fish kept at the highest water temperature (30 °C) had higher apoptosis rates than from those kept at 20 °C. Furthermore, cells from the fish irradiated with green light had effectively lower rates of apoptosis than those from the control light group.

3.6. Comet assay

We analyzed comet assays using a fluorescence microscope, in order to determine the effects of green light on damaged cells (Fig. 7), and the DNA damage response was determined by measuring the fraction of cells that had nuclear DNA comet tails (a phenomenon indicative of DNA breaks). The liver cells showed normal nuclear DNA for the fish kept at 20 °C, but cells with damaged nuclear DNA were visible in both the treatments kept at 30 °C (Fig. 7A). Furthermore, the cells from fish kept at 30 °C exhibited significantly higher comet tail lengths, higher percentages of DNA in the comet tail, and increased tail moments compared with those from the 20 °C treatments (Fig. 7B). However, cells from fish irradiated with green light had significantly decreased comet tail lengths, lower percentages of DNA in the comet tail, and decreased comets tail moments. This indicates that green light can play a role in reducing damage to nuclear DNA caused by thermal stress.

4. Discussion

This study investigated how irradiation of specific wavelengths regulates oxidative stress and apoptosis in heat-stress exposed olive flounder. We exposed them to acute temperature changes (20, 25, and 30 °C), and irradiated them with various light wavelengths in their experimental tanks (using a white fluorescent light bulb, 27 W; a blue LED, 450 nm; green LED, 530 nm; red LED, 630 nm). We subsequently analyzed the antioxidant gene and protein expressions, antioxidant enzyme activities, and changes in the oxidative stress. Furthermore, we measured change of plasma caspase-3 levels, in order to investigate how particular wavelengths influenced apoptosis at relatively high water temperatures.

Initially, we analyzed the olive flounder liver tissue for their SOD and CAT levels, activity, and mRNA expression, in order to investigate the effects of high water temperatures on their antioxidant capacity. The result showed that higher temperatures indeed

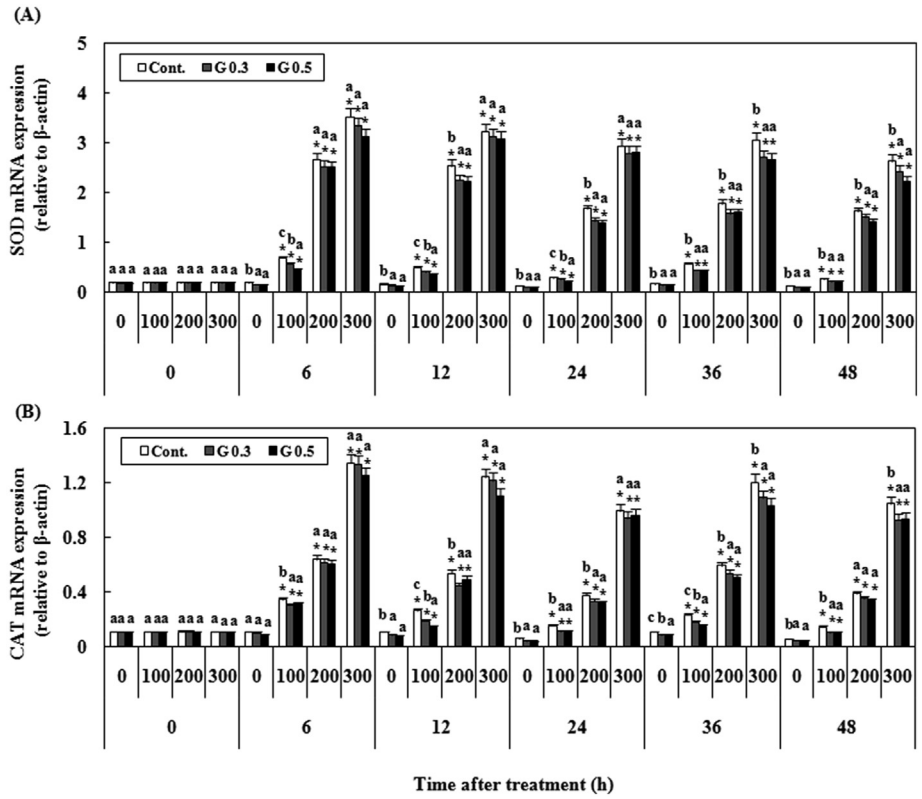

Fig. 5. SOD (A) and CAT (B) mRNA expression levels at white fluorescent light bulb (Cont.), conditions or green LED lights at 0.3 and 0.5 W/m^2, with different caspase-3 treatments (0, 100, 200, and 300 ng/μL), measured with a quantitative real-time PCR (qPCR). Total liver RNA (2 μg) was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels per sample. The lowercase letters with different characters denote significant differences between different wavelength LEDs with the same experimental time ($P < 0.05$). The asterisks (*) indicate significant differences among the different experimental timings per light treatment ($P < 0.05$). All values are represented as means ± SE ($n = 5$).

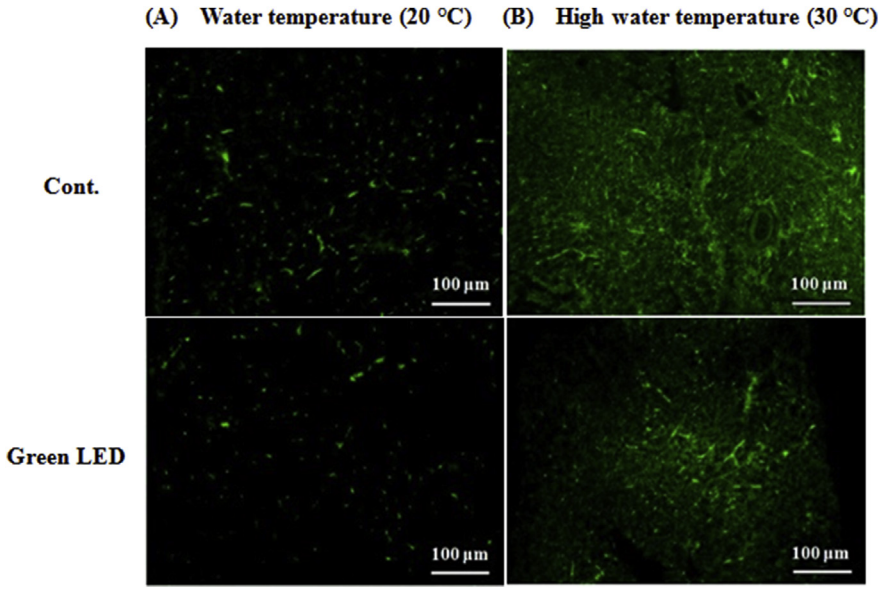

Fig. 6. TUNEL detection of liver cell apoptosis at different lighting conditions, after 24 h at 20 °C (A) or 30 °C (B), using a white fluorescent bulb (Cont.), or green LED light at 0.5 W/m^2. Cells were stained with acridine orange and subsequently observed with a fluorescent microscope. The green fluorescent cells indicate the apoptotic cells. Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly affected the antioxidant capacity. Fish kept under blue and green light conditions had significantly lower SOD and CAT levels, activity, and mRNA expression than those receiving white fluorescent light. In contrast, red light significantly increased these values, and the effects were stronger at higher light intensities (i.e. 0.5 vs. 0.3 W/m^2).

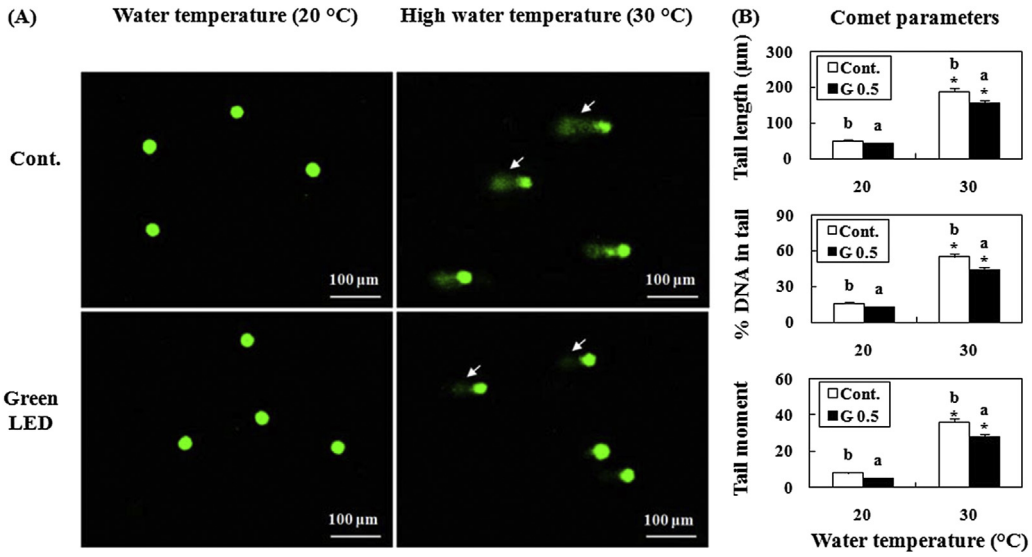

Fig. 7. Comet assay images (A) and comet assay parameters (B; comet tail length, percentage DNA in comet tail, and tail moment) kept at different thermal regimes, for 24 h, using a white fluorescent bulb (Cont.) and green LED lights of 0.5 W/m^2. White arrows indicate the damaged nuclear DNA (DNA breaks) of the liver cells, which were stained with SYBR-green. Scale bars = 100 μm. The lowercase letters with different characters are significantly differences between different lights regimes within the same temperature treatment ($P < 0.05$). The asterisks (*) indicate significant differences between the different temperature regimes per light treatment ($P < 0.05$). All values are represented as means ± SE ($n = 5$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our results are similar to work from Lushchak and Bagnyukova [31], in which goldfish (*Carassius auratus*) were exposed high water temperatures, after which the oxidative stress in the fish increased, as well as their SOD concentrations to remove this oxidative stress. Furthermore, Kim et al. [4] reported that goldfish exposed to high water temperatures (25 and 30 °C), exhibited higher oxidative stress factors and increases antioxidant enzymes concentrations, such as SOD, CAT, and GPX. However, the groups irradiated with green light had significantly lower antioxidant enzyme levels than the control groups, whereas the goldfish that had received red light had significantly increased antioxidant enzyme levels [4]. Overall, the results of both these studies support our findings that the antioxidant defense system was increased to protect in olive founder from reactive oxygen species (ROS) generation by thermal stress, and that irradiation with the shorter wavelengths from the visible light spectrum, such as green and blue light, effectively decreased ROS generation and oxidative stress in the fish.

Our study furthermore analyzed plasma H_2O_2 and LPO concentrations, in order to determine the degree of oxidative stress induced by the thermal environment. The groups exposed to 25 and 30 °C had higher plasma H_2O_2 and LPO concentrations than the control groups (20 °C). When comparing the different light groups, plasma H_2O_2 and LPO concentrations in the fish irradiated with green and blue light were lower than the control groups, whereas the opposite was true for the olive flounders receiving only red light. The latter treatment also showed an increase in oxidative stress at higher light intensities (i.e., 0.5 vs. 0.3 W/m^2).

These results are similar to those of Madeira et al. [32], which reported that thin-lipped mullets (*Liza ramada*), white seabream (*Diplodus sargus*), and European seabass (*Dicentrarchus labrax*) exposed to various water temperatures (24, 26, 28, 30, and 32 °C) had increased LPO levels at higher water temperatures. Furthermore 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 LPO and H_2O_2. In contrast, cinnamon clownfish exposed to red LED light displayed significantly increased LPO and H_2O_2 levels in their plasma. In sum, the results of our study are similar to those of previous studies that investigated oxidative caused by temperature stress, and green and blue LED light can play a role in reducing this temperature-induced oxidative stress and ROS generation.

These results are similar to the study of Jia et al. [33], who reported that juvenile turbot (*Scophthalmus maximus*) exposed to various concentrations (0.4 and 0.8 mM) of nitrite (NO_2^-), which is toxic to aquatic organisms, showed increased levels of oxidative stress, as well as increased mRNA expression of caspase-3, indicating an increase of apoptosis. In addition, Wang et al. [34] reported that caspase-3 activity in juvenile orange-spotted grouper (*Epinephelus coioides*) increased because of exposure to copper (100 μg/L). Overall, our results are similar to those of previous studies showing that stress, whether from toxins or acute water temperature changes, can increase the activity of caspase-3 levels and mRNA expression. Furthermore, it showed that irradiation with green light can reduce the oxidative stress, and hence inhibit caspase-3 activity.

Overall, we found that irradiation with short wavelength light can plays a role in reducing oxidative stress and apoptosis. Specifically green light was effective in reducing apoptosis. To confirm this reducing effect of green light irradiation in apoptosis in fish cells, we incubated olive flounder liver cells with various concentrations of caspase-3 (0, 100, 200, and 300 ng/μL). After 48 h of exposure to caspase-3, apoptosis was promoted, and we observed changes of SOD and CAT mRNA expression over time. SOD and CAT mRNA expression significantly increased at higher concentrations of caspase-3, although SOD and CAT mRNA expression was lower in cells from fish held at green light than those from the fish held at white fluorescent light. There were no differences among the groups held at different light intensities.

Cheng et al. [14] reported that pufferfish (*Takifugu obscurus*) blood cells incubated at a high temperature (34 °C) displayed increased oxidative stress that enhanced the mRNA expression of antioxidant genes such as SOD, CAT and GPX, as well as the mRNA expression of apoptosis-related genes P53, caspase-9, and caspase-3. Furthermore, Gorman et al. [35] reported that human HL-60 cells incubated high temperatures (42 °C) exhibited higher apoptosis

levels, whereas groups treated with antioxidants such as diphenyliodonium chloride (DPI), 1, 10-phenanthroline (Phen) showed lower rates of apoptosis. Huang et al. [36] reported that the antioxidant glutathione (GSSG) reduces the activity of caspase-3. 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, the TUNEL assays we performed showed that the groups held at high water temperatures (30 °C) clearly had increased apoptosis rates compared with the groups held at 20 °C. However, when we compared the different wavelength groups, those irradiated with green light showed significantly lower rates of apoptosis compared to the white fluorescent light groups.

These findings are similar to those of Yabu et al. [37], who reported that zebrafish embryos (*Danio rerio*) exposed to high water temperatures for 1 h showed higher rates of apoptosis during the warming treatment. In sum, our results found that high water temperature lead to oxidative stress and apoptosis, but the green light plays an important role in defending against oxidative stress damage and apoptosis when exposed to high water temperature.

Finally, the executed comet assays showed that liver cells of olive flounder displayed normally functioning nuclear DNA at 22 °C. At 30 °C, these cells showed significantly increased comet tail lengths, a larger percentage of DNA in the tails, larger comet tail moments, and damaged nuclear DNA. The cells form fish irradiated with green light showed less nuclear DNA damage than those that were with a white fluorescent bulb. The results of this study are similar to those of Villarini et al. [38], who reported oxidative stress caused by high water temperatures, as well as damaged DNA, in red blood cells of rainbow trout (*Oncorhynchus mykiss*). Furthermore, Anitha et al. [39] reported that in goldfish exposed to high water temperatures (34, 36, and 38 °C), the degree of DNA damage increased with increasing water temperatures, which is similar to our results.

Overall, the presented results are similar to those from previous studies, and show that relatively high water temperatures induced oxidative stress in fish, and damage of nuclear DNA that is caused by this oxidative stress. Nonetheless, green light can reduce the nuclear DNA damage, and protect cells.

In conclusion, 1) relatively acute raises in water temperature induced oxidative stress in olive flounder, and increased apoptosis in its cells. 2) Light with short wavelengths, particularly green light, effectively reduced the warming-induced oxidative stress and apoptosis, whereas red light increased the oxidative stress and apoptosis. Further studies are required to investigate how other wavelengths and light intensities induce such physiological activities effects in different fish varieties.

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