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Effects of UV Radiation on Oxidative Stress in Yellowtail Clownfish *Amphiprion clarkii*

Han Seok Ryu[1], Cheol Young Choi[1]*, Jin Ah Song[1], and Gyung-Suk Kil[2]

[1]*Division of Marine BioScience, College of Ocean Science and Technology, Korea Maritime and Ocean University, Busan 49112, Korea*
[2]*Division of Electronics Engineering, College of Engineering, Korea Maritime and Ocean University, Busan 49112, Korea*

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Abstract – We investigated oxidative stress under UV radiation (380 nm) at two intensities (0.2 and 0.4 W/m^2) for 14 days (0, 1, 3, 7 and 14) in the yellowtail clownfish *Amphiprion clarkii*. We analyzed mRNA expression and the activity of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)], levels of plasma hydrogen peroxide (H_2O_2), lipid peroxide (LPO), alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT). Furthermore, DNA damage was measured using comet assays and terminal transferase dUTP nick end labeling (TUNEL) assays were performed. As a result, the mRNA expressions and activities of antioxidant enzymes, the levels of plasma H_2O_2, LPO, AlaAT and AspAT were significantly higher in the group exposed to UV radiation than the control group. Finally, the comet and TUNEL assay showed that apoptosis was markedly higher at high UV intensity compared to the control group. These results indicate that UV radiation induces oxidative stress and decreases the antioxidant response in yellowtail clownfish.

Keywords – oxidative stress, ultraviolet radiation, yellowtail clownfish

1. Introduction

The rampant use of chlorofluorocarbon, known as "Freon gas", in recent times has been reducing the ozone levels in the stratosphere and this has caused destruction to the ozone layer. As a result, ultraviolet (UV) radiation levels reaching the surface of the sea at locations between 30–50° latitudes are increasing (Zagarese and Williamson 2001). Based on their wavelength, UV radiations are classified as UV-A (320–400 nm), UV-B (290–320 nm), and UV-C (100–290 nm) (Pfeifer and Besaratinia 2012). The depth to which UV radiation penetrates the surface of the sea varies with the UV type. Especially, UV-A can penetrate up to a depth of 23 m beneath the sea surface, whereas UV-B can penetrate up to 7–12 m (Lesser et al. 2001). The energy per photon of UV that penetrates seawater is higher than that of other wavelengths of light; therefore, the UV that penetrates seawater negatively affects the aquatic organisms, ranging from the primary producers to top-level predators, such as fish, in the ecosystem. Moreover, these radiations are known to damage molecules, in vivo, and induce oxidative stress (Zagarese and Williamson 2001). UV radiations can reach to the depths of coral reefs in coastal areas. The yellowtail clownfish *Amphiprion clarkii*, which inhabits the coral reefs at depths of 5–20 m can be directly affected by UV-A (Moyer 1976). These are subtropical fish species inhabiting the Pacific and Indian Oceans and coastal areas in Japan (Allen 1975; Fautin and Allen 1992). They are also known to inhabit the coastal area of Jeju Island in Korea (Choi et al. 2013).

UV radiation acts as a stress factor for fish and induces oxidation–reduction reactions, resulting in the production of reactive oxygen species (ROS), which causes oxidative stress in cells and tissues (Pourzand and Tyrrell 1999). Superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) are representative ROS (Roch 1999).

The overproduction of ROS in the body results in the denaturation of intracellular DNA and proteins, reduces the resistance of organisms to diseases, and induces physiological disturbances, such as decreased reproductive capacity or production of lipid peroxide (LPO) (Kim and Phyllis 1998; Pandey et al. 2003).

Therefore, several organisms, including fishes, activate

*Corresponding author. E-mail: choic@kmou.ac.kr

antioxidant mechanisms to protect themselves from oxidative stress, caused by the ROS generated in their body. These mechanisms are mediated by antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) (Mcfarland et al. 1999). These enzymes especially work in the liver and kidney of fish. SOD is primarily responsible for the removal of ROS by converting O_2^- to O_2 and H_2O_2. H_2O_2 produced by SOD is converted to non-toxic H_2O and O_2 by CAT, which is known to have an antioxidant action, and removes ROS (Kashiwagi et al. 1997; Basha and Rani 2003; Hansen et al. 2006).

The oxidative stress and ROS in the body directly increase the level of liver damage. In general, aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) are used as indicators to measure the level of liver damage (Nemcsók and Benedeczky 1990; Choi et al. 2015). Moreover, ROS are known to induce DNA damage and affect apoptosis in fish tissues (Häcker 2000; Kulms and Schwarz 2000).

In the present study, we investigated the effect of the UV-A wavelength that penetrates to the depth of yellowtail clownfish habitats to determine how much oxidative stress was induced in fish. We exposed the yellowtail clownfish to two intensities of UV-A (0.2 and 0.4 W/m^2), which we expected to affect the habitat of this fish at 5–20 m depth based on the observation of Lesser et al. (2000) that the UV intensity reaching 8 m depth was 0.260 W/m^2. Furthermore, we measured the expression and activity of SOD and CAT mRNA in order to investigate the induction of oxidative stress in yellowtail clownfish depending on the exposure time and the wavelength of UV. To determine the levels of oxidative stress in the body of the fish, we measured the plasma concentrations of H_2O_2 and lipid peroxidation (LPO). Moreover, we used comet assays and the terminal transferase dUTP nick end labelling (TUNEL) method to investigate the effects of DNA damage and apoptosis in liver tissues. We also measured AspAT and AlaAT levels, to determine the liver damage level caused by stress.

2. Materials and Methods

Fish samples and experimental conditions

Yellowtail clownfish (n = 200; length 5.2 ± 0.5 cm; mass 2.1 ± 0.5 g) were purchased from the Corea Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea), and were acclimated for 2 weeks in twelve 300 L circulation filter tanks in the laboratory. A white fluorescent bulb (27 W) was used for the control group, and light intensity at the water surface in the tanks was approximately 0.96 W/m^2. The water temperature was 27 ± 1°C and a 12-h light:12-h dark photoperiod (lights were switched on at 07:00 h and switched off at 19:00 h) was maintained. The experimental groups of fish were exposed to UV radiation (380 nm) at intensities of approximately 0.2 and 0.4 W/m^2 (Fig. 1). The UV source was set 20 cm above the surface of water and intensities were determined at the middle of water column in the tank, using a spectrometer (MR16; Rainbow Light Technology Co. Ltd., Taoyuan, Taiwan) and Photo-Radiometer (HD 2102.1; Delta OMH Co., Caselle di Selvazzano, Italy). We sampled three fishes from each of the groups at day 0, day 1, day 3, day 7, and day 14. The doses of UV radiation on day 0, day 1, day 3, day 7, and day 14 were 8.64, 25.92, 60.48, and 120.96 kJ/m^2, respectively, at an intensity of 0.2 W/m^2 and 17.28, 51.84, 120.96, and 241.62 kJ/m^2, respectively, at an intensity of 0.4 W/m^2. The control groups were sampled only on day 0. All the experiments were repeated thrice. The fishes were anesthetized using 2-phenoxyethanol (Sigma, St Louis, Mo, USA) and were decapitated prior to tissue 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 at 1,000 × g for 15 min), and stored at -80°C until the analysis. The liver tissues were collected from the fish, immediately frozen in liquid nitrogen, and stored at -80°C until the extraction of total RNA.

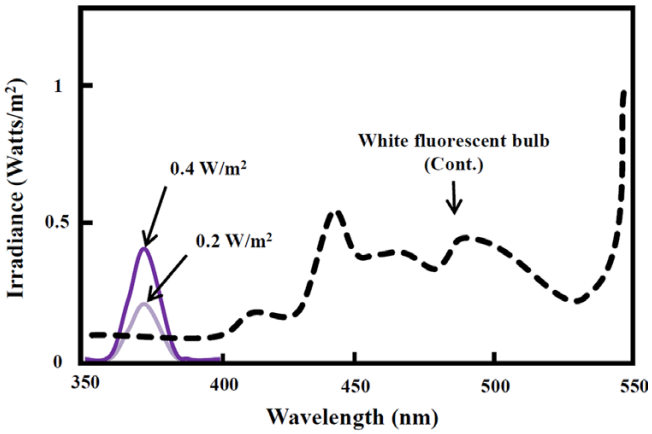

Fig. 1. Spectral profiles of UV radiation (UV 380 nm) and white fluorescent bulb (Cont.) used in the present study. Two different intensities were used (0.2 and 0.4 W/m^2) for each UV treatment

Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using TRI

Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. Subsequently, 2 μg total RNA was reverse transcribed in a 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 use in PCR and real-time quantitative polymerase chain reaction (qPCR) analysis.

Real-time qPCR analysis

The qPCR analysis was conducted to determine the relative expression of SOD and CAT, using the total RNA extracted from the liver of yellowtail clownfish. The qPCR primers were designed using the known yellowtail clownfish 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 iQ SYBR green supermix (Bio-Rad, USA), following the manufacturer's instructions. For control, β-actin gene was amplified along with each sample, and data were expressed as the difference between corresponding calculated β-actin threshold cycle (Ct) levels. The Ct values of the PCR products formed the basis for all analyses. The Ct levels were defined as the PCR cycle in which the fluorescence signal crossed a threshold during the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) of each sample and the internal control (β-actin) were calculated as follows: [ΔΔCt = 2^ - (ΔCt_{sample} - $\Delta Ct_{internal\ control}$)]. After the PCRs were completed, the qPCR data from three replicate samples were analysed using Bio-Rad software to determine the copy numbers of the transcript in each sample.

Analysis of plasma parameters

Plasma was separated from the whole blood, collected using heparin as an anticoagulant, by centrifugation (4°C, 1,500 × *g*, for 15 min), and was immediately stored at -20°C. The levels of SOD, CAT, and LPO were estimated by immunoassay using ELISA kits (SOD, CSB-E15929fh; CAT, CSB-E15928fh; Cusabio Biotech Co., Ltd., China; LPO, MBS285269; Mybiosource, USA). The H_2O_2 levels were measured using a peroxide detect kit (Sigma, USA). The levels of AlaAT and AspAT were determined with a dry multiplayer analytic slide method using a biochemical automatic analyser (Fuji Dri-Chem 4000, Fujifilm, Japan).

Comet assay

The comet assay is a simple and sensitive technique for quantitatively measuring the DNA damage in eukaryotic cells (Bajpayee et al. 2005). The liver cells (1×10^5 cells/mL) were examined using a CometAssay Reagent kit with single-cell gel electrophoresis assays (Trevigen, Gaithersburg, MD), according to the method described by Singh et al. (1988), with some modifications. The cells were immobilized in agarose gel on CometAssay comet slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Thereafter, the samples were electrophoresed at 18 V for 30 min, stained with SYBR Green (Trevigen) for 30 min in the dark, and then observed under a fluorescence microscope (excitation filter 465–495 nm; Eclipse C*i*, Nikon, Japan). At least 100 cells from each slide were analysed. For quantification of the results of comet assay, we analysed the percentage of DNA in the tail (tail intensity/total intensity) and tail moment (product of tail length and percentage of DNA in tail), which reflects the amount of DNA damage, using comet assay IV image analysis software (version 4.3.2; Perceptive Instruments Ltd., UK).

Terminal transferase dUTP nick end labelling (TUNEL) assay

To evaluate the apoptotic response of the fish liver cells to UV radiation, we performed the TUNEL assay using a commercially available *in situ* cell death detection kit (catalogue number, 11 684 795 910, Roche, Switzerland). Polylysine-coated slides were used to prevent any loss in the adherence of apoptotic cells to the slides. The fish liver tissue was

Table1. Primers used for qPCR amplification

Genes (accession no.)	Primer	DNA sequences
SOD (JN032591)	Forward	5′-CAC GAG AAG GCT GAT GAC-3′
	Reverse	5′-GAT ACC AAT GAC TCC ACA GG-3′
CAT (JN032592)	Forward	5′- GGG CAA ATT GGT CCT CAA-3′
	Reverse	5′-CGA TGT GTG TCT GGG TAG-3′
β-actin (JN039369)	Forward	5′-CCA ACA GGG AGA AGA TGA C-3′
	Reverse	5′-TAC AC CAG AGG CAT ACA-3′

washed and fixed with 4% buffered paraformaldehyde and was permeabilised with freshly prepared 0.1% Triton X-100 and 0.1% sodium citrate solution. The fixed and permeabilised liver tissue was then incubated with the TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. The slides were washed thrice with phosphate-buffered saline (PBS) and the incorporated biotin-dUTP was detected under a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon, Japan). For paraffin-embedded tissue sections, the slides were dewaxed and fixed according to standard protocols and then treated as described above. The green fluorescent cells indicated apoptosis.

Statistical analysis

All the data were analysed using the SPSS statistical package (version 19.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's post-hoc test was used to compare the differences in data ($P < 0.05$). The values are expressed as means ± standard error (SE).

3. Results

Changes in the mRNA expression and activity of SOD and CAT

The mRNA expression and activities of SOD and CAT were significantly increased over time in all the groups exposed to UV radiation (Fig. 2). The groups exposed to higher UV intensity (0.4 W/m^2) showed higher mRNA expression and enzyme activities in the plasma than those exposed to lower UV intensity (0.2 W/m^2). However, there were no significant differences in the mRNA levels and enzyme activities in the plasma of fish in the control group.

Changes in the levels of H_2O_2, LPO, AlaAT, and AspAT in the plasma

The levels of H_2O_2, LPO, AlaAT, and AspAT in the plasma were significantly increased over time in all the groups exposed to UV radiation (Fig. 3). The fish in the group exposed to higher UV intensity (0.4 W/m^2) had higher mRNA expression and enzyme activities in their plasma than those in the group exposed to lower UV intensity (0.2 W/m^2). However, there were no significant differences in the mRNA expression levels and enzyme activities in the plasma of fish in the control group.

Comet assay

The damage to liver tissue following 14 days of exposure to UV radiation was analysed using 100 randomly selected cells. Both the DNA content in the tail and tail moment increased significantly with the increase in exposure time in

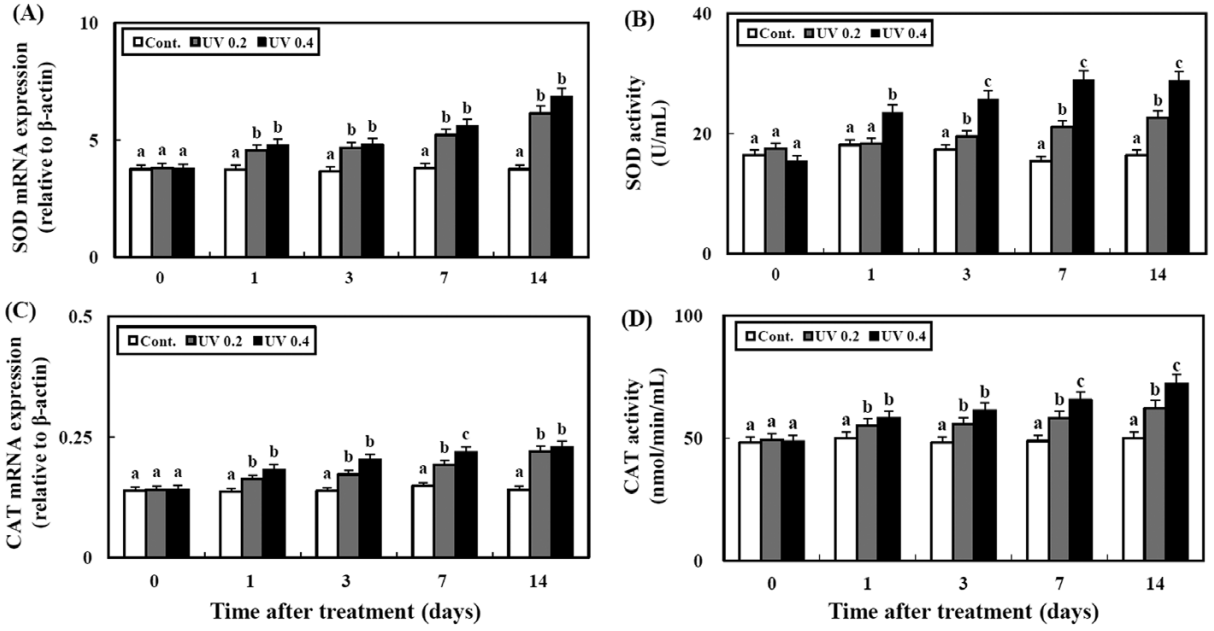

Fig. 2. Changes in the expression of superoxide dismutase (SOD) mRNA (A), SOD activity (B), catalase (CAT) mRNA (C), and CAT activity (D) in yellowtail clownfish exposed to UV radiation at different light intensities (0.2 and 0.4 W/m^2) and to white fluorescent bulb (Cont.), as measured with a biochemistry auto analyser. The lowercase letters indicate significant differences between the different UV intensities and control group for the same period of exposure ($P < 0.05$). All the values are means ± SE ($n = 9$)

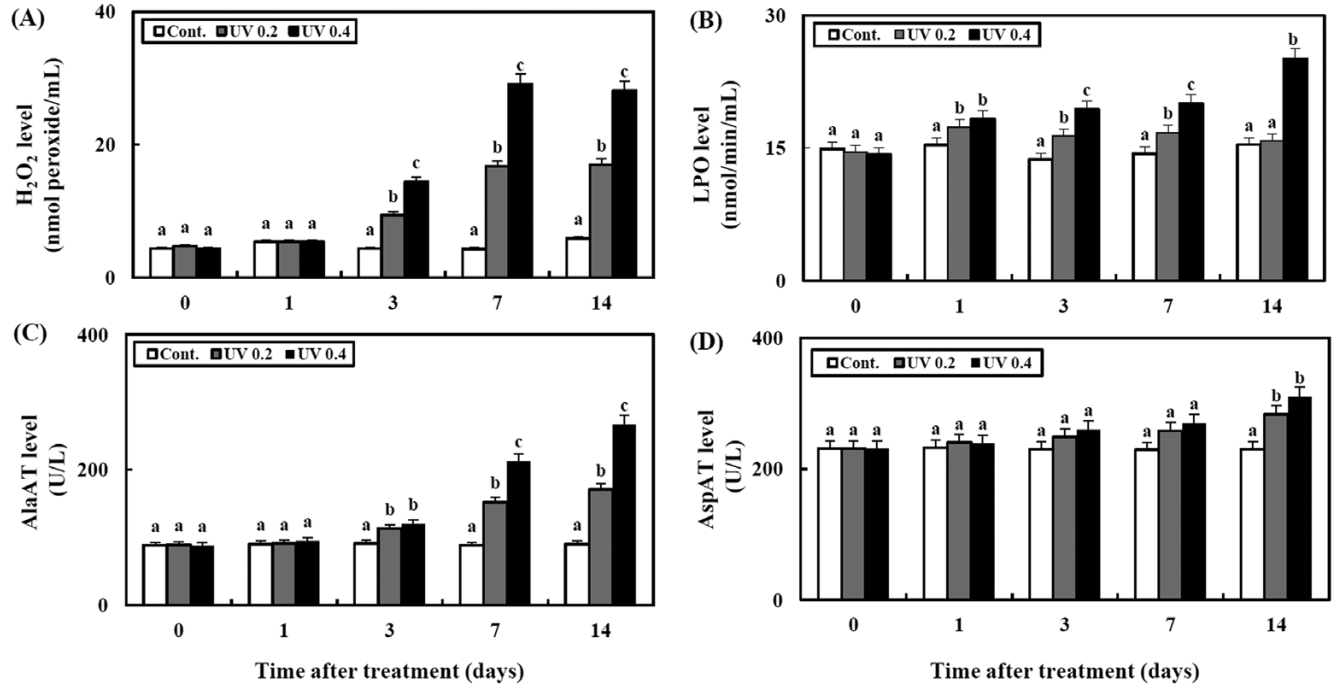

Fig. 3. Changes in the levels of plasma H_2O_2 (A), plasma lipid peroxide (LPO) (B), plasma alanine aminotransferase (AlaAT) (C), and plasma aspartate aminotransferase (AspAT) (D) in yellowtail clownfish exposed to UV radiation at different light intensities (0.2 and 0.4 W/m^2) and white fluorescent bulb (Cont.), as measured with a biochemistry auto analyser. The lowercase letters indicate significant differences between the different UV intensities and control group for the same period of exposure ($P < 0.05$). All the values are means ± SE ($n = 9$)

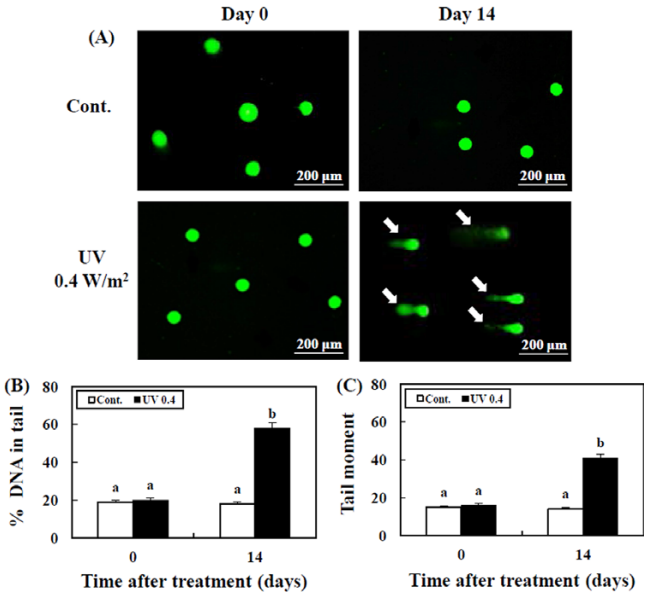

Fig. 4. Images of comet assay done using liver cells of yellowtail clownfish at the initial condition (at day 0) and after exposure to UV radiation (0.4 W/m^2 on day 14). White arrows in (A) indicate damaged nuclear DNA (DNA breaks) in liver cells, which have been stained with SYBR Gold. Scale bars = 200 µm. The lowercase letters indicate significant differences between the UV radiation and control groups for the same period of exposure ($P < 0.05$). All the values are mean ± SE ($n = 9$)

the experimental groups (0.4 W/m^2) compared to their respective values in the control group (Fig. 4). However, there was no significant difference in the control group over time.

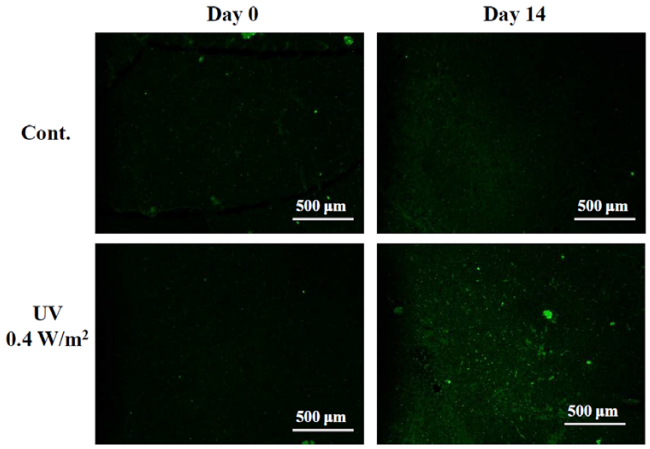

Fig. 5. Images of TUNEL assay done using liver cells of yellowtail clownfish at the initial condition (at day 0) and after exposure to UV radiation (0.4 W/m^2 at day 14). The cells were stained with acridine orange and visualized under a fluorescent microscope. The cells showing green fluorescence are apoptotic cells. Scale bars = 500 µm

TUNEL assay

The TUNEL assay was used to investigate the presence of apoptotic cells (Fig. 5). We observed significant visible differences among the labelled cells between the control group and the experimental group (0.4 W/m^2). After exposure to UV radiation, the frequency of apoptotic cells was higher than that in the control group.

4. Discussion

This study investigated the effects of exposure to UV-A irradiation at two different intensities (0.2 and 0.4 W/m^2) and for different times on oxidative stress and regulation in yellowtail clownfish.

We determined the mRNA expression and activities of SOD and CAT in yellowtail clownfish exposed to UV-A radiation. The expression of antioxidant genes and activities of antioxidant enzymes in the plasma were significantly increased after UV-A irradiation. In the experimental groups, the expression of SOD and CAT mRNA was not significantly different, but the activity was significantly higher in the experimental group irradiated with 0.4 W/m^2 UV-A compared to the experimental group irradiated with 0.2 W/m^2 UV-A. In a similar study, Lesser et al. (2001) examined the effects of UV on oxidative stress in the larvae of Atlantic cod *Gadus morhua* after 10 days of exposure to different wavelengths of UV and observed that the SOD activity was significantly higher in the experimental group exposed to UV. They also reported that as the wavelength of UV decreased, the SOD activity in the larvae increased. Charron et al. (2000) investigated the effects of UV on oxidative stress in the body of zebrafish *Brachydanio rerio* after UV exposure for 25 h, and found that the levels of SOD and CAT mRNA in the skin and muscle samples of the group exposed to UV were significantly higher than those in the skin and muscle of the group not exposed to UV. Likewise, our results showed that in yellowtail clownfish, oxidative stress was induced by UV-A exposure and there was a significant increase in the expression of antioxidant genes as well as in the activities of antioxidant enzymes in the plasma as a way to relieve the stress.

After UV-A irradiation, the concentrations of H_2O_2, LPO, AspAT, and AlaAT in the plasma of yellowtail clownfish were lower in the group exposed to 0.2W/m^2 UV-A than those of the group exposed to 0.4W/m^2 UV-A. However, there was no significant difference in AspAT concentration.

In a similar study, Zigman and Raffertyt (1994) determined H_2O_2 concentration in the lens of dogfish *Mustelus canis*, after exposure to UV, and found that the decomposition of H_2O_2 was reduced in the experimental group exposed to UV, which induces oxidative stress. Gouveia et al. (2015) reported that in silverside *Odontesthes argentinensis* oxidative stress increased the ROS and LPO levels after exposure to UV. They also reported that with the increase in the intensity of UV, the antioxidant capacity in silverside was decreased. Overall, as observed in previous studies, our results show that UV-A exposure induced oxidative stress in yellowtail clownfish, significantly increased the H_2O_2, LPO, and AlaAT levels in the plasma, and significantly decreased the antioxidant capacity. Also, based on these results, liver function was found to be degraded.

The degree of DNA damage in the liver tissue of yellowtail clownfish exposed to UV-A irradiation was determined using comet assay. The values of tail length and tail moment of DNA were significantly increased and DNA was found to be damaged in the nucleus of the liver cells of fish in the experimental group irradiated with 0.4 W/m^2 UV-A compared to those results obtained for fish in the control group. Sandrini et al. (2009) reported that the mRNA expression of *DDB2* and *XPC*, which are genes affecting DNA damage, was increased in the zebrafish *Danio rerio* upon exposure to UV. Dietrich et al. (2005) exposed the sperm cells of the rainbow trout *Oncorhynchus mykiss* to UV and performed the comet assay. They found that the values of tail length and tail moment of DNA increased with the increase in the time of exposure to UV. Overall, our results show that the expression of genes affecting DNA damage was increased in yellowtail clownfish after exposure to UV-A, resulting in DNA damage in the liver tissue.

We also determined cytotoxicity in the liver of yellowtail clownfish after UV-A irradiation using TUNEL assay. We noticed a large number of TUNEL-positive cells in fishes exposed to 0.4 W/m^2 UV-A. Yabu et al. (2001) observed the apoptosis of cells in zebrafish embryos exposed to UV using the TUNEL analysis. They reported the presence of several TUNEL-positive cells in the entire tissue, indicating the induction of cell death, upon UV exposure. The results of TUNEL assay clearly show that UV-A acts as a stress factor in yellowtail clownfish and induces apoptosis.

The results of this study demonstrate that UV-A is a stress factor in yellowtail clownfish, which not only causes DNA damage in liver cells, but also induces apoptosis. Moreover,

even low intensity (0.2 W/m^2) UV-A has a negative effect on the antioxidant defence mechanisms in the yellowtail clownfish and affected the physiology of the fish. Further studies are required to investigate the physiological changes caused by discursive wavelengths and intensities of UV in different fish species; the results of the present study can be used as basic research data for such studies.

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