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Effects of various LED light spectra on antioxidant and immune response in juvenile rock bream, *Oplegnathus fasciatus* exposed to bisphenol A

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ABSTRACT

Bisphenol A (BPA) is a monomer used in plastics and plasticizers. As an environmental toxin included in industrial wastewater, it contaminates the aquatic environment and is known to cause endocrine disruption in fish. Particular wavelengths of light-emitting diodes (LEDs) are known to affect the endocrine regulation of fish. The present study aimed to investigate the effects of green and red LED light on the antioxidant and immune systems in juvenile rock bream (*Oplegnathus fasciatus*) exposed to BPA. We used green and red LED exposure at two intensities (0.3 and 0.5 W/m^2) for 1, 3, and 5 days. We measured liver mRNA expression and plasma levels of antioxidant enzyme superoxide dismutase (SOD) and caspase-3. Furthermore, we measured plasma levels of hydrogen peroxide (H_2O_2), lipid peroxidation (LPO), melatonin, and immunoglobulin M (IgM). DNA damage and apoptotic activity were measured using comet and terminal transferase dUTP nick end labeling (TUNEL) assays, respectively. We found that SOD, H_2O_2, and LPO increased significantly, whereas melatonin and IgM decreased significantly, suggesting that BPA induces oxidative stress and reduces immune function. Likewise, both DNA damage and apoptotic activity increased following BPA exposure. However, we found that exposure to green LED light effectively reduced the detrimental effects induced by BPA, including decreasing DNA damage, apoptotic activity, SOD mRNA expression, and plasma levels of SOD, H_2O_2, and LPO. Likewise, the plasma levels of melatonin and IgM increased. Thus, our results indicate that green light conditions effectively reduces oxidative stress and promotes the immune function in juvenile rock bream.

1. Introduction

The recent increase in the use of plastics has resulted in an increase in environmental toxins. This occurs when producing and discarding plastics in the wastewater that then contaminates the aquatic environment (Bindhumol et al., 2003). These toxins are absorbed into the body of organisms, sometimes acting as endocrine-disrupting chemicals (Burridge, 2008). As an example, bisphenol A [BPA; 2,2-bis-(4-hydroxyphenyl)propane], which is one such environmental toxin, has been shown to act as an endocrine disruptor with functions similar to estrogenic hormones. In vertebrates, BPA has been shown to have a negative effect on antioxidant mechanisms, as well as to cause gender disturbances including feminization in males (Segner et al., 2003; Wu et al., 2011a). Wu et al. (2011b) reported that antioxidant hormone expression in the liver of medaka (*Oryzias latipes*) exposed to BPA significantly increased catalase (CAT) activity. In addition, Xu et al. (2015) reported that zebrafish (*Danio rerio*) gonads exposed to BPA showed significantly increased antioxidant enzyme activity, including superoxide dismutase (SOD) and glutathione peroxidase (GPX). Recently, a study also reporting negative effects of BPA showed that SOD activity and apoptosis significantly increased in the testis of rats exposed to BPA (40 mg/mL) due to increased oxidative stress caused by the BPA exposure (Wu et al., 2013).

In teleosts, a small portion of reactive oxygen species (ROS) are generated under normal conditions. However, undue stress caused by external environmental factors leads to increased oxidative

Abbreviations: BPA, bisphenol A; LED, light-emitting diodes; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; LPO, lipid peroxidation; IgM, immunoglobulin M; CAT, catalase; ROS, reactive oxygen species; TUNEL, terminal transferase dUTP nick end labeling; GPX, glutathione peroxidase.

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stress and free radicals (Li et al., 2009). Under these circumstances, an increase in lipid peroxidation (LPO) and damaged protein structures and nuclear DNA may be observed. Thus, ROS may have negative effects on cell viability caused by damages to the cell membrane as well as decreased immune system function through inhibited lysozyme activity (Wang et al., 2008). In particular, the overall result of excessive ROS induced by environmental stress exposure may be that cells enter induced apoptosis through loss of body homeostasis (Chandra et al., 2000). Apoptotic cells maintain their functionality although gradual detrimental characteristics, such as DNA fragmentation, may appear (Alnemri, 1997). The apoptotic response involves the activation by caspases, which are cysteine-dependent aspartate-specific proteases. In particular, caspase-3 plays a pivotal role in the apoptotic process by accompanying the biochemical and morphological changes (Hacker, 2000).

Vertebrates, including teleosts, remove ROS by initiating antioxidant mechanisms to cope with the oxidative stress caused by the external environmental factors. SOD plays a role in decomposing superoxide (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Basha and Rani, 2003; Hansen et al., 2006). The H_2O_2 generated by SOD is eventually detoxified as water and oxygen by the catalytic action of CAT and GPX (Shull et al., 1991). Melatonin (*N*-acetyl-5-methoxytryptamine) is also known as an antioxidant that removes ROS generated by external stress, as well as having a positive effect on immunity enhancement and biological rhythms (Reiter et al., 1997). This hormone also affects biological rhythms, maturity, and growth, and is adjusted by light in vertebrates including fish (Falcón et al., 2007).

Light is one of the most important external factors that regulate circadian rhythms (Jin et al., 2009). In organisms with photoreceptors, lights of various wavelengths induce/or inhibit not only physiological changes but also affect reproduction, growth, and behavior (Pierce et al., 2008). Recently, studies investigating the effect of exposure to light-emitting diodes (LEDs), including irradiation with specific wavelengths, have been carried out on fish (Choi et al., 2012). Kim et al. (2014) reported that antioxidant gene expression in goldfish exposed to thermal stress under green LED was significantly lower than that observed in fluorescent light control groups. Choi et al. (2012) reported that the expressions of SOD and CAT, which are both antioxidant genes, were significantly decreased. In addition, the concentrations of lysozyme and melatonin, which are associated with the immune system, were significantly increased under green LED compared with fluorescent light control groups in the clownfish (*Amphiprion melaniopus*) exposed to starvation stress (Choi et al., 2012). Based on these results, it has been suggested that green LED light reduces oxidative stress and enhances immune function. In addition to the positive effects on antioxidant activity and stress reduction, compared with metal halide bulbs, LEDs have other advantages including low power consumption and long life. Thus, LED light sources may confer many positive effects to the fish farming industry (Choi et al., 2015).

To confirm the effectiveness of different wavelengths on the regulation of oxidative stress and immune system enhancement, we exposed juvenile rock bream to green and red LED wavelengths at different intensities (0.3 and 0.5 W/m^2) for 1, 3, and 5 days following BPA exposure (10 and 50 ng/L). We analyzed the mRNA expression of SOD protein, as well as activity and the plasma concentrations of H_2O_2, LPO, melatonin, IgM, and lysozyme, to determine the change of oxidative stress and immune system function. In addition, we measured the nuclear DNA damage in liver cells by conducting a comet assay, and apoptotic activity was measured by analyzing the changes is expression of caspase-3 mRNA and activity and conducting a terminal transferase dUTP nick end labeling (TUNEL) assay.

2. Materials and methods

2.1. Experimental fish, treatments, and sample collection

For each experiment, juvenile rock bream (length, 10.6 ± 1.1 cm; mass, 8.7 ± 0.8 g) were purchased from a commercial aquarium (Jeju, Korea). They were allowed to acclimate to the laboratory for two weeks in three 300-L circulation filter tanks. Each tank (each experimental group) contained nine fish. The fish were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea). During the experimental period, the water temperature and salinity were maintained at 19 °C and 30 psu, respectively. The photoperiod was set to a 12-h light (L):12-h dark (D) period (lights on 07:00 h and lights off 19:00 h, respectively). All fish were fed a commercial feed twice daily (at 09:00 and 17:00 h).

We established one control group, exposed to 0 ng/L BPA, and two experimental groups, exposed to 10 and 50 ng/L BPA, respectively. No fish died as a result of the BPA exposure. Following the BPA exposure, both the control (0 ng/L BPA) and experimental groups (10 and 50 ng/L BPA, respectively) were exposed to green (520 nm) and red (630 nm) LED lights (in addition to a white fluorescent bulb) placed 40 cm above the surface of water, for 1, 3, and 5 days. Each LED group was exposed to two different intensities (0.3 and 0.5 W/m^2) (Daesin LED Co., Kyunggi, Korea). The fish were reared under these conditions with daily feeding of a commercial feed until the day prior to sampling. A spectral analysis of the lights was performed using a spectrometer (MR-16, Rainbow Light Technology Co., Pingzhen, Taiwan). The fish were anesthetized with 200 mg/L 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, 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, 1,000 × *g* for 5 min) and stored at −80 °C until further analysis. To collect the liver tissue and blood, the fish were euthanized [after 0 (immediately after light irradiation), 1, 3, and 5 days of exposure, respectively] by spinal transaction under dim white light, using an attenuated white fluorescent bulb.

2.2. Total RNA extraction and cDNA synthesis

Total RNA were extracted from liver tissue (*in vivo* and *in vitro* cultured) from all experiment groups using the TRIzol kit following the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). The concentrations and purities of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. 3 μg of total RNA was reverse transcribed in a total volume of 20 μL, using an oligo-d(T)$_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) following the manufacturer's protocol. The resulting cDNA was diluted and stored at −20 °C for subsequent use in polymerase chain reaction (PCR) and quantitative PCR.

2.3. Real time quantitative PCR (RT-qPCR)

In this study, we followed the recommendations of the minimum information for publication of RT-qPCR experiments guidelines (Bustin et al., 2009). Total RNA was extracted from the liver tissue, using a TRIzol kit (Gibco/BRL, USA) according to the manufacturer's instructions. Total RNA was synthesized to cDNA by reverse transcription, which was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. RT-qPCR was performed using cDNA. RT-qPCR was conducted to determine the relative expression levels of SOD (GenBank accession no. JN593103), caspase-3 (JQ315116), and β-actin (FJ975145) mRNA, using cDNA extracted from both *in vitro* cultured and *in vivo* liver cells. The primers used for the qPCR are presented in Table 1. These primers were designed for each gene

Table 1
Primers used for QPCR amplification.

Genes (accession no.)	Primer	Sequences
SOD (EF690700)	Forward	5′ – TGA CCT GAC CTA CGA CTA TG – 3′
	Reverse	5′ – GCC TCC TGA TAT TTC TCC TCT – 3′
Caspase-3 (JQ315116)	Forward	5′ – CGA AGG TTT ACA ATG ACC AGA CA 3′
	Reverse	5′ – GCA GAA CAC AGA CGA ATG AGG – 3′
β-actin (AB039726)	Forward	5′ – CAG AGC AAG AGA GGT ATC C – 3′
	Reverse	5′ – TCG TTG TAG AAG GTG TGA TG – 3′

using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database, to ensure the primer specificity. The PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) following the manufacturer's instructions. The RT-qPCR was performed as follows: 95 °C for 5 min, followed by 50 cycles each consisting 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 calculated corresponding β-actin threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using the $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta Ct = 2^{-(\Delta Ct_{sample} - \Delta Ct_{internalcontrol})}$].

2.4. Western blot analysis

Total protein isolated from *in vivo* juvenile rock bream liver tissue was extracted using a protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin, pH 7.4), sonicated, and quantified using the Bradford method (Bio-Rad, USA). A total of 25 μg of total protein was loaded per lane onto Mini-PROTEAN® TGX™ gels (Bio-Rad, USA). For reference, a protein ladder (Bio-Rad, USA) was also used. Following electrophoresis at 180 V, the gels were immediately transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad, USA) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Thereafter, the membranes were blocked with 5% milk in 0.04% Tris-buffered saline with Tween (TTBS) for 45 min, followed by a TTBS wash. The membranes were incubated with SOD antibodies (1:4,000 dilution, NBP1-47443, Novus Biologicals, Littleton, CO, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:4,000 dilution; Bio-Rad, USA) for 60 min. As an internal control, β-tubulin primary antibody (1:4,000 dilution; ab6046, Abcam, Milton, CB, UK) was followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:4,000; Bio-Rad, USA) for 60 min. Bands were detected using a sensitive electrochemiluminescence system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDoc XRS+ System (Bio-Rad, USA).

2.5. In vitro *culture of liver cells*

The culture of rock bream liver was performed using enzymatic and mechanical procedures. The liver tissue was quickly removed and placed in 3 mL of ice-cold dispersion buffer (pH 7.4, Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride, containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone; GIBCOBRL, Rockville, MD, USA). The isolated liver samples were then transferred to 6 mL fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma. USA). The connective tissue and other impurities were removed, and the liver tissues were chopped into small pieces with a pair of scissors. The minced liver tissue was transferred to a flask and incubated for 10 min at room temperature with slow stirring. The dispersed liver tissue was filtered and a culture medium (neuro basal medium without L-glutamine, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 1% fetal bovine serum; Gibco-BRL, USA; to adjust the medium's osmolarity to rock bream plasma osmolarity, 353 mOsm/L) was added. The cell suspension was centrifuged at 800 × *g* for 10 min, and the cells were then resuspended in fresh culture medium.

The liver cells (1.2×10^6 cells/800 μL/well) were applied to a 24-well tissue culture plate, which was started at 23:00 and then sampled at 12-h (L): 24-h (D) intervals. The *in vitro* cultured liver cells were exposed to red (630 nm) and green (520 nm) LEDs (Daesin LED Co., Korea), in addition to a white fluorescent bulb. The LEDs were placed 40 cm above the surface of the cell culture plate following a light regime of 12-h (L):12-h (D) (lights on 07:00 h and lights off at 19:00 h). The spectral analysis of the lights was performed using a spectroradiometer (DeltaOhm Co., Padova, ITALY), and the temperature was maintained using an LED heat prevention system.

2.6. Plasma parameter analysis

2.6.1. Plasma H_2O_2 and LPO levels

H_2O_2 concentrations (nmole peroxide/mL) were measured using the modified methods of Nourooz-Zadeh et al. (1994) and PeroxiDetect kit (Sigma-Aldrich, USA). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve.

LPO (nmole/mL) was quantified by measuring plasma malondialdehyde(MDA) and 4-hydroxynonenal (4-HNE), the degradation products of lipid peroxidation of polyunsaturated fatty acids, according to the manufacturer's instructions (Fish Lipid peroxide ELISA kit; MBS013426, MyBioSource Inc., San Diego, CA, USA). The absorbance was read at 450 nm using a plate reader.

2.6.2. Plasma melatonin, IgM, and caspase-3 levels

Plasma levels of melatonin, IgM, and caspase-3 were analyzed using the immunoassays Fish Melatonin ELISA kit (Catalog no. MBS013211; MyBioSource, USA), Fish Immunoglobulin M ELISA kit (MBS042385; MyBioSource, USA), and Fish Caspase-3 ELISA kit (MBS012786; MyBioSource, USA), respectively. An anti-antibody that was specific to the melatonin antibody was pre-coated onto a microplate. Next, 100 μL tissue supernatant, 50 μL HRP-conjugate, and 50 μL antibody were added to each well. These were mixed and then incubated for 2 h at 37 °C. Following the last wash, any remaining Wash Buffer was aspirated or decanted off, and 50 μL of each of substrates A and B were added to each well. These substrate solutions were then incubated for 15 min at 37 °C in the dark, during which time they changed from colorless to dark blue. Following incubation, 50 μL stop solution was added to each well, changing the color from blue to yellow. The absorbance of the solution was measured at each step at 450 nm, using a plate reader.

2.7. Comet assay

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells at 5 days (the peak value of all parameters compared to day 0). The

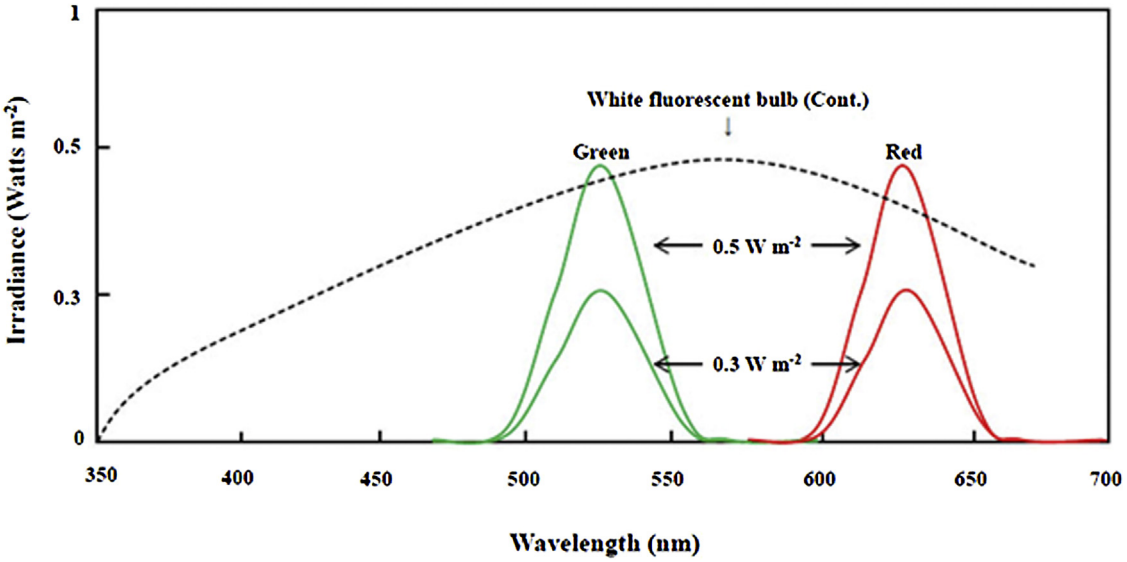

Fig. 1. Spectral profiles of the red (630 nm) and green (520 nm) light-emitting diodes (LEDs) used in this study. Each LED light source was set to two different intensities (0.3 W/m^2; 0.5 W/m^2). The dashed line represents the spectral profile of white fluorescent bulb (simulated natural photoperiod). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

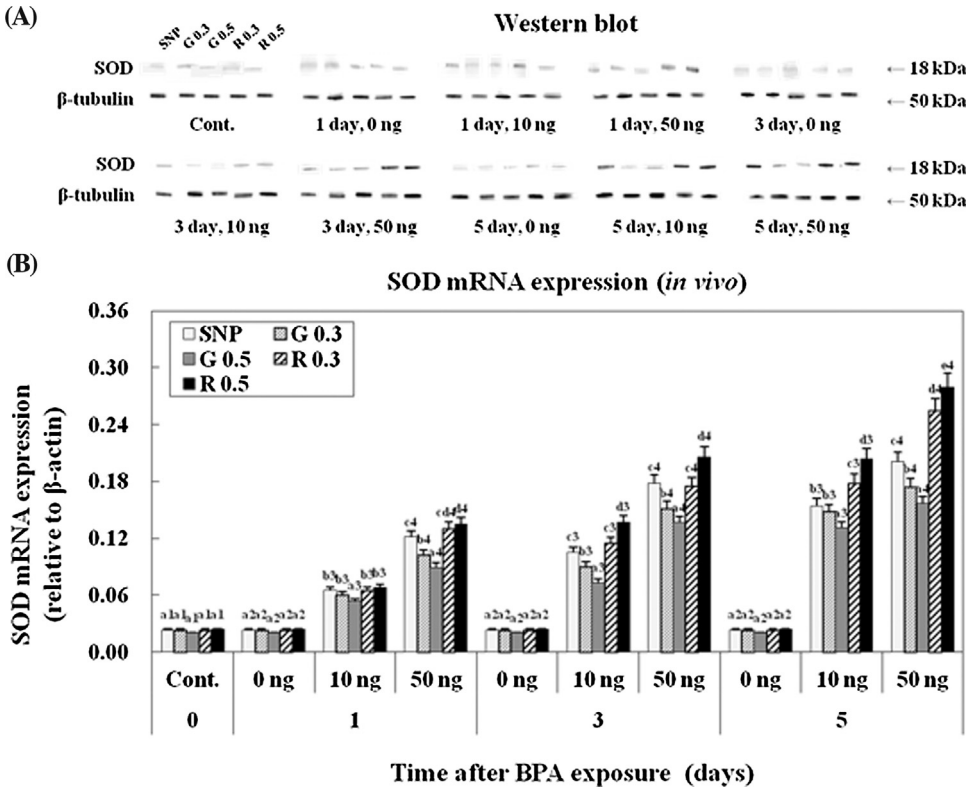

Fig. 2. SOD protein (A) and SOD mRNA expression (B) in liver during exposure to two concentrations (10 and 50 ng/L) of BPA, followed by 1, 3, and 5 days of green and red LED treatment at two intensities (0.3 and 0.5 W/m^2) in juvenile rock bream. SOD mRNA expression levels are expressed in relation to β-actin mRNA levels. The numbers indicate significant ($p < 0.05$) differences among the different exposure periods and BPA concentrations. The lower-case letters indicate significant differences among different parameter values within the same BPA concentrations and exposure periods ($p < 0.05$). All values are means ± SE ($n = 5$).

rock bream liver cells (1×10^5 cells/mL) were examined using a CometAssay® Reagent kit for single cell gel electrophoresis assay (Trevigen Inc., Gaithersburg, MD, USA). Cells were immobilized in an agarose gel on CometAssay® comet slides, and immersed in freshly prepared alkaline unwinding solution for 20 min. Subsequently, the slides were electrophoresed at 15 V for 30 min, stained with SYBR® Green (Trevigen Inc., USA) for 30 min in the dark, and read using a fluorescence microscope (excitation filter 465–495 nm, Eclipse Ci; Nikon, Tokyo, Japan). At least 100 cells from each slide were analyzed at Zeitgeber time 8 (15:00). For the comet assay quantification analysis, we analyzed the tail length (distance of DNA migration from head) and % DNA in tail (percentage of DNA in tail; tail intensity/total intensity in tail) using comet assay IV image anal-

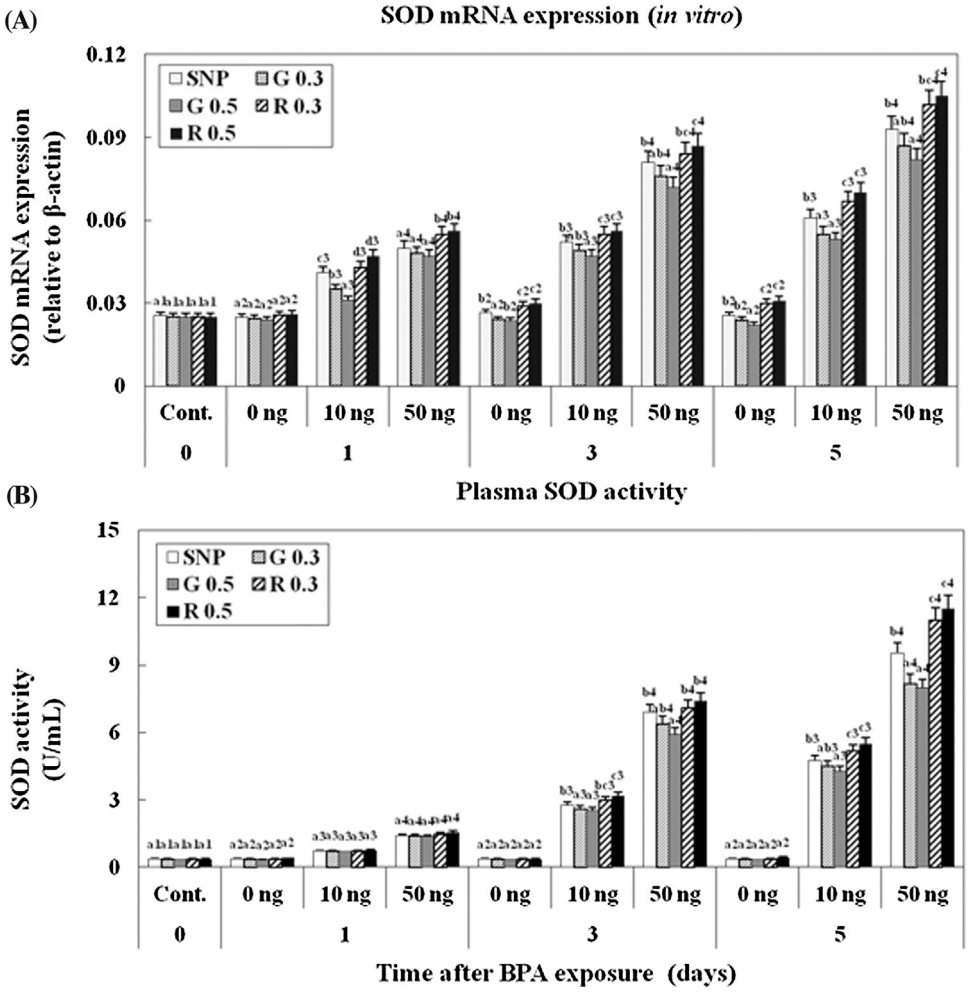

Fig. 3. SOD mRNA expression in liver cells (A) and plasma activity (B) during exposure to two concentrations (10 and 50 ng/L) of BPA, followed by 1, 3, and 5 days of green and red LED treatment of two intensities (0.3 and 0.5 W/m^2) in rock bream. SOD mRNA expression levels are expressed in relation to β-actin mRNA levels. The SOD activity change in plasma was measured using a microplate reader. The numbers indicate significant ($p < 0.05$) differences among the different exposure periods and BPA concentrations. Lower-case letters indicate significant differences among different parameter values within the same BPA concentration and exposure periods ($p < 0.05$). All values are means ± SE ($n = 5$).

ysis software (version 4.3.2, Perceptive Instruments Ltd., Bury Saint Edmunds, UK).

2.8. TUNEL assay

To evaluate the apoptotic response of the red and green LED spectra, we performed the TUNEL technique, using the commercially available *in situ* cell death detection kit fluorescence (11 684 795 910; Roche Co., Basel, Switzerland). To avoid apoptotic cells losing adherence to the slides, the slides were coated with polylysine. After rearing for 1, 3, and 5 days under red and green LED spectra while exposed to 50 mg/L BPA, the livers were washed and fixed with 4% buffered paraformaldehyde and permeabilized with freshly prepared 0.1% Triton X-100, 0.1% sodium citrate solution. The livers were then incubated with 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 C*i*; Nikon, Japan). For the paraffin-embedded tissue sections, slides were dewaxed and fixed according to standard protocols and were then treated as described above. Cells fluorescing in green, indicate apoptotic cells.

2.9. 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) (Fig. 1).

3. Results

3.1. Change in expression and activity of SOD

In this study, we examined the change of protein and mRNA expression and activity of SOD in juvenile rock bream liver tissue (*in vivo*) and cell (*in vitro* cultured) exposed to BPA under different LED light sources and intensity (Figs. 2 and 3). We found that SOD mRNA expression and plasma activity increased significantly over time and at higher concentrations of BPA in all experimental groups. Within experimental groups exposed to the same BPA concentrations, SOD mRNA expression and activity tended to decrease with increasing intensity of green LED. In contrast, SOD mRNA expression in the liver and SOD plasma activity tended to increase with increasing intensity of red LED.

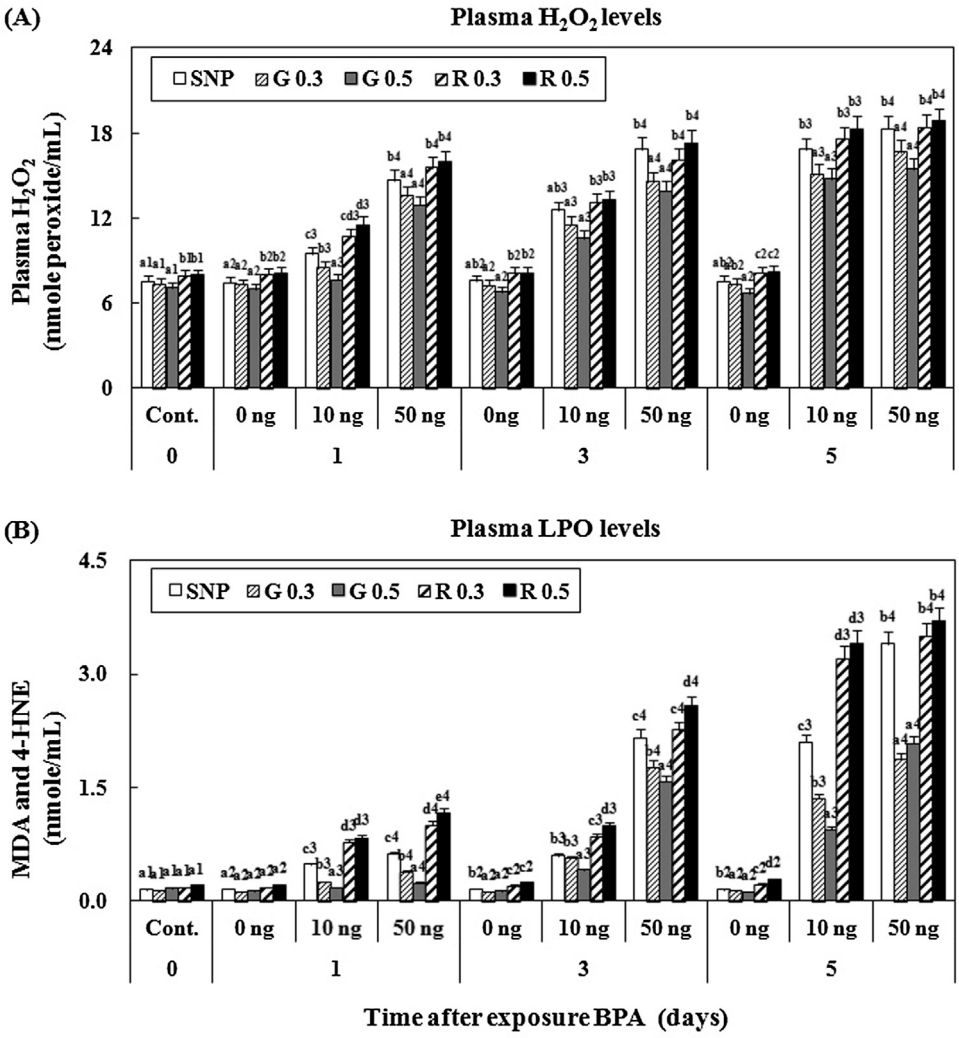

Fig. 4. Changes in plasma levels of H_2O_2 (A) and LPO (B) during exposure to two concentrations (10 and 50 ng/L) of BPA, followed by 1, 3, and 5 days of green and red LED treatment at two intensities (0.3 and 0.5 W/m^2) in rock bream liver. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured by microplate reader. The numbers indicate significant ($p < 0.05$) differences among the different exposure periods and BPA concentrations. The lower-case letters indicate significant differences among the different parameter values at the same BPA concentration and exposure periods ($p < 0.05$). All values are means ± SE ($n = 5$).

3.2. Change in levels of plasma H_2O_2 and LPO

We examined the change in H_2O_2 and LPO levels under different LED light sources and intensities (Fig. 4). The levels of both H_2O_2 and LPO increased significantly over time and at higher concentrations of BPA, in all groups. Within experimental groups exposed to the same concentration of BPA, the levels of H_2O_2 and LPO tended to decrease with increasing intensity of green LED, whereas it tended to increase with increasing intensity of red LED.

3.3. Change in melatonin and IgM plasma activity

The plasma melatonin and IgM activities measured to evaluate the immune system disturbance following exposure to BPA are presented in Fig. 5. The plasma melatonin and IgM activity decreased significantly over time and at higher concentrations of BPA, in all experimental groups. Within experimental groups exposed to the same BPA concentration, the plasma melatonin and IgM activities tended to increase with increasing intensity of green LED, whereas it tended to decrease with increasing red LED intensity.

3.4. Change in expression and activity of caspase-3

We examined the change in caspase-3 liver mRNA expression and plasma activity of juvenile rock bream exposed to BPA under different LED light sources and intensities (Fig. 6). Caspase-3 mRNA expression and activity increased significantly over time and with higher concentrations of BPA, in all experimental groups. Within experimental groups exposed to the same BPA concentration, increasing intensity of green LED resulted in no change in caspase-3 mRNA expression or activity, whereas a tendency to an increase was observed with increasing intensity of red LED.

3.5. Comet assay

Liver tissue DNA damage following a 5-day exposure to BPA was analyzed using 100 randomly selected cells. For the comet assay quantification analysis, we analyzed the tail length and % DNA in tail. The DNA content in the tail and tail length both increased significantly with higher BPA concentrations, in all experimental groups (Fig. 7). Within groups exposed to the same BPA concentration (0 or 50 ng/L BPA), the % DNA in the tail and tail length was signifi-

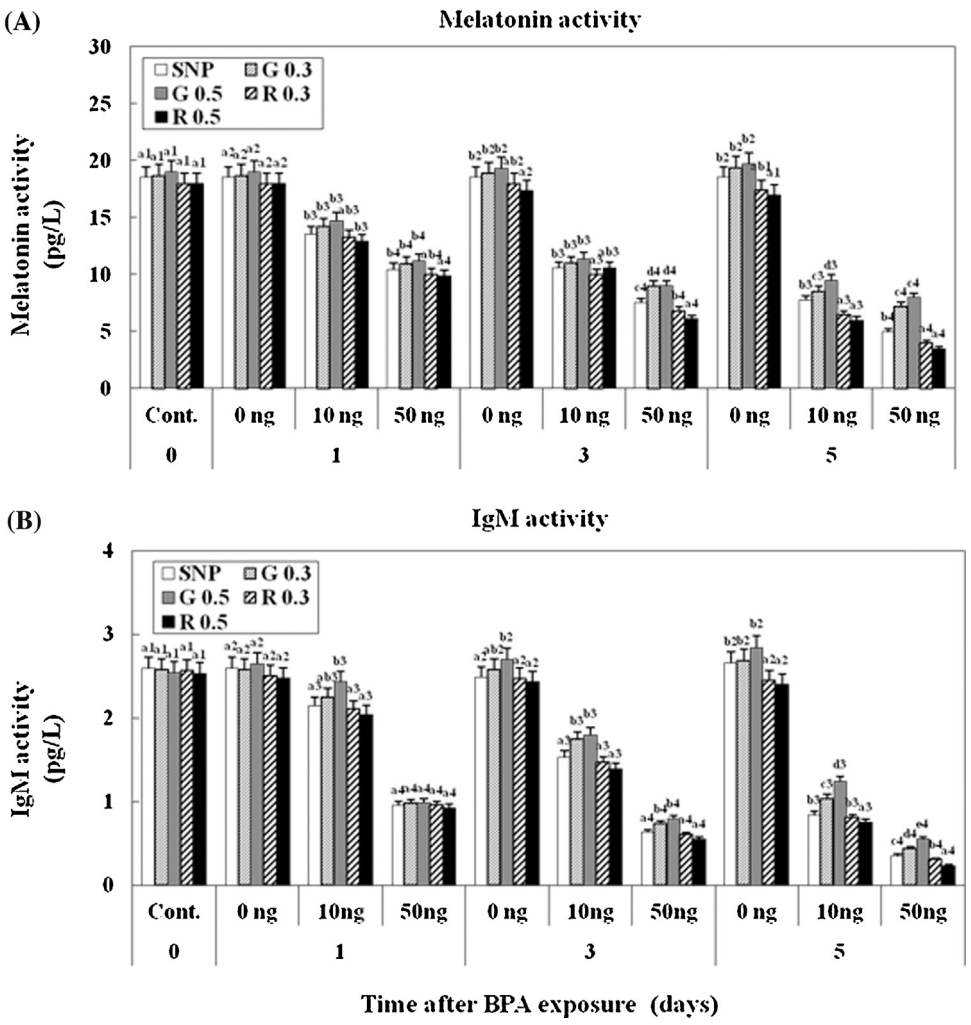

Fig. 5. Plasma activity of melatonin (A) and IgM (B) after exposure to two BPA concentrations (10 and 50 ng/L), followed by 1, 3, and 5 days of green and red LED treatment of two intensities (0.3 and 0.5 W/m^2) in rock bream liver, as measured by a microplate reader. The numbers indicate significant ($p < 0.05$) differences among the different exposure periods and the BPA concentrations. The lower-case letters indicate significant differences among the different parameter values at the same BPA concentration and exposure period ($p < 0.05$). All values are means ± SE ($n = 5$).

cantly lower in the group exposed to green LED, compared to that exposed to red LED.

3.6. TUNEL assay

A TUNEL assay was used to investigate the presence of apoptotic cells (Fig. 8). There were significant visible differences among the labeled cells as shown by the TUNEL assay between the control (non-BPA treated) and the experimental groups (simulated natural period, red LED, and green LED) exposed to 50 mg/L BPA. The frequency of apoptotic cells decreased after exposure to green LED compared to in the other experimental groups. In contrast, more apoptotic cells were detected after exposure to red LED.

4. Discussion

In this study, we investigated changes in juvenile rock bream regulation of oxidative stress and immune system function after irradiation of green and red LED wavelengths at two different intensities (0.3 and 0.5 W/m^2) following BPA exposure (10 and 50 ng/L). We did this by measuring stress related hormones and enzyme activities, and investigating the expression of immune indicators, including IgM and melatonin after 1, 3, and 5 days. In addition, we measured the expression of caspase-3 and conducted comet and TUNEL assays, to investigate the effects of specific wavelengths on BPA-induced cell damage and apoptotic activity.

The SOD protein levels and SOD mRNA expression and plasma activity in all experimental groups increased significantly with increasing BPA concentration and exposure time (Figs. 2 and 3), suggesting that BPA induces oxidative stress. In contrast, the SOD mRNA expression and plasma activity tended to be lower, regardless of light intensity (0.3 or 0.5 W/m^2), in the green wavelength, whereas they tended to increase with increasing light intensity (0.3 W/m^2 → 0.5 W/m^2) in the red wavelength. In support of our results, Kim et al. (2014) found that oxidative stress was induced in goldfish (*Carassius auratus*) exposed to high temperature conditions (30 °C). They found that this oxidative stress was significantly decreased in fish exposed to green wavelengths and increased in fish exposed to red wavelengths. Similar to previous reports, the oxidative stress increased significantly in juvenile rock bream exposed to BPA, but was moderated by the effect of green LED, in this study. We suggest that light of green wavelengths may effectively decrease oxidative stress induced by BPA toxicity.

We found that the plasma concentrations of H_2O_2 and LPO were similar to the results we found in SOD expression (Fig. 4). An increase in H_2O_2 concentration suggests decreasing antioxidant capacity and ability to remove the H_2O_2, as a result of BPA exposure. LPO is suggested to increase due to the continuous reac-

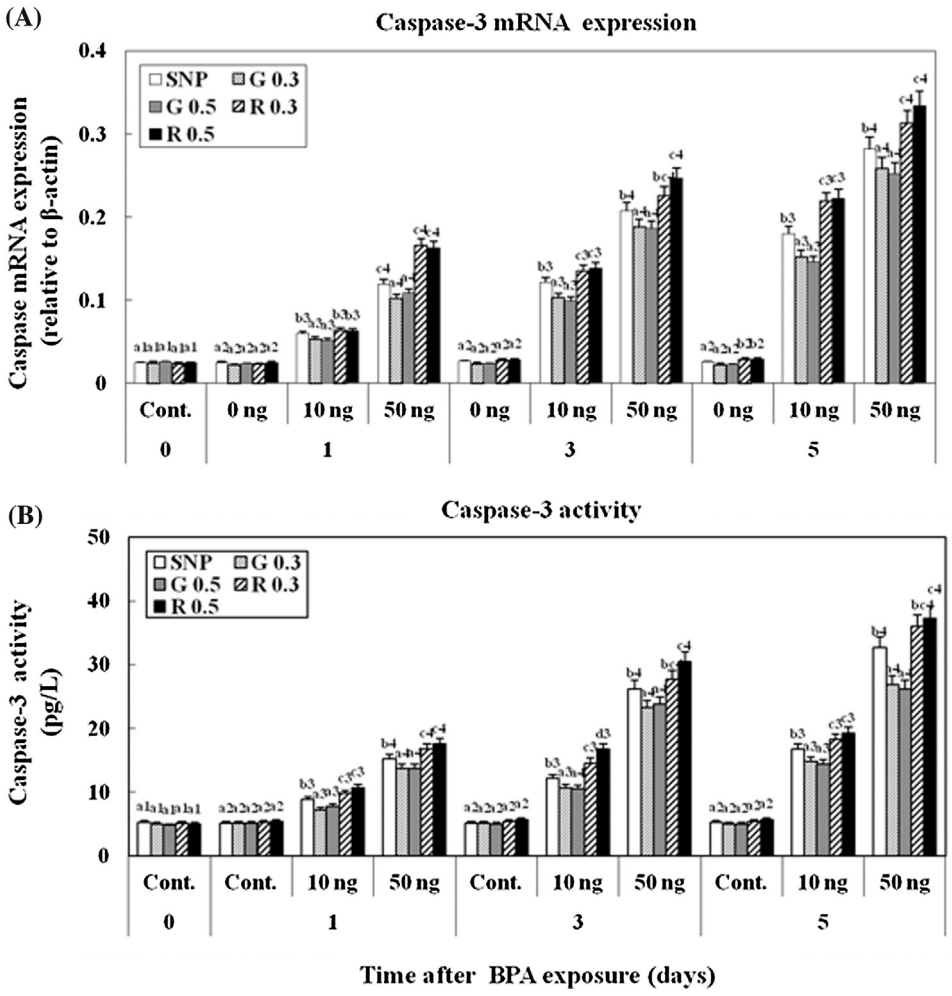

Fig. 6. Change in caspase-3 mRNA expression in rock bream liver (A) and plasma activity (B) after exposure to two BPA concentrations (10 and 50 ng/L), followed by 1, 3, and 5 days of green and red LED treatment at two intensities (0.3 and 0.5 W/m^2), as measured by a microplate reader. The numbers indicate significant ($p < 0.05$) differences among the different exposure periods at the BPA concentrations. The lower-case letters indicate significant differences among the different parameter values at the same BPA concentration and exposure period ($p < 0.05$). All values are means ± SE ($n = 5$).

tion between unsaturated fatty acids and ROS. However, the plasma H_2O_2 and LPO concentrations decreased in both green wavelength groups. This effect was probably due to the antioxidant effect of this type of light. Similar to the results found here, *D. rerio* exposed to BPA (at 10, 100, and 1,000 μg/L), showed a significant increase in plasma concentration of LPO with increasing BPA concentration (Wu et al., 2011a). Mohanty et al. (2013) reported that the LPO plasma levels increased significantly with increasing cadmium concentrations in Indian carp (*Labeo rohita*). We found that oxidative stress increased significantly in juvenile rock bream exposed to BPA, but the negative effect was decreased after exposure to green LED. Thus, these results add additional support for the role of green wavelength lights as modulators of oxidative stress induced by BPA toxicity.

In accordance with the immune response results, the plasma concentrations of both melatonin and IgM also decreased with increasing BPA concentrations and exposure time (Fig. 5). However, compared with the group exposed to simulated natural period, the plasma concentrations of melatonin and IgM in the green wavelength group tended to increase, whereas they tended to decrease in the red wavelength groups. Choi et al. (2013) also reported that selenium (Se) acts as a toxic substance when presented in excess in the water. Exposure to Se increases the oxidative stress and decreases immunity in *C. auratus*, even though Se is an essential trace element. Xie et al. (2008) reported that the number of T lymphocytes, which are involved in cell immune function, was significantly increased in chickens irradiated with green wavelength light. In accordance with this, the results found in our study suggest that green wavelength light may enhance juvenile rock bream immunity by reducing the detrimental effects of BPA.

To investigate the effects of different wavelengths on apoptotic activity in juvenile rock bream, we measured the liver mRNA expression and plasma activity of caspase-3. Both mRNA expression and plasmatic activity increased significantly following BPA exposure (Fig. 6). In particular, caspase-3 expression and activity in the green wavelength groups were significantly decreased during the experiment period. Recently, Jia et al. (2015) reported that juvenile turbot (*Scophthalmus maximus*) exposed to nitrite (NO_2^-), which is a metabolite of ammonia and is absorbed into the body inducing toxicity, had accelerated apoptotic activity through increased expression of caspase-3 mRNA. Our results also confirm that caspase-3 activity increased in juvenile rock bream following BPA exposure and that BPA acts as a factor inducing oxidative stress in the body. As observed in the other measured parameters, the green wavelength acted as a factor reducing oxidative stress, effectively inhibiting caspase-3 activity in the fish liver.

We analyzed the level of liver cell DNA damage caused by BPA exposure, using a comet assay. We found that the % DNA in the tail and tail length both increased significantly in the group exposed to 50 ng/L BPA (Fig. 7). However, within experimental groups, these

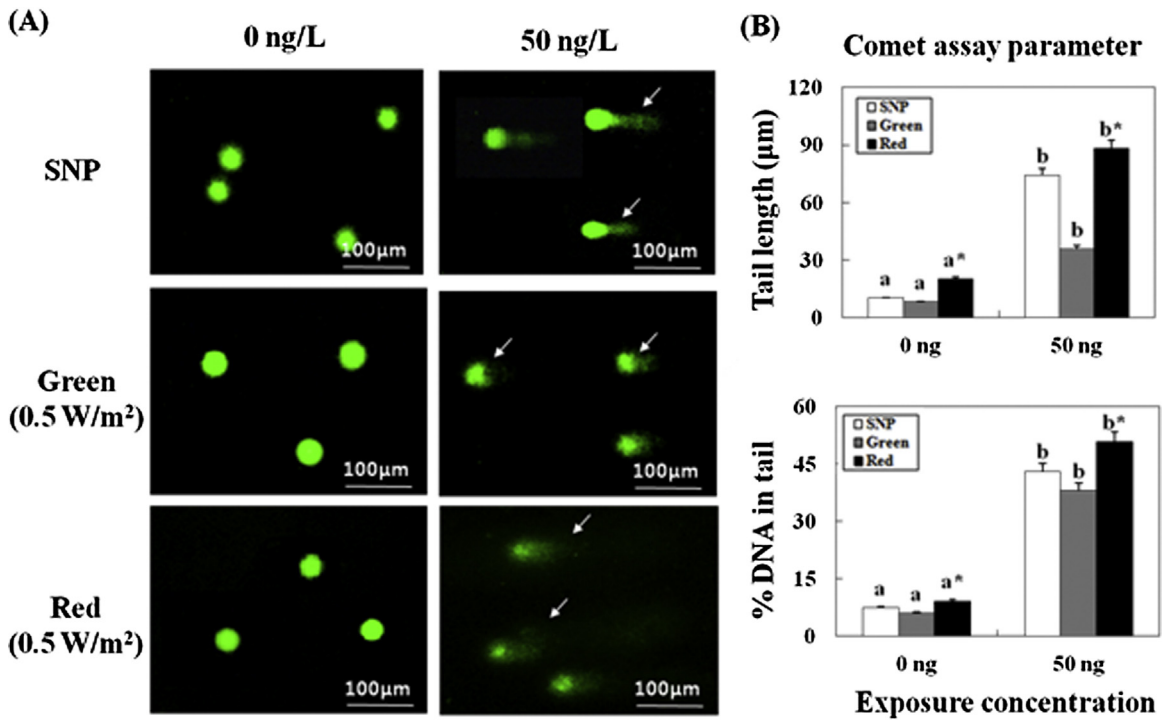

Fig. 7. Comet assay images (A) and comet assay parameter for tail length and % DNA in tail (B) following two different BPA exposure concentrations (0 and 50 ng/L) combined with simulated natural photoperiod (SNP), green LED (0.5 W/m^2), and red LED (0.5 W/m^2), as measured by fluorescence microscopy. White arrows in (A) indicate damaged nuclear DNA (DNA breaks) in the liver cells, which have been stained with SYBR Green. Scale bars = 100 μm. The lowercase letters in (B) represent the presence of significant differences within the same experimental groups and within the same temperature treatment ($p < 0.05$). The asterisks (*) indicate the presence of significant differences between different experimental groups within treatments of the same concentration ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

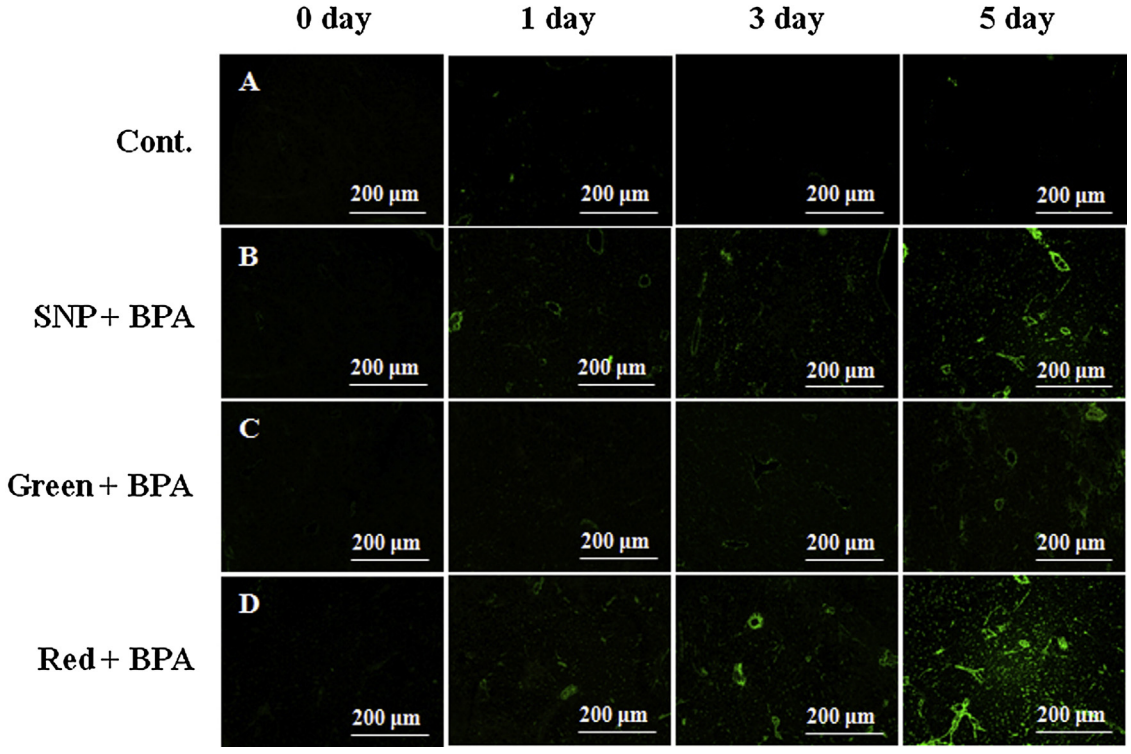

Fig. 8. TUNEL detection of rock bream liver cell apoptosis under different lighting conditions for 1, 3, and 5 days, using a white fluorescent bulb (Cont.) (A), as well as experimental groups (simulated natural photoperiod (SNP; B), green LED (C), and red LED (D), all with 50 mg/L BPA treatment). Cells are stained with acridine orange and visualized with a fluorescent microscope. Cells fluorescing in green indicate apoptotic cells. Scale bars = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values were significantly lower the green LED group (tail length: 7.9 ± 0.3; % DNA in tail: 5.8 ± 0.2), compared to the red LED group. Çavaş and Könen (2007) reported that the plasma concentration of H_2O_2 increased in *C. auratus* exposed to glyphosate (at 5, 10, and 15 ppm), which is used as a herbicide. They found that glyphosate-induced H_2O_2 resulted directly in nuclear DNA damage. In our study, we found that the nuclear DNA damage could be decreased through exposure to green wavelength LED light, reducing the BPA-induced H_2O_2 damage.

The apoptotic process of BPA-exposed juvenile rock bream was also investigated using a TUNEL assay. In accordance with the results presented above, we found that the BPA-induced increase in apoptotic activity could be moderated by exposure to green wavelength (Fig. 8). Jia et al. (2015) reported that when rats were exposed to BPA (200 mg/kg), the apoptotic activity increased in the liver. Our results support that hepatocyte apoptosis is increased by BPA exposure and that this effect can be reduced by exposure to green LED wavelengths.

In summary, we found that (1) if BPA is present even at trace amounts (10 ng/L) in the water, it may act as a factor that increases oxidative stress, antioxidant enzymes, and reduces immunity in juvenile rock bream. (2) The green wavelength light can effectively decrease this BPA-induced oxidative stress, by increasing antioxidant capacity and immunity, as well as suppressing nuclear DNA damage and apoptotic activity. In future studies, we suggest that more variation in the light wavelengths and intensities are investigated to further elucidate their antioxidant and immune function augmenting effects in fish.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Alnemri, E.S., 1997. Mammalian cell death proteases: a family of highly conserved aspartate specific cysteine proteases. J. Cell. Biochem. 64, 33–42.
Basha, S.P., Rani, U.A., 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxicol. Environ. Saf. 56, 218–221.
Bindhumol, V., Chitra, K.C., Mathur, P.P., 2003. Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicology 188, 117–124.
Burridge, E., 2008. Chemical Profile: Bisphenol A. European Chemistry News http://www.icis.com/resources/news/2008/10/13/9162868/chemical-profile-bisphenol-a.
Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: Minimum Information for Publication of Quantitative Real-Time PCR experiments. Clin. Chem. 55, 611–622.
Çavaş, T., Könen, S., 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22, 263–268.
Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29, 323–333.
Choi, C.Y., Shin, H.S., Choi, Y.J., Kim, N.N., Lee, J., Kil, G.S., 2012. Effect of LED light spectra on starvation-induced oxidative stress in the cinnamon clownfish *Amphiprion melanopus*. Comp. Biochem. Physiol. A 163, 357–363.
Choi, Y.J., Kim, N.N., Shin, H.S., Park, M.S., Kil, G.-S., Choi, C.Y., 2013. Effects of exposure to waterborne selenium on the antioxidant and immune activity in goldfish, *Carassius auratus*. Mol. Cell. Toxicol. 9, 365–373.
Choi, C.Y., Shin, H.S., Kim, N.N., Yang, S.-G., Kim, B.-S., Yu, Y.M., 2015. Time-related effects of various LED light spectra on reproductive hormones in the brain of the goldfish *Carassius auratus*. Biol. Rhythm Res. 46, 671–682.
Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo–pituitary axis in fish. Trends Endocrinol. Metab. 18, 81–88.
Hacker, G., 2000. The morphology of apoptosis. Cell. Tissue Res. 301, 5–17.
Hansen, B.H., Romma, S., Garmo, O.A., Olsvik, P.A., Andersen, R.A., 2006. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. Comp. Biochem. Physiol. A 143, 263–274.
Jia, R., Han, C., Lei, J.L., Liu, B.L., Huang, B., Huo, H.H., Yin, S.T., 2015. Effects of nitrite exposure on haematological parameters, oxidative stress and apoptosis in juvenile turbot (*Scophthalmus maximus*). Aquat. Toxicol. 169, 1–9.
Jin, Y.X., Chen, R.J., Sun, L.W., Liu, W.P., Fu, Z.G., 2009. Photoperiod and temperature influence endocrine disruptive chemical-mediated effects in male adult zebrafish. Aquat. Toxicol. 92, 38–43.
Kim, N.N., Choi, Y.J., Shin, H.S., Lim, J.R., Han, J.M., Choi, J.Y., Lee, J., Kil, G.-S., Choi, C.Y., 2014. The effect of LED light spectra on antioxidant system by thermal stress in goldfish, *Carassius auratus*. Mol. Cell. Toxicol. 10, 47–58.
Li, Z.H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., Randak, T., 2009. Responses of antioxidant status and Na^+-K^+ ATPase activity in gill of rainbow trout, *Oncorhynchus mykiss*, chronically treated with carbamazepine. Chemosphere 77, 1476–1481.
Mohanty, B.P., Mahananda, M.R., Pradhan, S., 2013. Cadmium induced toxicity and antioxidant activities in *Labeo Rohita* (Hamilton). Environ. Ecol. Res. 1, 41–47.
Nourooz-Zadeh, J., Tajaddini-Sarmadi, J., Wolff, S.P., 1994. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal. Biochem. 220, 403–409.
Pierce, L.X., Noche, R.R., Ponomareva, O., Chang, C., Liang, J.O., 2008. Novel function for period 3 and Exo-rhodopsin in rhythmic transcription and melatonin biosynthesis within the zebrafish pineal organ. Brain Res. 1223, 11–24.
Reiter, R.J., Carneiro, R.C., Oh, C.S., 1997. Melatonin in relation to cellular antioxidative defense mechanisms. Horm. Metab. Res. 29, 363–372.
Segner, H., Caroll, K., Fenske, M., Janssen, C.R., Maack, G., Pascoe, D., Schäfers, C., Vandenbergh, G.F., Watts, M., Wenzel, A., 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicol. Environ. Saf. 54, 302–314.
Shull, S., Heintzl, N., Muniraj-Manohar, M.P., Janssenl, Y.M.W., Marshli, J.P., Mossmann, B.T., 1991. Differential regulation of antioxidant enzymes in response to oxidants. J. Biol. Chem. 226, 24398–24403.
Wang, F., Yang, H., Gao, F., Liu, G., 2008. Effects of acute temperature or salinity stress on the immune response in sea cucumber, *Apostichopus japonicus*. Comp. Biochem. Physiol. A 151, 491–498.
Wu, M., Xu, H., Shen, Y., Qiu, W., Yang, M., 2011a. Oxidative stress in zebrafish embryos induced by short-term exposure to bisphenol A, nonylphenol, and their mixture. Toxicol. Environ. Chem. 30, 2335.
Wu, M., Xu, H., Yang, M., Xu, G., 2011b. Effects of chronic bisphenol A exposure on hepatic antioxidant parameters in medaka (*Oryzias latipes*). Toxicol. Environ. Chem. 93, 270–278.
Wu, H.J., Liu, C., Duan, W.X., Xu, S.C., He, M.D., Chen, C.H., Wang, Y., Zhou, Z., Yu, Z.P., Zhang, L., Chen, Y., 2013. Melatonin ameliorates bisphenol A-induced DNA damage in the germ cells of adult male rats. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 752, 57–67.
Xie, D., Wang, Z.X., Dong, Y.L., Cao, J., Wang, J.F., Chen, J.L., Chen, Y.X., 2008. Effects of monochromatic light on immune response of broilers. Poult. Sci. 87, 1535–1539.
Xu, H., Dong, X., Zhang, Z., Wu, X., 2015. Long-term effects on plasma androgen and antioxidative enzymes in testis after exposure to bisphenol A in male zebrafish (*Danio rerio*). In: Environmental Protection and Sustainable Ecological Development: Proceedings of the 2014 International Conference on Environmental Protection and Sustainable Ecological Development (EPSED 2014), Wuhan, Hubei, China, October 24–26, 2014. CRC Press, p. 31.