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Effects of melatonin injection or green-wavelength LED light on the antioxidant system in goldfish (*Carassius auratus*) during thermal stress

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ABSTRACT

We tested the mitigating effects of melatonin injections or irradiation from green-wavelength light-emitting diodes (LEDs) on goldfish (*Carassius auratus*) exposed to thermal stress (high water temperature, 30 °C). The effects of the two treatments were assessed by measuring the expression and activity levels of the antioxidant enzymes, superoxide dismutase and catalase, plasma hydrogen peroxide, lipid hydroperoxide, and lysozyme. In addition, a comet assay was conducted to confirm that high water temperature damaged nuclear DNA. The expression and activity of the antioxidant enzymes, plasma hydrogen peroxide, and lipid hydroperoxide were significantly higher after exposure to high temperature and were significantly lower in fish that received melatonin or LED light than in those that received no mitigating treatment. Plasma lysozyme was significantly lower after exposure to high temperature and was significantly higher after exposure to melatonin or LED light. The comet assay revealed that thermal stress caused a great deal of damage to nuclear DNA; however, treatment with melatonin or green-wavelength LED light prevented a significant portion of this damage from occurring. These results indicate that, although high temperatures induce oxidative stress and reduce immune system strength in goldfish, both melatonin and green-wavelength LED light inhibit oxidative stress and boost the immune system. LED treatment increased the antioxidant and immune system activity more significantly than did melatonin treatment.

1. Introduction

Recent studies have examined the impact of global warming on the global ecosystem [1–3]. The Intergovernmental Panel on Climate Change (IPCC) reported that the global temperature has increased by an average 0.85 °C (0.65 °C–1.06 °C) over the last 133 years, between 1880 and 2012 [4]. An increase in global temperature also increases water temperature, which, in turn, affects the physiology of fish, including their growth, propagation, metabolism, osmoregulation [5], and immune system function [6]. In addition, increasing water temperature could increase reactive oxygen species (ROS) generation, thereby inducing oxidative stress [7]. ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$), lead to increased levels of lipid hydroperoxide (LPO) and negatively affect cell viability by causing membrane damage and enzyme inactivity [8,9]. In addition, ROS can break the double strand of DNA, which may play a role in cell death, mutagenesis, and carcinogenesis [10]. Organisms protect themselves from oxidative stress by operating an antioxidant defense system that reduces cell damage caused by ROS [11]. The defense system uses enzymes that have antioxidant effects, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-*S*-transferase (GST) [12,13]. The first step of ROS elimination is performed by SOD, which converts intracellular O_2^- into H_2O_2 and molecular oxygen (O_2). Then, CAT breaks down H_2O_2,

Abbreviations: CAT, catalase; H_2O_2, hydrogen peroxide; LED, light-emitting diode; LPO, lipid hydroperoxide; SOD, superoxide dismutase.

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thus reducing its toxic effect [14]. Antioxidant activity is known to increase in the liver, kidneys, intestines, and gills, as well as increasing by small amounts in other fish tissues, due to exposure to stress [15,16].

In addition, water temperature may affect immune system function [17]. The immune system is the physical defense against infection that acts by recognizing and attacking pathogens and helping to maintain homeostasis and health [18,19]. In particular, lysozyme is an important indicator of immune system function that helps to protect against invasion by pathogens and to mitigate the damage from stress [20,21].

Melatonin is a hormone that is primarily secreted by the pineal organ and that plays a crucial role in the regulation of the seasonal and circadian rhythms of physiology and behavior in most vertebrates, including fish. It is involved in feeding, reproduction, and stress responses [22]. Recently, several studies have shown that melatonin is a direct scavenger of free radicals and a highly effective antioxidant [23,24]. Melatonin also increased the expression and activity of antioxidants such as SOD, CAT, and GPX and removed internally generated ROS [25]. Therefore, it augments antioxidant defenses [26].

Light-emitting diodes (LEDs) manufactured to output specific wavelengths are used effectively in the fish farming industry [27–29]. Red wavelengths of LED light negatively affected the physiological function of fish and induced oxidative stress, while green wavelengths increased the antioxidant capacity of the fish, in part by increasing the production of antioxidants [28]. Another study suggested that it may be possible to control oxidative stress in fish via treatment with specific-wavelength LEDs, because green-wavelength LEDs effectively inhibit thermally induced oxidative stress and help protect fish from the effects of harmful ROS [30].

Although separate studies have reported that green wavelength LEDs inhibit oxidative stress and that melatonin, which is a strong antioxidant, scavenges free radicals [29,31], to date no studies have compared these two treatments on the antioxidant capacity of fish. The present study examined the antioxidant and immune system activity of goldfish (*Carassius auratus*) that were exposed to high levels of thermal stress. The levels and expression of antioxidant enzymes and immune response were compared for melatonin treatment and irradiation with green-wavelength LED.

We measured mRNA expression, concentrations and activity of the antioxidant enzymes SOD and CAT, and changes in oxidative stress in goldfish by measuring plasma H_2O_2 and LPO concentration. We also confirmed via a comet assay that high thermal conditions induced free radicals that damaged the nuclear DNA.

2. Materials and methods

2.1. Experimental fish and melatonin injections

Goldfish ($n = 480$, length 12.0 ± 0.4 cm; mass 15.1 ± 0.5 g) were purchased from a commercial fish farm (Busan, Korea) and maintained in eight 100-L circulation filter tanks prior to the experiments, which were conducted in a laboratory. During the experiments, the fish were randomly divided into three groups: a control, a group that received melatonin injections, and a group that was exposed to green-wavelength LED light. The control and melatonin injection groups were exposed to light produced by a white fluorescent bulb, while the green-wavelength LED group was exposed to light produced by a green (530-nm wavelength) LED (Daesin LED Co., Kyunggi, Korea). The light sources were placed 50 cm above the water's surface, and the irradiance at the water's surface was maintained at approximately 4.5 μmol/m^2/s. The photoperiod consisted of a 12-h light (L):12-h dark (D) cycle, intended to match natural conditions; lights were turned on at 07:00 and turned off at 19:00. The goldfish were reared using an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimatize to the conditions for 24 h. The water temperature was then increased from 22 °C to 30 °C in daily increments of 2 °C. The fish received commercial feed once per day until the day prior to sampling. Sampling was performed at the experimental temperatures (22 °C and 30 °C) and the experiments were started at 07:00. We sampled at the following Zeitgeber time (ZT) intervals: ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24.

The fish were anesthetized with tricaine methanesulfonate (MS-222; Sigma, USA) prior to receiving the melatonin injection. The melatonin (Sigma, USA) was dissolved in 0.9% physiological saline, and each fish was injected with either 5 or 10 μg melatonin/g body mass, in a volume of 10 μL/g body mass. After injection, fish were reared at the temperatures of 22 °C or 30 °C during the experimental period for 24 h. All fish were anesthetized using tricaine methanesulfonate (Sigma) and decapitated prior to tissue collection. Liver samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until total RNA was extracted for analysis. Plasma samples were separated by centrifugation (4 °C, 10000×*g*, 5 min) and stored at −80 °C until analysis.

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), according to the manufacturer's instructions. Then, a 2-μg sample of total RNA was reverse transcribed in a total reaction volume of 20 μL, using an oligo-$(dT)_{15}$ anchor and M-MLV reverse transcriptase (Promega, USA), according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4 °C for use in PCR and real-time qPCR analysis.

The qPCR analysis was conducted to determine the relative expression levels of the antioxidant enzymes SOD and CAT using total RNA extracted from the livers of goldfish. The primers for qPCR were designed using known goldfish sequences (Table 1). We conducted 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), according to the manufacturer's instructions. β-actin was also amplified as a control for each sample, and all data were expressed as the change with respect to the corresponding calculated β-actin threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was 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) of each sample and the internal control (β-actin) were calculated [$\Delta\Delta Ct = 2^{\hat{}} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$]. After PCR was completed, the qPCR data from three replicate samples were analyzed using Bio-Rad to estimate the transcript copy numbers of each sample.

2.3. SOD and CAT activity analysis

The liver tissues were homogenized in 1 × PBS. The homogenates were centrifuged at 5000×*g* for 5 min at 4 °C. The

Table 1
Primers used for QPCR amplification.

Genes (accession no.)	Primer	DNA sequences
SOD (JQ776518)	Forward	5′-ACA ACC CTC ATA ATC AAA CTC A-3′
	Reverse	5′-GCA ACA CCA TCT TTA TCA GC-3′
CAT (JQ776513)	Forward	5′-ATC TTA CAG GAA ACA ACA CCC-3′
	Reverse	5′-CGA TTC AGG ACG CAA ACT-3′
β-actin (AB039726)	Forward	5′-TTC CAG CCA TCC TTC CTA T-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′

supernatant was used for the analyses. SOD and CAT activity were determined using the fish SOD ELISA kit (CSB-E15929fh, Cusabio Biotech Co., Ltd., China) and the fish catalase ELISA kit (CSB-E15928fh, Cusabio Biotech Co., Ltd., China).

2.4. Western blot analysis

The total protein isolated from the livers of goldfish was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. A total of 30 μg protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used as a reference. Samples were electrophoresed at 180 V, and the gels were immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Next, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min, after which they were washed in TBS. Membranes were incubated with SOD (1:2000 dilution, NBP1-47443, Novus Biologicals, USA) and CAT (1:2000 dilution, sc-58332, Santa Cruz Biotechnology, USA) antibodies, followed by incubation with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (SOD and CAT dilution 1:2000, Bio-Rad) for 60 min. β-Tubulin (dilution 1:5000, ab6046, Abcam, UK) was used as the internal control. 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 density was estimated using Image Lab™ Software, version 3.0 (Bio-Rad).

2.5. In vitro culture of liver cells

The goldfish liver cells culture was performed using both enzymatic and mechanical procedures. The liver tissue was quickly removed 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 tissues were then transferred to 6 mL fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma). The connective tissues and other impurities were removed, and the liver tissues were chopped into small pieces using scissors. The liver cells and the minced liver tissue were transferred to a flask and incubated for 10 min at room temperature with slow stirring. The mixture of dispersed liver cells and tissues was filtered, and the 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 in fresh culture medium. The liver cells (1.2×10^6 cells 800 μL/well) were placed in a 24-well tissue culture plate.

For the experiments, the fish were randomly divided into three groups: control, melatonin treatment (0.1 and 1.0 μg/μL), and green-wavelength LED treatment. The control and melatonin

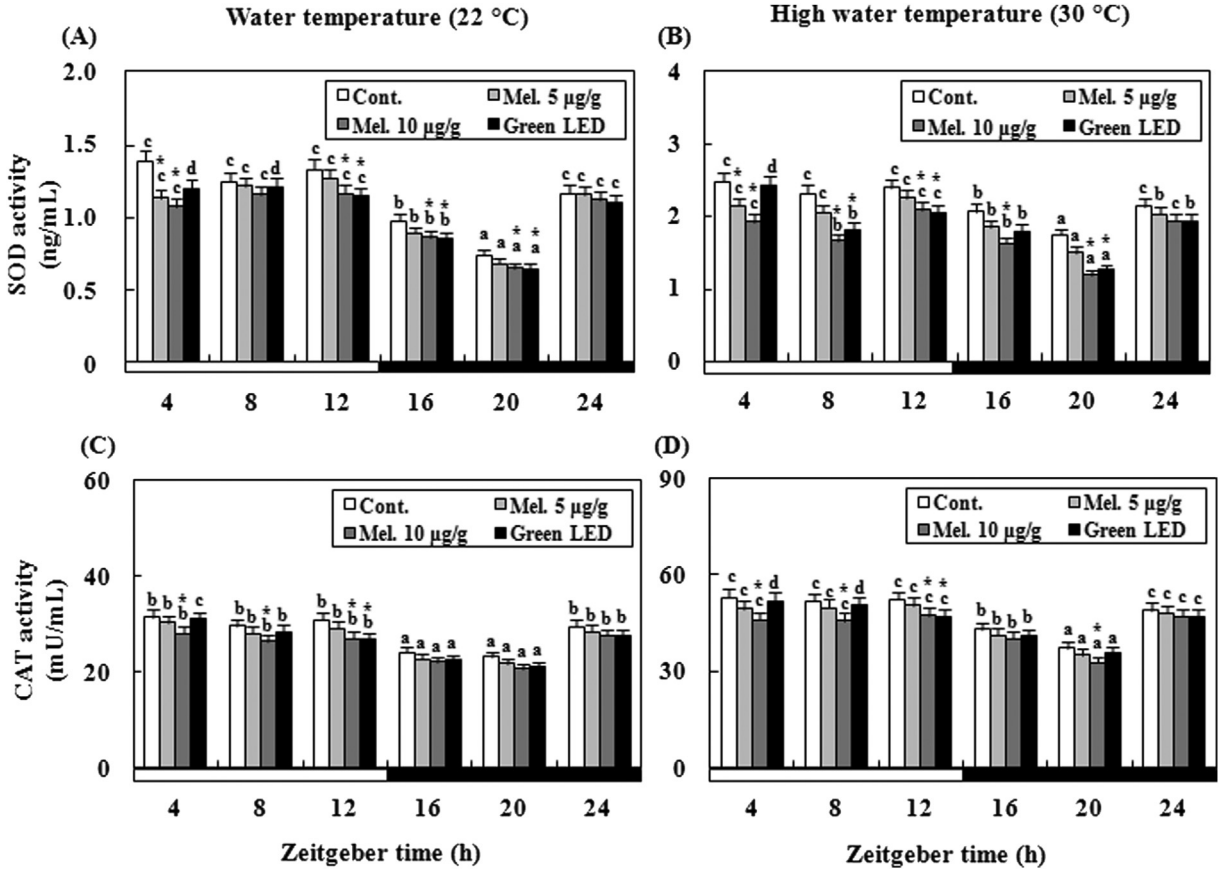

Fig. 1. Changes in SOD activity during thermal changes at 22 °C (A) and 30 °C (B), and CAT activity during thermal changes at 22 °C (C) and 30 °C (D), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

treatment groups were exposed to light produced by a white fluorescent bulb, and the green-wavelength LED group was exposed to light produced by a green (530 nm) LED (Daesin LED Co. Kyunggi, Korea). The light sources were placed 50 cm above the water surface, and the irradiance at the surface was maintained at approximately 4.5 μmol/m^2/s. The photoperiod consisted of a 12-h light (L):12-h dark (D) cycle with the photophase lasting from 07:00 to 19:00 (lights on at 07:00, and lights off at 19:00) and sampled at ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24.

2.6. Plasma parameter analysis

Plasma samples were separated by centrifugation (4 °C, 10000×*g*, 5 min), and analyzed by examining the H_2O_2, LPO, and lysozyme levels.

H_2O_2 concentrations were measured using a modified version of the methods described by Nourooz-Zadeh et al. [32], and a Perox-iDetect kit (Sigma). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve. The concentrations are expressed as nmol peroxide/mL.

LPO was quantified by measuring the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs), and hydroperoxides, according to the manufacturer's instructions (Fish Lipid Peroxide ELISA Kit, MyBioSource Inc., San Diego, CA, USA). The absorbance was read at 450 nm using a plate reader. LPO was expressed as nmol/mL.

Lysozyme levels were determined via a turbidimetric assay performed according to the method described by Ellis [33]. Briefly, 50 μL test plasma was added to 50 μL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.2). The absorbance at 450 nm was measured using a spectrophotometer, and levels were expressed as ng/mL.

2.7. Comet assay

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells [34]. The liver cells (1×10^5 cells/mL) of goldfish were examined using a CometAssay® Reagent kit for single-cell gel electrophoresis assay (Trevigen Inc., USA), according to the method described by Singh et al. [35], with some modifications. Cells were immobilized in an agarose gel on CometAssay® comet slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Next, slides were electrophoresed at 15 V for 30 min. The samples were stained with SYBR® Green (Trevigen, Inc.) for 30 min in the dark and then read using a fluorescence microscope (excitation filter 465–495 nm; Eclipse C*i*, Nikon, Japan). At least 100 cells from each slide were analyzed at ZT8 (15:00). For a quantification analysis of the comet assay, we analyzed the tail length (distance of DNA migration from head), percentage of DNA in tail (tail intensity/total intensity in tail), and tail moment (amount of DNA damage, product of tail length, and percentage of DNA in tail) using comet assay IV image analysis software (version 4.3.2, Perceptive Instruments Ltd., UK).

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A two-way ANOVA followed by

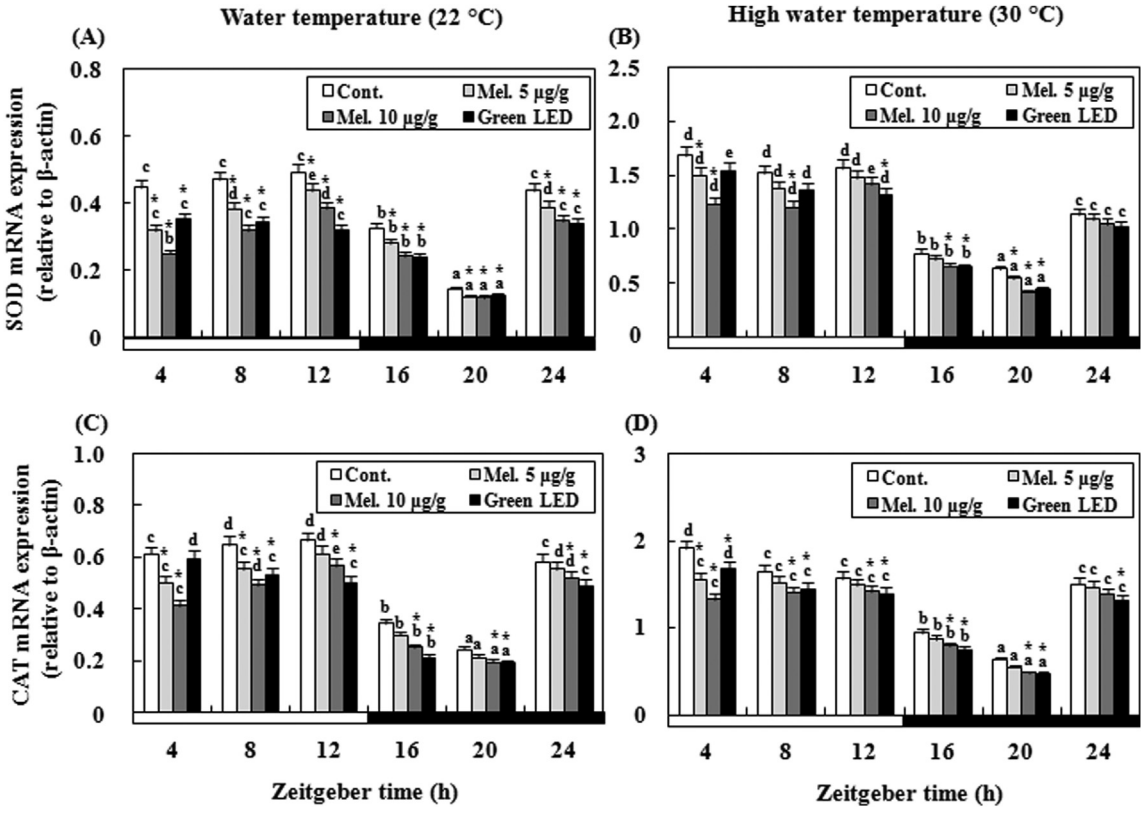

Fig. 2. Changes in SOD mRNA expression during thermal changes at 22 °C (A) and 30 °C (B), and CAT mRNA expression during thermal changes at 22 °C (C) and 30 °C (D), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass) and green-wavelength LED light, as measured by 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 in the same sample. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

Tukey's post hoc test was used to compare differences in the data ($P < 0.05$). The values are expressed as the mean ± standard error (SE).

3. Results

3.1. Expression and activity of antioxidant enzymes SOD and CAT in the liver

The present study investigated changes in the activity and mRNA levels of the antioxidant enzymes SOD and CAT in liver tissues of goldfish in response to high water temperature. Antioxidant mRNA levels and activity were significantly higher in fish that experienced high water temperature (30 °C) water than in the groups maintained at 22 °C (Figs. 1 and 2). In addition, values were significantly lower during the scotophase than during the photophase. Levels were significantly lower in the groups that received melatonin, and were significantly lower in the group that received 10 μg melatonin/g than in the group that received 5 μg/g. The fish that were exposed to green-wavelength LED treatment also showed significant decreases in the activity and mRNA levels of SOD and CAT. In particular, at ZT12, the levels in the green-wavelength LED treatment group were significantly lower than those observed in the melatonin injection groups.

In vitro experiments that used liver cells culture revealed that SOD and CAT mRNA expression levels were significantly higher at 30 °C than at 22 °C. In addition, expression levels during the scotophase were significantly lower than those during the photophase. SOD and CAT mRNA expression levels fell significantly in both the melatonin treatment and the green LED spectra groups (Fig. 3). The mRNA expression levels in the 1 μg/μL melatonin treatment group were significantly lower than those in the 0.1 μg/μL melatonin treatment group. Meanwhile, mRNA expression levels in the green-wavelength LED treatment group were similar to those seen in the melatonin treatment groups.

3.2. Western blotting of antioxidant enzymes SOD and CAT in the liver

SOD and CAT protein expression levels were significantly higher in the 30 °C treatment group than in the 22 °C treatment group (Fig. 4A and B). In addition, expression levels during the scotophase were significantly lower than those during the photophase. SOD and CAT protein expression in both the melatonin injection and green-wavelength LED groups significantly decreased relative to the control (Fig. 4).

3.3. Plasma H_2O_2 and LPO concentrations

The plasma H_2O_2 concentrations observed in the 30 °C groups were significantly higher than in the 22 °C groups, although they were significantly lower during the scotophase than during the photophase (Fig. 5A and B). Plasma H_2O_2 concentrations in the melatonin injection and green-wavelength LED groups were also significantly decreased.

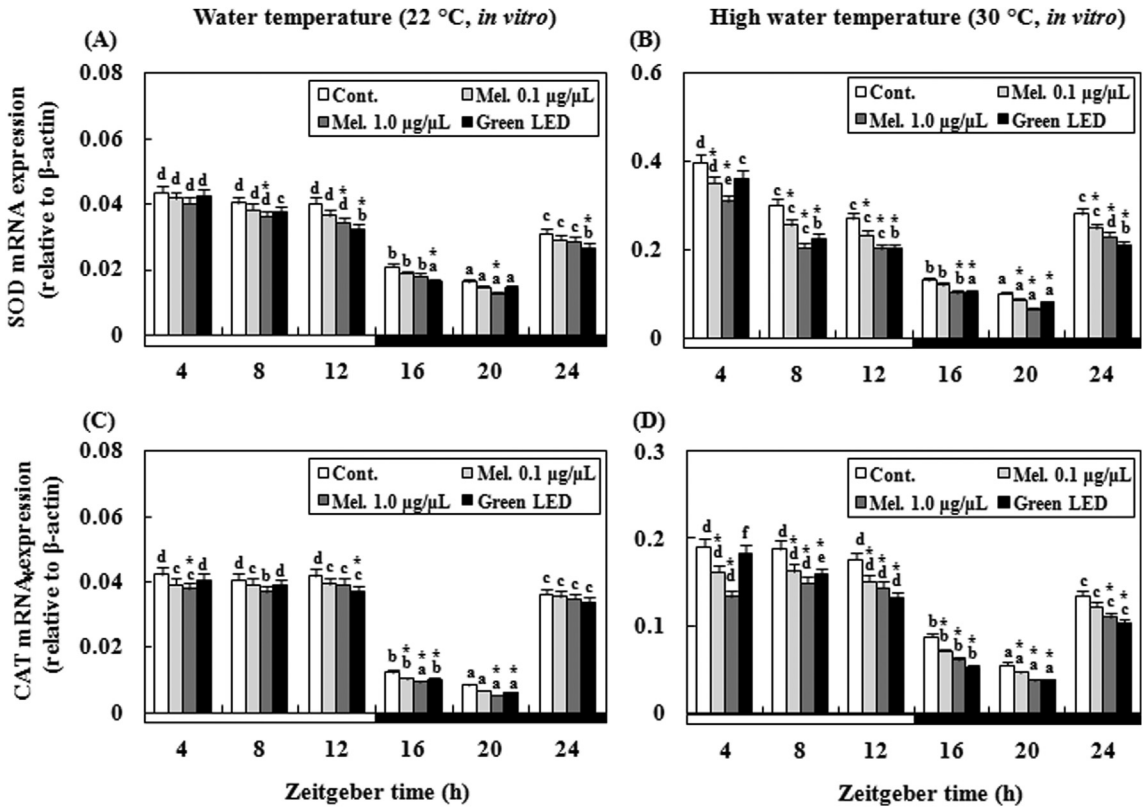

Fig. 3. Changes in SOD mRNA expression during thermal changes at 22 °C (A) and 30 °C (B), CAT mRNA expression during thermal changes at 22 °C (C) and 30 °C (D) of each experimental groups in goldfish (*in vitro*): control, melatonin treatment (0.1 and 1.0 μg/μL) and green-wavelength LED light, as measured by quantitative real-time PCR (qPCR). Results are expressed as normalized fold expression levels, relative to the β-actin levels in the same sample. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

The variation in the plasma LPO concentrations was similar to that of the variation in the plasma H_2O_2 concentration (Fig. 5C and D). The plasma LPO concentrations of the 30 °C treatment group were significantly lower than those of the 22 °C group. Concentrations were significantly lower during the scotophase than during the photophase. In particular, the plasma LPO concentrations at ZT12 were significantly lower in the group irradiated by green-wavelength LEDs than in the melatonin injection groups.

3.4. Plasma lysozyme levels

The plasma lysozyme levels of the 30 °C group were significantly lower than those of the 22 °C group (Fig. 6). However, the levels were significantly higher in the melatonin injection and green-wavelength LED groups than in the control. In particular, after ZT12, the plasma lysozyme levels were significantly higher in the group exposed to light from green-wavelength LED light than in melatonin injection-treated groups.

3.5. Comet assay

A total of 100 cells were randomly chosen for analysis at ZT8, using a fluorescence microscope (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). At 22 °C, the liver cells possessed normal nuclear DNA, but at 30 °C, cells with damaged nuclear DNA were visible in all groups (Fig. 7A). The samples exhibited significantly higher tail length, percentage DNA in tail, and tail moment at 30 °C than at 22 °C (Fig. 7B). The cells of the control groups at 30 °C sustained high levels of damage to their nuclear DNA and exhibited long tails, high percentages of DNA in the tail, and high tail moment. However, the groups that received melatonin injection or were exposed to green-wavelength LED light exhibited significantly decreased tail length, percentage DNA in the tail, and tail moment, indicating that they sustained a low level of damage to their nuclear DNA. Therefore, even after exposure to 30 °C, the nuclear DNA in these individuals

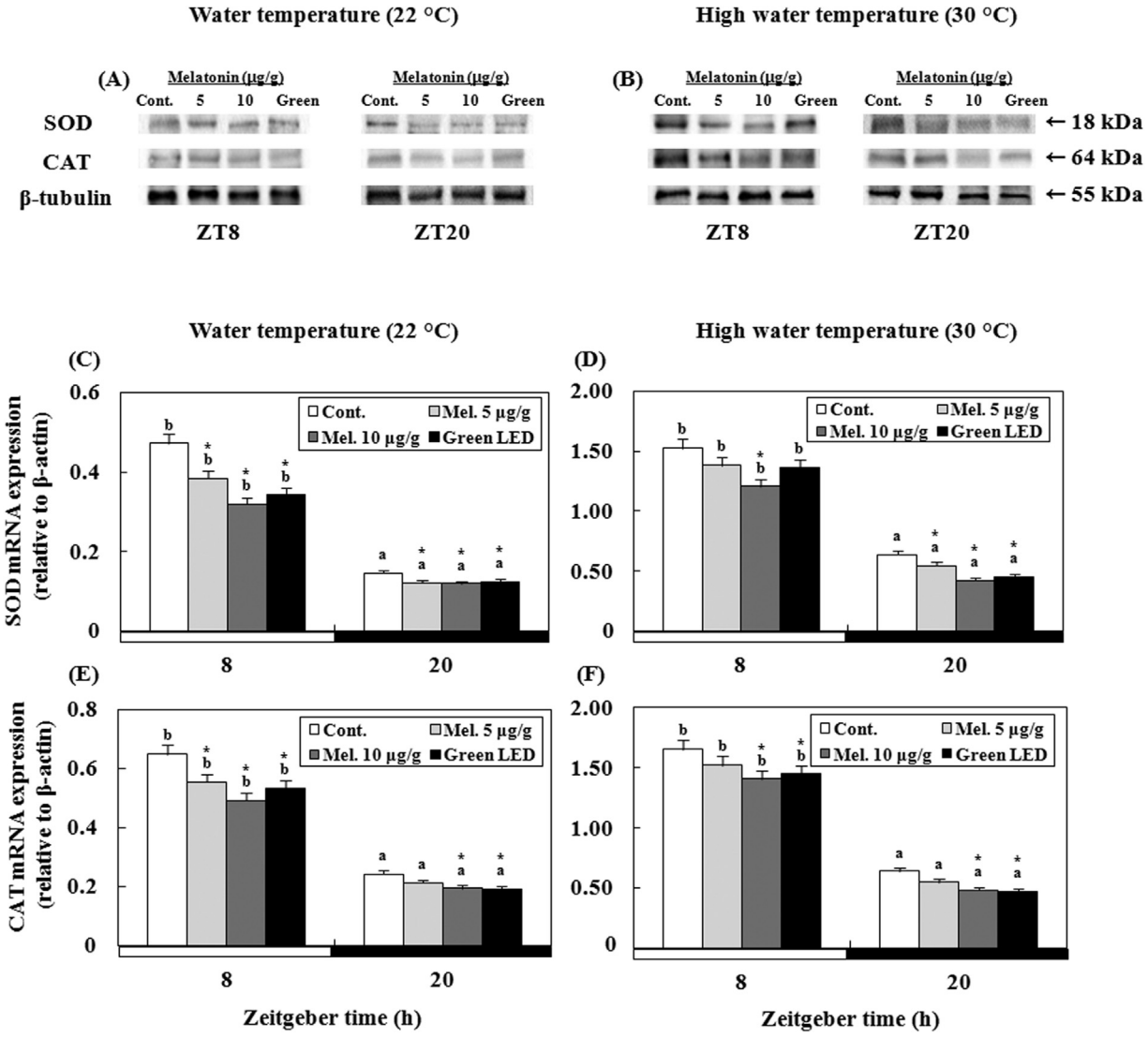

Fig. 4. Changes in SOD and CAT protein expression during thermal changes at 22 °C (A) and 30 °C (B), and SOD and CAT mRNA expression during thermal changes at 22 °C (C and E) and 30 °C (D and F), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light. Western blots of the expression of antioxidant enzymes [SOD (18 kDa) and CAT (64 kDa)] in the liver of goldfish after thermal change, and β-tubulin (55 kDa) was used as an internal control. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

was only slightly damaged and could be repaired.

4. Discussion

In order to confirm that melatonin and green-wavelength LED light increase antioxidant production and promote immune function, and to compare the responses to these two treatments, we exposed goldfish to thermal stress, analyzed the resulting changes in antioxidant enzyme and protein expression, and tracked changes in oxidative stress over time. Changes in the activity, mRNA, and protein expression of SOD and CAT indicate responses to high levels of environmental stress.

We analyzed these changes in order to confirm the effects of thermal stress on antioxidant enzymes in goldfish liver tissue. Experimental groups kept at 30 °C demonstrated significantly higher activity, mRNA, and protein expression levels for SOD and CAT, compared with groups maintained at 22 °C (Figs. 1–4). However, melatonin-injected groups showed a significant decrease in the activity, mRNA, and protein expression levels for SOD and CAT relative to that of the control. In particular, groups that received 10 μg melatonin/g body mass showed significantly decreased activity, mRNA, and protein expression levels for SOD and CAT, compared with groups that received 5 μg melatonin/g body mass. The results for groups that were exposed to green-wavelength LED light were similar to those of the melatonin-injection groups.

This study demonstrated that activity levels of the antioxidant enzymes SOD and CAT were affected by thermal stress and were significantly affected during photophase on ZT8. In particular, during the photophase, activity levels in the green-wavelength LED treatment group were significantly lower on ZT12 than in the melatonin-injected groups. The results of the experiment using cultured liver cells treated with melatonin were similar to those of the *in vivo* experiment (Fig. 3).

This results of this study are in agreement with those of Shin et al. [28], who reported that yellowtail clownfish (*Amphiprion clarkii*) injected with melatonin exhibited reduced expression levels of antioxidant enzymes; therefore, they concluded that melatonin acts as a strong antioxidant by scavenging ROS. In addition, the antioxidant enzyme activities of liver cells in climbing perch (*Anabas testudineus*) were significantly lower in melatonin-treated groups than in untreated groups, under a 24-h photoperiod [31]. Thus, melatonin was determined to be a strong antioxidant that effectively protected the cell membrane and scavenged for internally generated free radicals [36]. Therefore, the results of this study are similar to those of previous studies, which reported that green-wavelength LED light can effectively decrease oxidative stress and that melatonin, which is mainly secreted during the scotophase, acts as a powerful antioxidant.

In order to confirm that oxidative stress was experienced by goldfish subjected to high temperatures, we analyzed the plasma H_2O_2 levels and the LPO concentration of the fish. Groups exposed to 30 °C showed exhibited significantly higher plasma H_2O_2 levels and LPO concentrations than did groups maintained at 22 °C. However, H_2O_2 levels and LPO concentrations were significantly

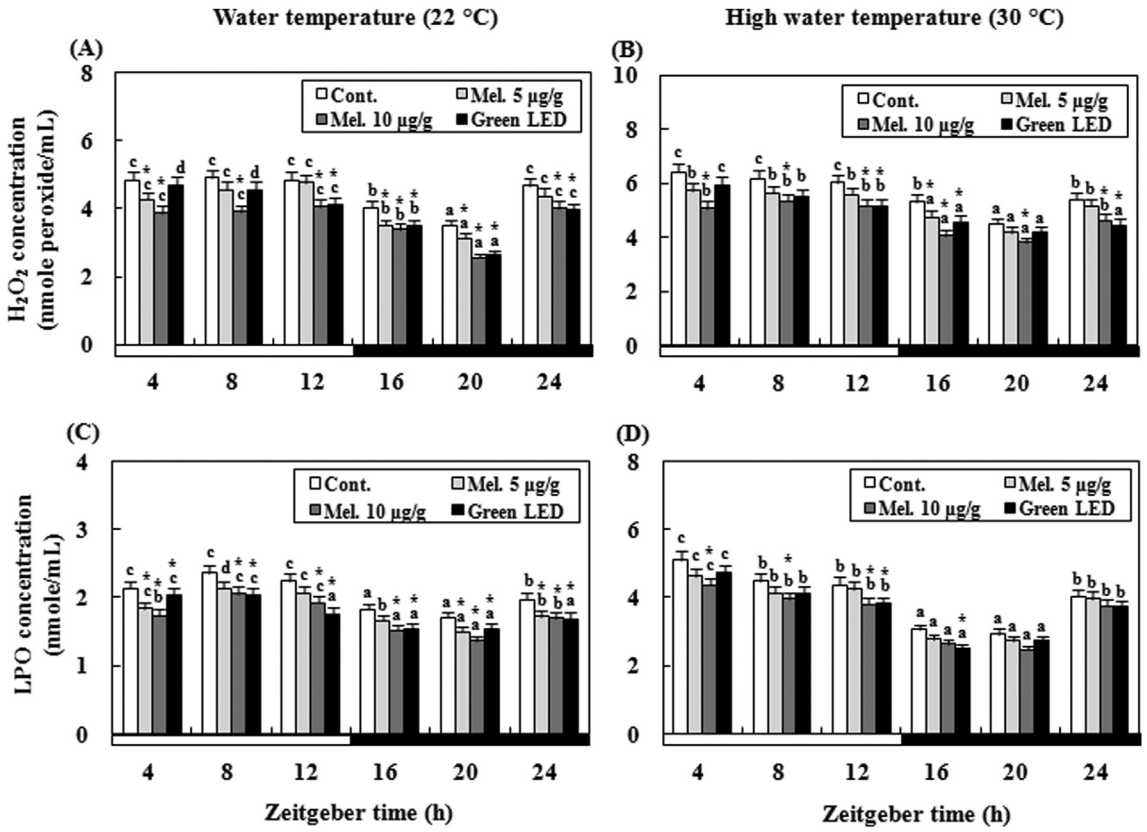

Fig. 5. Changes in plasma H_2O_2 levels during thermal changes at 22 °C (A) and 30 °C (B), and plasma LPO levels during thermal changes at 22 °C (C) and 30 °C (D), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates the presence of significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

lower in melatonin-injected groups. Fish that were exposed to green-wavelength LED treatment exhibited results similar to those of melatonin-injected fish (Fig. 5).

The results of this study are similar to those of previous study in which Nakano et al. [37] reported that oxidative stress, thus the level of plasma LPO, was increased when coho salmon (*Oncorhynchus kisutch*) were exposed to thermal stress. According to Choi et al. [29], the results showed that oxidative stress was induced in starved cinnamon clownfish (*Amphiprion melanopus*); nevertheless, in the present study, the groups exposed to green-wavelength LED light exhibited significantly reduced levels of plasma LPO and lower H_2O_2 concentrations. Gülcin et al. [38] reported that after rainbow trout (*Oncorhynchus mykiss*) received a melatonin injection, their LPO levels decreased significantly over time. Thus, melatonin plays a role in reducing LPO levels. The present study confirmed the reports of previous studies that green-wavelength LED light is effective at preventing cell damage due to thermal stress and can protect fish in the absence of melatonin treatments.

In addition, this study investigated the activity level of plasma lysozyme to determine the effect of heat stress on the immune systems of goldfish, as lysozyme content is an indicator of non-specific immune function. The groups that experienced 30 °C exhibited significantly decreased lysozyme activity compared with the 22 °C groups, whereas groups that received melatonin injections or green-wavelength LED treatment exhibited significantly increased lysozyme activity. In particular, after ZT12, green-wavelength LED groups exhibited higher lysozyme activity than the melatonin-injected groups (Fig. 6).

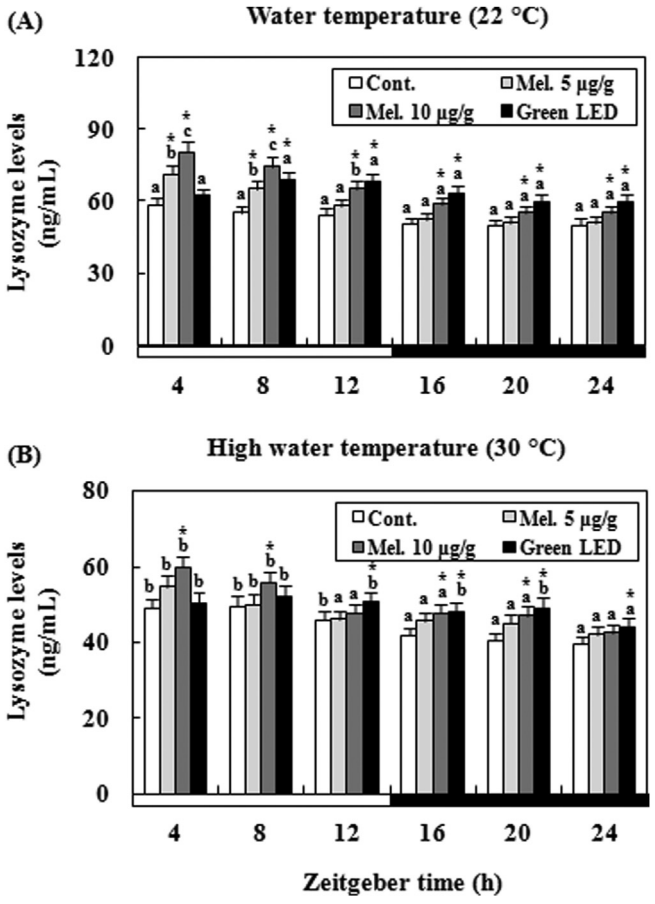

Fig. 6. Changes in plasma lysozyme levels during thermal changes at 22 °C (A) and 30 °C (B), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

Wang et al. [39] reported that acute changes in salinity caused internal oxidative stress and reduced lysozyme activity and thus directly decreased the efficacy of the immune system in sea cucumber (*Apostichopus japonicus*). In addition, Choi et al. [29] reported that starvation induces oxidative stress, but treatment with green-wavelength LED light decreased oxidative stress and increased immune function in cinnamon clownfish. The direct effect of melatonin on lysozyme activity remains unclear. However, Esteban et al. [40] reported that lysozyme activity in the circadian rhythm was significantly higher during the scotophase than during the photophase in sea bass (*Dicentrarchus labrax* L.); thus, melatonin, a hormone secreted during the scotophase, may affect lysozyme activity. In addition, Cuesta et al. [41] reported that in gilthead sea bream (*Sparus aurata* L.), the injection of melatonin causes immune-relevant genes and activity to be upregulated; thus, melatonin increased immune function. Therefore, our work confirmed that of previous studies that both green-wavelength LED and melatonin treatment increased both lysozyme activity and immune capacity.

A comet assay at ZT8 was used to investigate oxidative nuclear DNA damage. The comet assay clearly identified damage to nuclear DNA due to increased free radicals caused by stress (Fig. 7). Goldfish cells possess normal nuclear DNA at 22 °C, whereas cells with damaged nuclear DNA were observed in all groups at 30 °C (Fig. 7A). In addition, *in vitro* analysis using liver cells showed that the groups at 30 °C exhibited significantly higher tail length, percentage DNA in the tail, and tail moment than did those at 22 °C (Fig. 7B). However, cells from fish treated with melatonin injections or green-wavelength LED light showed significantly decreased tail length, percentage DNA in tail, and tail moment. They appeared to incur hardly any damage compared with the fish in the high-temperature groups.

Anitha et al. [42] reported that nuclear DNA was damaged by the increased water temperatures when goldfish were exposed to water temperatures of 34 °C, 36 °C, and 38 °C. In addition, Villarini et al. [43] reported that high temperatures induced oxidative stress and damaged the DNA of rainbow trout (*O. mykiss*) erythrocytes, but antioxidant treatment reduced the level of damage to the nuclear DNA.

The results of the present study are similar to those of previous studies that showed oxidative stress is induced through exposure to high temperatures, in that thermal stress damaged the nuclear DNA of goldfish liver cells, but our results showed that treatment with melatonin or green-wavelength LED light played a role in protecting and repairing damaged nuclear DNA.

In conclusion, our results indicate that acute changes in water temperature induce oxidative stress in goldfish and reduce their liver cells' antioxidant capacity and immune function; however, melatonin injection and exposure to green-wavelength LED light effectively controlled (or reduced) the oxidative stress induced by thermal stress. In other words, these treatments can increase the antioxidant capacity and boost the immune system of fish. In particular, the effect of exposure to green-wavelength LED light was comparable to that of treatment with 10 μg melatonin/g body mass; therefore, irradiation with green-wavelength LED light should be considered a potential replacement for melatonin treatment.

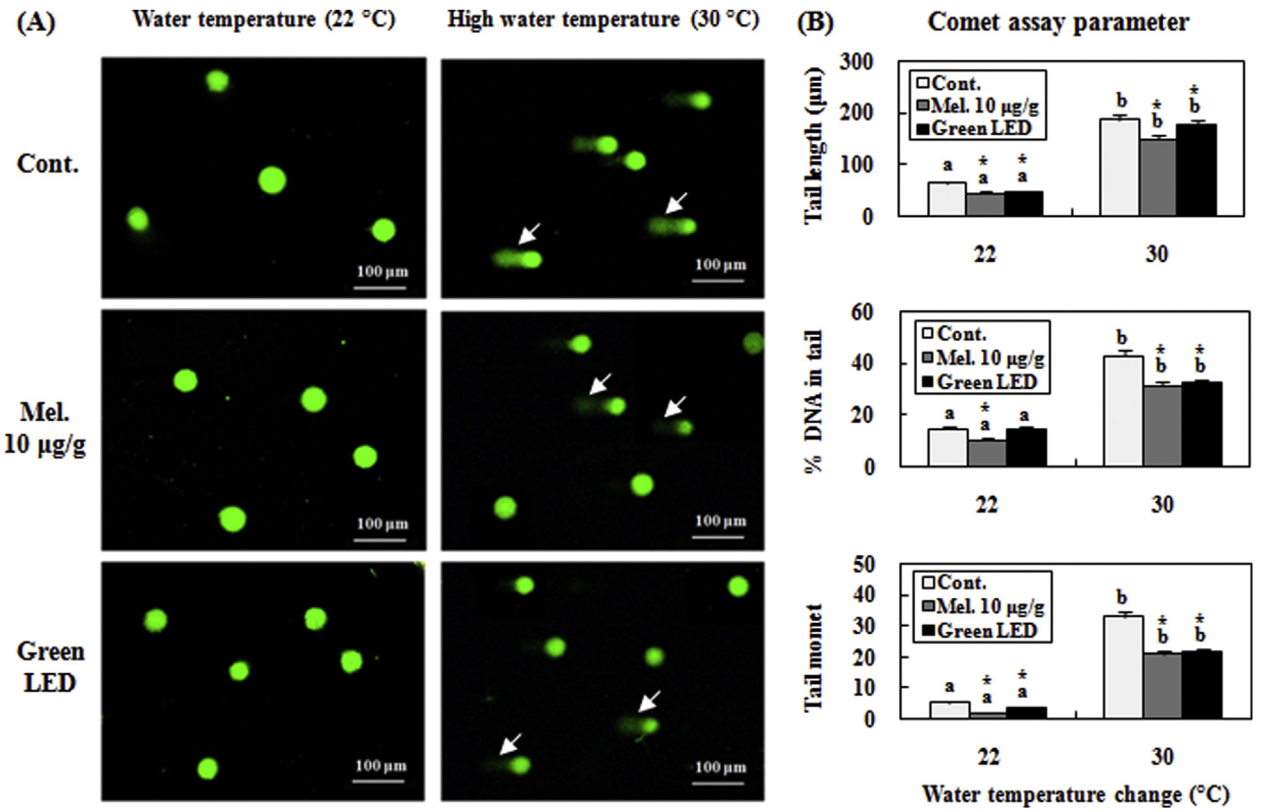

Fig. 7. Comet assay images (A), comet assay parameter for tail length, percentage DNA in tail, and tail moment (B) during thermal change at ZT8 (15:00), in each experimental goldfish group: control, 10 μg/g body mass of melatonin injection, and green-wavelength LED light, as measured by fluorescence microscopy. White arrows indicate the damaged nuclear DNA (DNA breaks) of liver cells, which are stained with SYBR-green. Scale bars = 100 μm. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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