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Variations in the antioxidant system and circadian rhythms of goldfish, *Carassius auratus*, exposed to ammonia: profile of the effects of green LED spectra

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ABSTRACT

The present study evaluated effects of green light emitting diode (LED) spectra on oxidative stress and circadian rhythms in goldfish exposed to various concentrations (0.25 and 0.5 mg/L) of NH₃, under a white fluorescent bulb (control; simulated natural period) and green LED light. We measured mRNA expression and activity of antioxidant enzymes (superoxide dismutase and catalase) and mRNA expression of circadian rhythms (*period 2*), in addition to levels of plasma hydrogen peroxide, cortisol and melatonin. Damage to nuclear DNA was assessed using the comet assay. All stress indicators and melatonin were significantly lower in the green LED group than in the control group. With an increase in the concentration of ammonia, the observed effects became even more significant and generally increased with time. Comparatively, damage to the nuclear DNA was greater in the 0.5 mg/L NH₃ group, and lower in the green LED group. The *Period 2* mRNA expression reduced as increasing ammonia treatment but increased as green LED exposed. We have suggested that Green LED reduced levels of oxidative stress, which suggests an antioxidant effect against NH₃ toxicity. Additionally, ammonia is affected the circadian rhythms and the green LED wavelength is able to regulate effectively the circadian rhythm.

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1. Introduction

Stress factors in marine creatures such as fish can be mainly classified as physical factors and chemical factors. Physical factors include salinity, stocking density and water temperature (Beckmann et al. 1990) and chemical factors include the concentration of ammonia (NH₃), heavy metals and disinfectants in the water (Yang & Yeo 2004). Ammonia, which is present as NH₄⁺ ions and NH₃ in water, is a final product of nitrogen metabolism in teleosts. High concentrations of ammonia have toxic effects on the nervous system, retard the growth rate, cause diseases, disturb ion homeostasis and eventually induce damage to the gills

(Benli et al. 2008; Romano & Zeng 2013). The flow of ammonia in the aquatic environment from sewage, industrial waste, and manure, can lead to a rapid increase in its concentration to dangerous levels, particularly in marine areas with high population densities (Wilkie 1997; Randall & Tsui 2002; Dosdat et al. 2003).

Moreover, ammonia induces oxidative stress in fish by increasing levels of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen (1O_2) (Roch 1999). Excess ROS production induced by environmental stress can result in the loss of structure and function of nucleic acids and proteins, thereby leading to a number of physiological disorders, such as reduced fertility and resistance to diseases (Choi et al. 2013; Sinha et al. 2014).

Organisms generally have antioxidant defence mechanisms against oxidative stress and ROS-induced toxicity that helps to maintain homeostasis. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) function mainly in the liver and kidneys (Basha & Rani 2003; Hansen et al. 2006). SOD and CAT can directly remove ROS. SOD first removes free radicals by converting O_2^- to O_2 and H_2O_2 ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). H_2O_2 is then converted by CAT to the non-toxic products, H_2O and O_2 (Kashiwagi et al. 1997).

The hypothalamic-pituitary-interrenal (HPI) axis is also activated to maintain homeostasis in organisms exposed to the stress of toxicity (Bonga 1997; Flik et al. 2006). To regulate the hormones released in response to stress, corticotropin-releasing hormone (CRH) is secreted by the hypothalamus and it acts on the anterior pituitary to stimulate the secretion of adrenocorticotrophic hormone (ACTH) (Bonga 1997). ACTH, which is derived from the precursor protein, pro-opiomelanocortin, stimulates the synthesis and release of cortisol in interregal cells of the head kidney (Bonga 1997; Flik et al. 2006). Cortisol levels indicate the magnitude of the stress response. Cortisol is secreted into the plasma and affects several physiological functions (Beato et al. 1996; Bonga 1997). It binds to specific receptors present on cell membranes, such as the glucocorticoid receptor (GR), and exerts direct cellular effects. In addition, cortisol directly increases levels of glucose, which is an important energy source and metabolic substrate in vertebrates (Mommsen et al. 1999; Begg & Pankhurst 2004).

Variations in the wavelength of light and photoperiod are important environmental factors that affect the physiological functions, such as maturation, growth and regulation of stress and immunity, and circadian rhythms in the aquatic organisms, particularly teleosts (Pierce et al. 2008; Shin et al. 2011, 2012; Choi et al. 2013; Kim et al. 2014). In circadian rhythms response, melatonin is mainly produced in the pineal organ and retina, and its concentration in the plasma is higher during the night than the day. Moreover, the hormone not only acts as a neuroendocrine messenger in the regulation of the circadian rhythm but also affects seasonal biological rhythms (Reiter 1991). Furthermore, *Period* (*Per*) is a key protein in the circadian system of animals. Four types of *Per* (*Per 1*, *2*, *3*, and *4*) have been reported in zebrafish *Danio rerio* (Delaunay et al. 2000). In zebrafish, *Per2* is a circadian oscillator that is rapidly induced by light information from the suprachiasmatic nucleus (SCN) and appears to be involved in light-dependent clock resetting (Vallone et al. 2004).

Several recent studies have focused on the wavelengths of light emitting diodes (LEDs) compared to those of natural light or a white fluorescent bulb (control; simulated natural period). LEDs emit light of a specific wavelength and it has been used as a novel light source in various studies (Migaud et al. 2007; Shin et al. 2011; Kim et al. 2014). Choi et al. (2012) and Kim et al. (2014) in their studies on clownfish, *Amphiprion melanopus* and goldfish, *Carassius*

auratus, respectively, reported that green LED wavelengths effectively promote antioxidant function, by suppressing levels of oxidative stress induced by fasting and rapid temperature change. Thus, recent research has shown the use of LED wavelengths to study circadian rhythms. In addition, previous studies reported that specific LED wavelengths are able to regulate circadian rhythm by regulating the expression of clock genes *period 2* (*Per2*) and levels of melatonin (Shin et al. 2011; Park et al. 2013).

Thus, the aim of the present study was to confirm the antioxidant effects and circadian rhythms (biorhythms) of green LED light in goldfish, under conditions of ammonia toxicity. Goldfish were exposed to two concentrations of ammonia (0.25 and 0.5 mg/L) and green LED irradiation, after which the antioxidants SOD and CAT, as well as the changes in mRNA expression and activity in plasma were measured. In addition, comparative analysis of active oxygen (H_2O_2) and cortisol concentrations, as they relate to oxidative stress was conducted. In addition, the comet assay was selected to confirm the effects of green light (including an overall reduction in DNA damage) under conditions of ammonia toxicity. Furthermore, to investigate the effects of ammonia exposure or green LEDs on the circadian rhythms, we measured the levels of plasma melatonin levels and *Per2* mRNA expression.

2. Materials and methods

2.1. Experimental fish and ammonium chloride treatment

Goldfish ($n = 130$, mean 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 investigation, which was conducted in a laboratory. The water temperature was maintained at 22 ± 1 °C and the photoperiod included 12-h light and 12-h dark periods (lights on at 07:00 h and off at 19:00 h). Fish were randomly allocated to experimental groups: a control group, and four other groups exposed to NH_3 in a white fluorescent bulb (control, simulated natural period; 27 W, wavelength range 350–650 nm) and under green (530 nm wavelength) LED light (Daesin LED Co., Kyunggi, Korea). The light sources were placed 50 cm above the surface of the water. Irradiance at the water's surface was maintained at approximately 0.5 W/m². Experimental groups were treated with waterborne ammonium chloride (NH_4Cl ; Daejung Chemicals & Metals Co., LTD., Kyunggi, Korea) at concentrations of 0.25 and 0.5 mg/L. Tissues were sampled from five fish of each experimental group at each of the following time periods: 0, 6, 12, 24, 48 and 72 h. All tissues (brain and liver) were collected, immediately frozen in liquid nitrogen and stored at -80 °C until total RNA was extracted. In addition, blood was collected from the caudal vein of each fish sample using a 3-mL syringe coated with heparin. Plasma samples were separated from blood by centrifugation (4 °C, $10,000 \times g$, for 10 min) and stored at -80 °C until further analysis.

2.2. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

Total RNA was extracted from each sample using TRI Reagent[®] (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. Total RNA (2 µg) was then reverse transcribed in a total reaction volume of 20 µL, 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 and stored at 4 °C for subsequent use in 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'
<i>Per2</i> (EF690697)	Forward	5'- CTG GAG CCG CAA AGT TTC-3'
	Reverse	5'- CTG GAT GTC TGA GTC TAA-3'
β -actin (AB039726)	Forward	5'-TTC CAG CCA TCC TTC CTA T-3'
	Reverse	5'-TAC CTC CAG ACA GCA CAG-3'

polymerase chain reaction (PCR) and qPCR. The qPCR was conducted to determine relative mRNA expression levels of the antioxidant enzymes (SOD, GenBank Accession No. JQ776518 and CAT, GenBank Accession No. JQ776513) and *Per2* (GenBank Accession No. EF690697), using total RNA extracted from the liver and brain. The primers for qPCR were designed using known goldfish sequences and are presented in Table 1. The qPCR amplification was conducted 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. For each sample, β -actin was also amplified as a control and all changes were expressed with respect to the corresponding β -actin cycle threshold (Ct) levels. All analyses were based on Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold at the exponential phase of the amplification curve. The calibrated Δ Ct value ($\Delta\Delta$ Ct) of each sample and the internal controls (β -actin) were calculated [$\Delta\Delta$ Ct = $2^{-(\Delta$ Ct_{sample} - \DeltaCt_{internal control})]. Following the PCR, qPCR data from three replicates were analysed, using a Bio-Rad program to estimate the number of transcript copies in each sample.}

2.3. SOD and CAT activity

Liver tissues were homogenized in 1 × PBS. The homogenates were centrifuged at 5,000 × *g* for 5 min at 4 °C. The supernatant was then removed and the pellet was used for further analysis. SOD activity was determined using a fish superoxide dismutase (SOD) ELISA kit (706002, Cayman Chemical, Ann Arbor, MI, USA) and was expressed as U/mL.

Plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, for 10 min), and CAT levels were analysed. Variations in CAT levels in plasma following NH₃ exposure were measured using a fish catalase (CAT) ELISA immunoassay kit, according to the manufacturer's instructions (CSB-E15928fh, Cusabio, Wuhan, China). CAT levels were expressed as U/mL.

2.4. Western blot analysis

Total protein isolated from liver samples was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo, Waltham, MA, USA) according to the manufacturer's instructions. Protein (30 μ g per lane) was loaded 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 μ m polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. The membranes were then blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min, after which they were washed

in TBS. Separate membranes were incubated with SOD (1:4,000 dilution, NBP1-47443, Novus Biologicals, Littleton, CO, USA) and CAT (1:4,000 dilution, sc-58332, Santa Cruz Biotechnology, TX, USA) antibodies, respectively, and then incubated with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (SOD and CAT; dilution 1:2000, Bio-Rad) for 60 min. The internal control was β -tubulin (dilution 1:5000, ab6046, Abcam, Cambridge, UK). Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) following 30 s of exposure in a Molecular Imager® from ChemiDoc™ XRS+ Systems (Bio-Rad, Hercules, CA, USA). Membrane images were scanned using a high-resolution scanner and band density was estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad).

2.5. Plasma H₂O₂, cortisol, and melatonin assays

Plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, for 10 min) and analysed via evaluation of H₂O₂, cortisol and glucose levels.

Plasma H₂O₂ concentrations were measured using a PeroxiDetect™ kit (Sigma, St. Louis, MO, USA) according to the methods described by Nouroozadeh et al. (1994). Absorbance was read at 560 nm and the concentration of H₂O₂ was interpolated from a standard curve and expressed in nmol peroxide/mL.

Plasma cortisol and melatonin concentrations were measured using a fish cortisol ELISA kit (CSB-E08487f; Cusabio, China) and fish Melatonin ELISA kit (MBS-013211; MyBioSource, USA). Absorbance was read at 450 nm and cortisol concentration was interpolated from a standard curve and expressed in ng/mL (cortisol) and pg/mL (melatonin).

2.6. Comet assay

The comet assay is a relatively simple, sensitive technique for quantitatively measuring DNA damage in eukaryotic cells (Bajpayee et al. 2005). Liver cells (1 × 10⁵ cells/mL) were examined using a Comet Assay® Reagent Kit for single cell gel electrophoresis (Trevigen, Gaithersburg, MD, USA), according to the methods described by Singh et al. (1988). Cells were immobilized in agarose gel on Comet Assay® slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Slides were then electrophoresed at 15 V for 30 min. Samples were stained with SYBR® Green (Trevigen) for 30 min in the dark, and read using a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon, Japan). At least 100 cells from each slide were analysed at 0 and 72 h, respectively. For quantification analysis of the comet assay, we analysed the tail length (distance of DNA migration from the head), % DNA in the tail (tail intensity/total intensity × 100) and tail moment (extent of DNA damage; product of tail length and % of DNA in the tail) using the comet assay IV image analysis software (version 4.3.2, Perceptive Instruments Ltd., UK).

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey *post hoc* test was used to compare differences in the data. All statistical significance tests were done with a one-tailed homoscedastic (equal variance) *t*-test ($p < 0.05$). The values were expressed as the mean ± SE of three independent measurements.

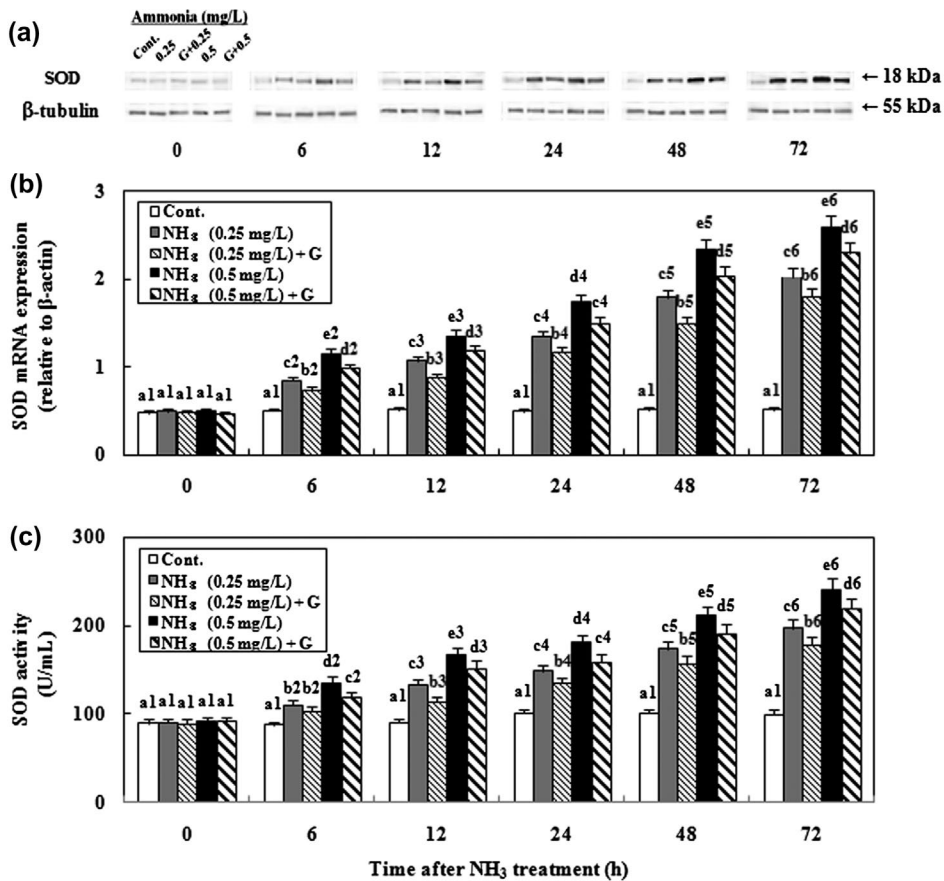


Figure 1. Changes in SOD expression and activity of goldfish exposed to NH₃ and green LED light. (a) Western blot of SOD protein expression in the liver after NH₃ treatment. The internal control was β -tubulin (55 kDa). (b) SOD mRNA expression levels in the liver of goldfish following NH₃ treatment are expressed in relation to β -actin mRNA levels and based on quantitative real-time PCR data. Total RNA (2 μ g) prepared from liver samples was reverse-transcribed and amplified using gene-specific primers. The results are expressed as normalized fold expression (relative to the control data) in relation to β -actin levels for the same sample. (c) Changes in SOD activity in plasma were measured by a microplate reader. Notes: Numbers indicate significant differences among different time points with a similar light source and NH₃ concentration ($p < 0.05$). Lower case letters indicate significant differences among different light sources and NH₃ concentrations at the same time point ($p < 0.05$). All values represent means \pm SE ($n = 5$). G, green LED; Cont., control.

3. Results

3.1. Expression and activity of SOD and CAT

Following NH₃ exposure, we evaluated the changes in mRNA expression and activity of the antioxidant enzyme SOD in the liver, (Figure 1(b) and (c)). Western blot analysis revealed a protein with SOD enzyme-specific immunoreactivity and a mass that corresponded to the predicted mass of goldfish SOD (18 kDa; Figure 1(a)). After 72 h, SOD mRNA expression and activity were significantly higher following exposure to NH₃ at a concentration of 0.5 mg/L compared to that of 0.25 mg/L NH₃. Expression and activity of SOD mRNA were also

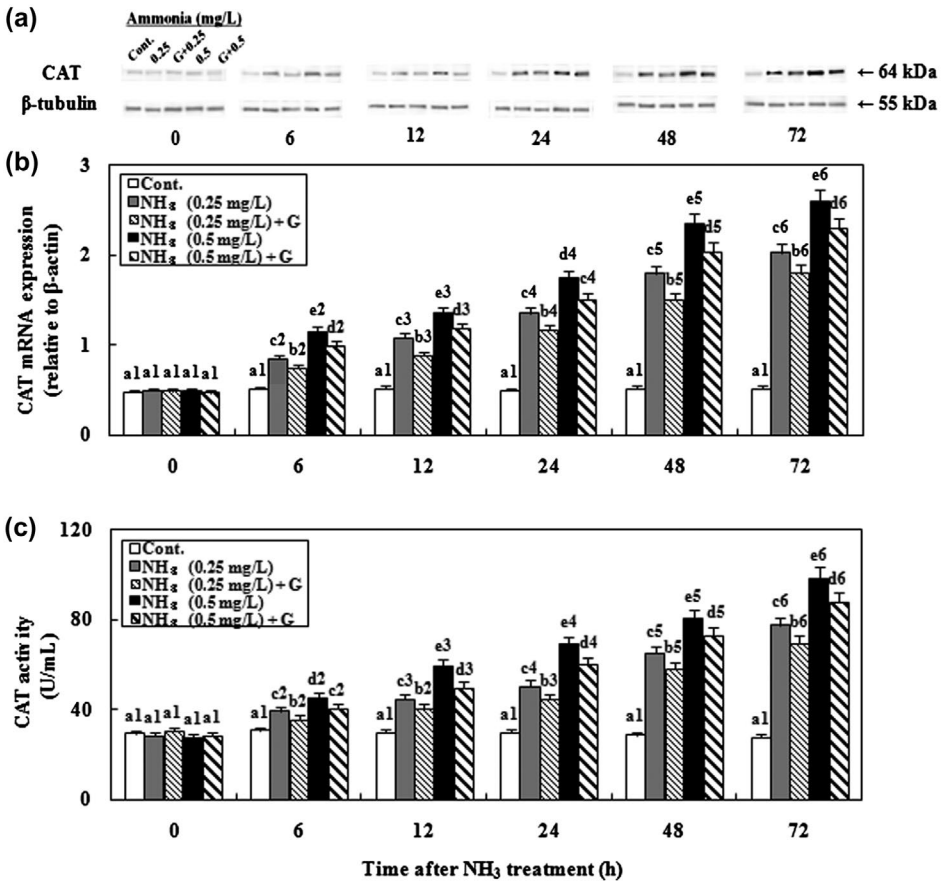


Figure 2. Changes in CAT expression and activity in plasma of goldfish exposed to NH₃ and green LED light. (a) Western blot of CAT protein expression in the liver of goldfish following NH₃ treatment. The internal control was β -tubulin (55 kDa). (b) Levels of CAT mRNA expression are expressed in relation to β -actin mRNA levels in the liver of goldfish following NH₃ treatment and based on quantitative real-time PCR data. The results are expressed as normalized fold expression (relative to the control data) as compared to β -actin levels for the same sample. (c) Changes in CAT activity in plasma were measured by a microplate reader.

Notes: Numbers indicate significant differences among different time points with the same light source and NH₃ concentration ($p < 0.05$). Lower case letters indicate significant differences among different light sources and NH₃ concentrations at the same time point ($p < 0.05$). All values represent means \pm SE ($n = 5$). G, green LED; Cont., control.

significantly lower under green LED light conditions than under control conditions. The expression pattern of the SOD protein resembled that of SOD mRNA in goldfish liver.

Figure 2(b) and (c) illustrate the variation in expression and activity of CAT mRNA in fish liver, following exposure to NH₃. Western blot analysis revealed a protein with CAT enzyme-specific immunoreactivity and a mass that corresponded to the predicted mass of goldfish CAT (64 kDa; Figure 2(a)). After 72 h, CAT mRNA expression and activity in the liver were significantly higher following exposure to NH₃ at a concentration of 0.5 mg/L compared to that of 0.25 mg/L NH₃. Furthermore, expression and activity of CAT were significantly lower under green LED light conditions than under control conditions. The expression pattern of the CAT protein resembled that of CAT mRNA in goldfish liver.

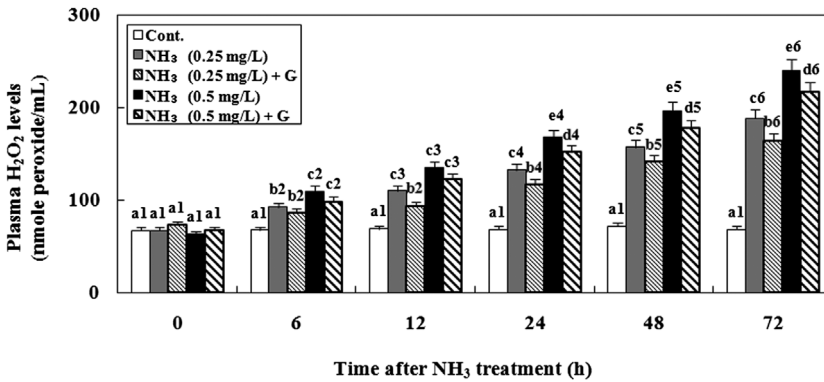


Figure 3. Plasma H₂O₂ level of goldfish exposed to NH₃ under conditions of green light and a white fluorescent bulb, as measured by a microplate reader.

Notes: Numbers indicate significant differences among different time points with the same light source and NH₃ concentration ($p < 0.05$). Lower case letters indicate significant differences among different light sources and NH₃ concentrations at the same time point ($p < 0.05$). All values represent means \pm SE ($n = 5$). G, green LED; Cont., control.

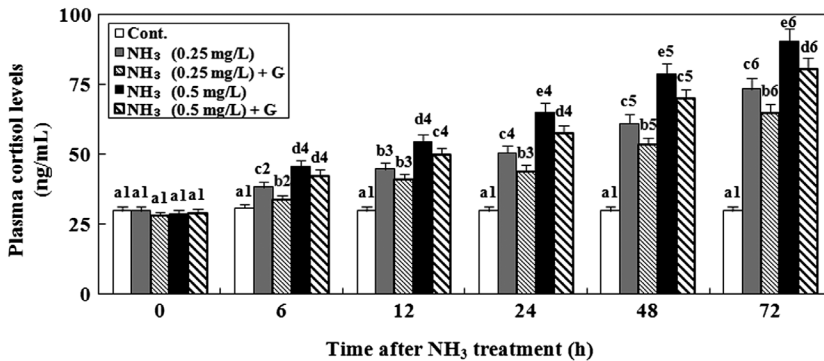


Figure 4. Plasma cortisol of goldfish exposed to NH₃ under conditions of green light and a white fluorescent bulb (Cont.), as measured by a microplate reader.

Notes: Numbers indicate significant differences among different time points with a similar light source and NH₃ concentration ($p < 0.05$). Lower case letters indicate significant differences among different light sources and NH₃ concentrations at the same time point ($p < 0.05$). All values represent means \pm SE ($n = 5$). G, green LED; Cont., control.

3.2. Plasma H₂O₂ levels

The ELISA kit revealed that after 72 h, plasma H₂O₂ was significantly increased following exposure to NH₃ (Figure 3). Plasma levels in the groups exposed to a concentration of 0.5 mg/L NH₃ were significantly higher than those in groups exposed to 0.25 mg/L NH₃. The level of H₂O₂ in all experimental groups was significantly lower under conditions of green LED light than under control.

3.3. Plasma cortisol levels

Figure 4 shows the variations in plasma cortisol levels following exposure to NH₃. In comparison to the control, cortisol was significantly increased. Plasma cortisol levels were

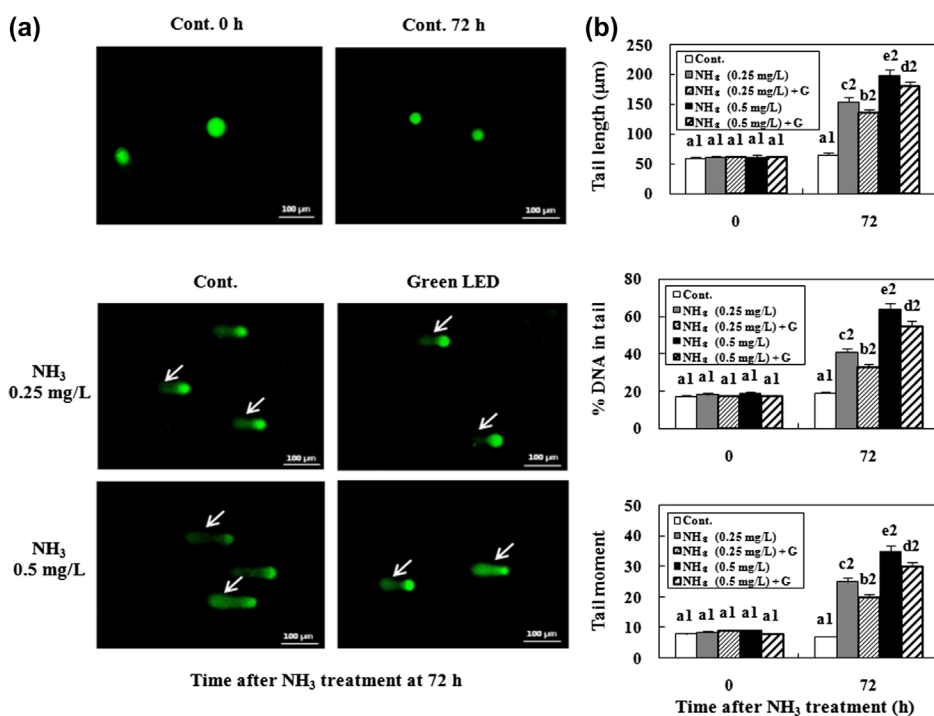


Figure 5. Comet assay images (a), tail length, percentage DNA in tail, and tail moment (b) following exposure to NH_3 at 0 and 72 h in each experimental group: control; 0.25 mg/L NH_3 ; and 0.5 mg/L NH_3 . Notes: Control and green LED light was measured by fluorescence microscopy. White arrows indicate damaged nuclear DNA (DNA breaks) of liver cells, which were stained with SYBR-green. Scale bars = 100 μm . Lower case letters and numbers indicate significant differences within the same experimental group and at the same temperature, respectively ($p < 0.05$). G, green LED; Cont., control.

significantly higher in groups exposed to 0.5 mg/L NH_3 than those exposed to 0.25 mg/L NH_3 . In addition, plasma cortisol levels were also significantly lower in groups exposed to green LED light than those exposed to control.

3.4. Comet assay

DNA damage was determined by measuring the fraction of cells with nuclear DNA comet tails (a phenomenon indicative of DNA breaks). Cells (100 cells) were randomly selected for analysis at 0 and 72 h, using a fluorescence microscope. At 0 h, liver cells possessed normal nuclear DNA. In addition, no nuclear DNA damage was visible at 72 h (Figure 5(a)). The comet tails in the groups exposed to 0.5 mg/L NH_3 were significantly longer than those in the groups exposed to 0.25 mg/L NH_3 . Furthermore, groups exposed to the control exhibited significantly longer tails, higher percentages of DNA in the tail and greater tail moments than those exposed to green LED light (Figure 5(b)). The cells of the groups exposed to 0.25 mg/L NH_3 and green LED light sustained lower levels of nuclear DNA damage, exhibited shorter tails, had lower percentages of DNA in the tail and shorter tail moments.

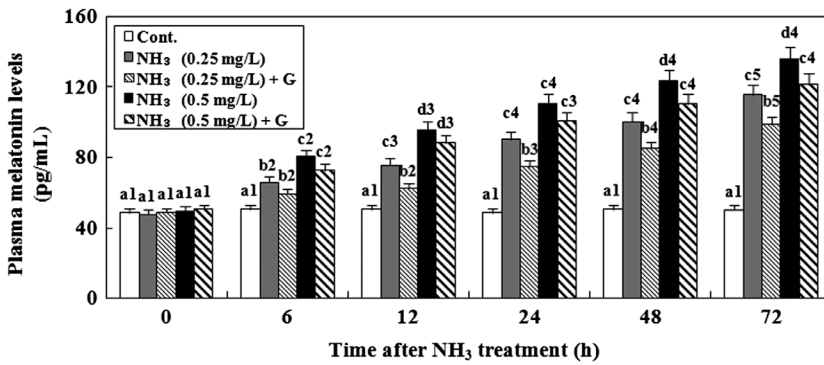


Figure 6. Plasma melatonin of goldfish exposed to NH_3 under conditions of green light and a white fluorescent bulb (Cont.), as measured by a microplate reader.

Notes: Numbers indicate significant differences among different time points with a similar light source and NH_3 concentration ($p < 0.05$). Lower case letters indicate significant differences among different light sources and NH_3 concentrations at the same time point ($p < 0.05$). All values represent means \pm SE ($n = 5$), G, green LED; Cont., control.

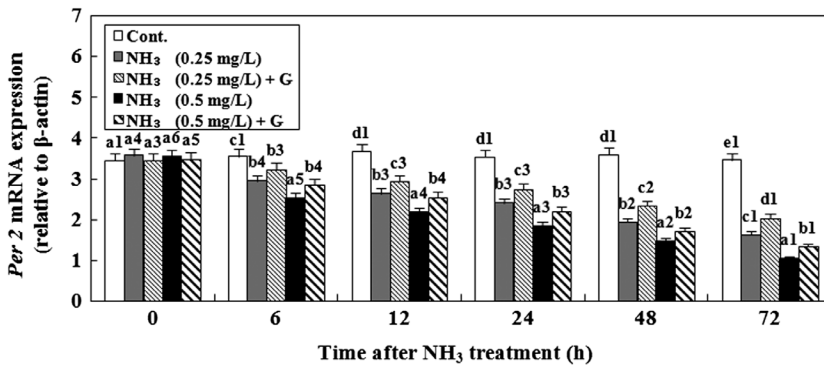


Figure 7. Changes in *Per2* mRNA expression in the brain of goldfish, exposed to NH_3 and green LED light. NH_3 treatment are expressed in relation to β -actin mRNA levels and based on quantitative real-time PCR data.

Notes: Numbers indicate significant differences among different time points with a similar light source and NH_3 concentration ($p < 0.05$). Lower-case letters indicate significant differences among different light sources and NH_3 concentrations at the same time point ($p < 0.05$). All values represent means \pm SE ($n = 5$), G, green LED; Cont., control.

3.5. Plasma melatonin levels and *Per2* mRNA expression

Following NH_3 exposure, we evaluated the changes in plasma melatonin and *Per2* mRNA expression in the liver, (Figures 6 and 7). The plasma melatonin levels was significantly increased following exposure to NH_3 (Figure 6). Plasma levels in the groups exposed to a concentration of 0.5 mg/L NH_3 were significantly higher than those in groups exposed to 0.25 mg/L NH_3 . The level of melatonin in all experimental groups was significantly lower under conditions of green LED light than under control.

In contrast, after 72-h exposure to NH_3 , expression of *Per2* mRNA was significantly decreased in comparison to the control (Figure 7). In particular, *Per2* mRNA expression was significantly low following exposure to NH_3 at a concentration of 0.5 mg/L compared to that

of 0.25 mg/L NH_3 and was also significantly high under green LED light conditions than under non-green LED light condition after exposure to NH_3 .

4. Discussion

The present study confirmed the effects of green LED light in goldfish exposed to ammonia toxicity (at concentrations of 0.25 and 0.5 mg/L). Following exposure to irradiation by the white fluorescent bulb (27 W) and green LED light (0.5 W/m²), samples of the brain, liver and plasma were taken at 0, 6, 12, 24, 48 and 72 h.

As the ammonia concentration and exposure time was increased, SOD and CAT mRNA and protein expression and activity were also significantly increased. However, in the goldfish exposed to green LED light, antioxidant gene and protein expression and activity were significantly reduced.

The findings of the present study are consistent with those of a previous study on rainbow trout, *Oncorhynchus mykiss* and carp, *Cyprinus carpio* exposed to ammonia, which reported that SOD and CAT activities were significantly increased in the gills and liver (Sinha et al. 2014). Kim et al. (2014) also reported that green light effectively reduced oxidative stress in goldfish, which is consistent with the results of the present study. Choi et al. (2012) reported that green LED irradiation significantly reduced oxidative stress (induced by starvation for 12 d) in clownfish. In other words, in comparison to the control, green LED irradiation significantly reduces the expression of antioxidant genes and proteins. Therefore, in clownfish exposed to the stress of starvation, the wavelength of green LED can reduce oxidative stress to levels that are almost the same as the control group (Choi et al. 2012).

Thus, the present study corroborates the results of previous studies, which report that antioxidant gene expression and activity are significantly increased by ammonia-induced oxidative stress, and that the green LED wavelength effectively reduces oxidative stress.

Proliferation of ROS due to external environmental stress promotes the denaturation of nucleic acids and protein and increases levels of H_2O_2 (Kim and Phyllis 1998; Pandey et al. 2003). We measured plasma H_2O_2 concentrations of goldfish to confirm the changes induced by the stress of ammonia toxicity. Our results revealed that with an increase in the concentration of ammonia, the levels of H_2O_2 were significantly increased; however, these levels were significantly lower in groups exposed to green LED light, compared to either those exposed to the control.

A previous study reported significant increases in the levels of H_2O_2 in the gills and liver of rainbow trout and carp exposed to ammonia (Sinha et al. 2014). Another study reported that the levels of plasma H_2O_2 were significantly increased in goldfish exposed to high concentrations of selenium (which is toxic to fish) (Choi et al. 2015). In addition, Kim et al. (2014) reported relatively low levels of H_2O_2 and oxidative stress in goldfish exposed to green and blue LEDs (generally shorter wavelengths), in comparison to those exposed to fluorescent light. The fish in that study had been raised in a series of tanks, in which the temperature was artificially regulated and the light source was either control, green LED or blue LED.

The findings of the present study are similar to those of previous studies that report significantly increases plasma H_2O_2 levels of goldfish exposed to ammonia; the effective reduction of those levels by green LED irradiation; and subsequent inhibition of the damage induced by oxidative stress.

Neuroendocrine responses to stressors (such as toxicity) in vertebrates like fish entail activation of the hypothalamus-pituitary-interrenal axis (HPI), which plays an important role in adaptation to stress (Bonga 1997; Flik et al. 2006). In the present study, we observed variations in cortisol and glucose levels following activation of the HPI axis, which effectively regulated the stress response to ammonia toxicity. The levels of plasma cortisol were significantly increased in the fish as the concentration of ammonia was increased. On the other hand, plasma cortisol levels were significantly lower in the groups exposed to green LED light than those exposed to the control.

In a previous report, the expression of CRH, ACTH and GR mRNA; the levels of hormones (such as ACTH and cortisol) under control of the HPI axis were all significantly increased in goldfish exposed to high concentrations (3 and 4 mg/L) of selenium (Choi et al. 2015). Cortisol promotes the synthesis of glucose and thereby increases plasma glucose levels *in vivo* to maintain glucose homeostasis (Mommensen et al. 1999; Begg & Pankhurst 2004). The results of the present study are consistent with those of previous studies that state that ammonia-induced oxidative stress in goldfish directly increases levels of plasma cortisol and that green LED light plays a key role in reducing oxidative stress in goldfish.

In addition, the present study confirmed the incidence of nuclear DNA damage following exposure to ammonia toxicity using the comet assay. Although nuclear DNA damage was not evident in the group that had not been exposed to ammonia, nuclear DNA damage was observed in all other groups treated with ammonia. According to Guilherme et al. (2012), DNA damage is increased in the European eel, *Anguilla anguilla* L. following exposure to the glyphosate herbicide at different concentrations (58 and 116 g/L). In the present study, nuclear DNA damage was significantly increased as the concentration of ammonia increased from 0.25 to 0.5 mg/L, and significantly reduced by green LED irradiation. Thus, green LED irradiation appears to be one mechanism by which oxidative stress can be effectively reduced.

To investigate the effects of ammonia on circadian rhythm of goldfish, we observed the plasma melatonin levels and *Per2* mRNA expression exposed to green LEDs. The plasma melatonin concentration in goldfish was significantly higher in the ammonia treatment group. However, after exposed to the green LEDs, the plasma melatonin concentration was significantly lower than under only ammonia treatment. These results are consistent with those of a previous study where goldfish exposed to stress indicator, such as high water temperatures, increased their plasma melatonin concentration but lower melatonin levels during green LED exposure (Choi et al. 2014; Kim et al. 2014). Shin et al. (2011) and Choi et al. (2014) reported that melatonin, which is both a circadian regulation hormone and antioxidant, increased during stress condition but a melatonin decrease induced oxidative stress in green LED lights.

The circadian rhythms-related genes, *Per2*, as opposed to melatonin levels, reduced as ammonia treatment concentration increases and with the passage of time. The circadian factor, CLOCK-BMAL heterodimers, is inhibited by increased melatonin (Gerstner & Yin 2010), and these findings have been hypothesized that *Per2* act as a feedback with CLOCK-BMAL heterodimers. So, we have suggested that *Per2* act also decreased by increased melatonin after ammonia treatment. However, in the goldfish exposed to green LED light, *Per2* expression was significantly increased than non-green LEDs exposure. Similar to this study, Choi et al. (2014) was reported that environment change reduced the circadian rhythms, but specific wavelength (green LEDs light) increased the levels of *Per2* mRNA. So, we suggested that ammonia treatment confuses the circadian rhythm of goldfish but the green LED lights reduced stress, thus stabilizing and maintaining circadian rhythms.

In conclusion, we confirmed that (1) ammonia is toxic to goldfish and induces oxidative stress even at relatively low concentrations; (2) green LED light can effectively reduce oxidative stress and DNA damage induced by ammonia toxicity; (3) furthermore, ammonia toxicity is affected the circadian rhythms, however the green LED wavelength is able to regulate effectively the circadian rhythm.

Further studies are required to determine the appropriate wavelength and light intensity required to induce physiological changes *in vivo* in various species. The findings of the present study could form the basis for further related research.

Disclosure statement

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