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Effect of Harmful Environmental Factors on the Oxidative Stress of Two Marine Teleosts

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Effect of Harmful Environmental Factors on the Oxidative Stress of Two Marine Teleosts

A dissertation

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List of Abbreviations

ANOVA	analysis of variance
AlaAT	alanine aminotransferase
AspAT	aspartate aminotransferase
BrO^-	hypobromite ion
cDNA	complementary deoxyribonucleic acid
CAT	catalase
$CHBr_3$	bromoform
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
H_2O_2	hydrogen peroxide
HOBr	hypobromous acid
HSP	heat shock protein
LPO	lipid peroxide
mRNA	messenger ribonucleic acid
O_3	ozone
$^1O_2$	singlet oxygen
O_2^-	superoxide
OH^-	hydroxyl radical
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qPCR	real-time quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TRO	total residual oxidant

TUNEL	terminal transferase dUTP nick end labeling
UV	ultraviolet

외부 유해 환경 노출이 두 종류 해산어류의 산화스트레스에 미치는 영향

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초 록

어류를 포함한 생명체가 급격한 외부 환경변화에 노출될 경우 체내에서 강력한 산화물질인 ROS가 생성된다. ROS는 H_2O_2, O_2^-, OH^- 및 $^1O_2$ 등이 대표적이며, 이들은 산화·환원 반응을 통해 세포의 생존에 악영향을 줄 수 있다. 이 과정에서 간세포 손상 지표인 AlaAT 및 AspAT가 증가할 수 있으며, DNA가 파괴되거나 세포사멸이 발생하기도 한다. 또한, 이들은 세포의 정상적인 활동을 방해하는 LPO를 증가시키거나 스트레스 지표인 HSP70의 발현을 증가시키기도 하는 것으로 알려져 있다. 이러한 ROS를 제거하기 위하여 체내에서는 항산화 방어 메커니즘이 작동되는데, 이 과정에서 발현되는 항산화 효소인 SOD와 CAT는 유독성 물질인 ROS에 단계적으로 작용하여 최종적으로 체내에 무해한 H_2O와 O_2로 전환시키는 역할을 한다.

본 연구는 두 종류의 해산어류인 넙치 *Paralichthys olivaceus*와 흰동가리 *Amphiprion clarkii*를 인위적으로 외부 환경요인인 TRO와 UV에 각각 노출시킨 후, 어체 내에서 발생되는 생리학적 변화와 산화스트레스 반응과 어떠한 항산화 방어 메커니즘이 작동하는지를 확인하기 위하여 수행되었다.

1. 넙치 *Paralichthys olivaceus*의 산화스트레스에 미치는 TRO의 영향

본 연구에서는 TRO에 노출된 넙치의 체내에서 유도된 산화스트레스 및 세포사멸 정도를 확인하기 위하여 넙치를 서로 다른 농도의 TRO (20, 40 및 60 μg/L)에 노출시킨 후 14일 동안 생리학적 변화를 관찰하였다.

TRO가 미치는 독성의 영향을 확인하기 위하여 우선 14일간 넙치의 생존율을 관찰하였으며, TRO에 의하여 유도되는 스트레스를 확인하기 아가미와 간 조직에서 항산화 유전자인 SOD 및 CAT mRNA 발현을 분석하였다. 아가미와 간 조직에서 스트레스 지표인 HSP70 mRNA 발현과 활성을 측정하였으며, 혈장 내에서는 H_2O_2 농도를 측정하였다. 또한, TRO에 의한 넙치 아가미 조직 내 DNA의 손상 정도를 알아보기 위하여 TUNEL assay를 실시하였다.

그 결과, 대조구와 TRO 20 실험구에서는 폐사한 개체가 관찰되지 않았지만, TRO 40 및 60 실험구에서는 각각 14일과 7일째에 모든 개체가 폐사하였다. TRO 40 및 60 실험구 넙치의 아가미와 간 조직에서 항산화 유전자인 SOD, CAT 및 HSP70 mRNA 발현 및 활성이 유의적으로 증가하였으며, TRO 40 실험구에 비하여 TRO 60 실험구에서 더 높은 mRNA 발현 및 활성이 관찰되었다. 산화스트레스 지표인 혈장 내 H_2O_2 농도 역시 TRO 40에 비하여 TRO 60 실험구에서 더 높은 수치가 관찰되었다. 또한, 넙치 아가미에서 DNA의 손상 정도를 확인하기 위한 TUNEL assay 실험 결과, TRO 농도가 높아지고 노출 시간이 길어질수록 넙치 아가미에서 DNA의 손상이 눈에 띄게 증가한 것이 관찰되었다.

본 연구 결과를 종합하여 보면, 20 μg/L 농도의 TRO는 넙치의 생존율, 산화스트레스 및 세포사멸에 유의적으로 영향을 미치지는 못하였으나, 40과 60 μg/L 농도의 TRO에서는 넙치의 폐사율이 증가되었으며 산화스트레스를 유도하였을 뿐만 아니라 세포사멸까지도 유발하는 것으로 판단되었다.

2. 흰동가리 *Amphiprion clarkii*의 산화스트레스에 미치는 UV의 영향

본 연구에서는 UV에 의해 흰동가리 체내에서 유도되는 산화스트레스 및

세포사멸 정도를 확인하기 위하여 흰동가리를 2가지 세기의 UV(0.2 및 0.4 W/m^2)에 노출시킨 후 14일 동안 흰동가리의 생리학적 변화를 관찰하였다.

UV에 의하여 흰동가리 체내에서 생성된 ROS를 제거하는 역할을 하는 항산화 유전자인 SOD 및 CAT mRNA 발현과 활성을 간 조직에서 분석하였으며, 산화스트레스 지표인 H_2O_2와 LPO 농도는 혈장에서 측정하였다. 또한, UV에 의한 조직의 손상 정도를 알아보기 위하여 간 손상 지표인 AlaAT 및 AspAT 농도는 혈장 내에서 측정하였으며, 간 조직 내 DNA 손상 정도는 TUNEL assay와 comet assay를 실시하여 측정하였다.

그 결과, UV에 노출된 실험구의 흰동가리에서 항산화 유전자인 SOD 및 CAT mRNA 발현 및 활성뿐만 아니라 산화스트레스의 지표인 H_2O_2, LPO, 간 손상 지표인 AlaAT와 AspAT의 혈장 내 농도가 유의적으로 증가하였으며, UV의 세기가 세질수록 더 높은 수치가 관찰되었다. 또한, 흰동가리 간 조직에서 DNA의 손상 정도를 확인하기 위한 TUNEL assay와 comet assay 실험 결과, 강한 세기의 UV에 노출된 흰동가리의 간에서 DNA 손상이 눈에 띄게 증가한 것이 관찰되었다.

본 연구 결과를 종합하여 보면, 흰동가리는 0.2 및 0.4 W/m^2 세기의 UV에 노출 시 체내에서 ROS의 일종인 H_2O_2가 증가하였고 산화스트레스 지표인 LPO가 유발되었는데, 이러한 현상은 UV에 의하여 유도된 산화스트레스에 대응하기 위하여 흰동가리 체내에서 항산화 효소인 SOD와 CAT가 유의적으로 증가한 것으로 보인다. 또한, 강한 세기의 UV는 흰동가리에게 스트레스 요인으로 작용하여, 간 조직에서는 DNA 손상이 발생하였을 뿐만 아니라 세포사멸까지도 유도된 것으로 판단된다. 즉, 0.2 및 0.4 W/m^2 세기의 UV는 흰동가리 체내에서 산화스트레스를 유발시킨 것으로 사료되며, UV의 세기가 세질수록 더 강한 스트레스 요인으로 작용하는 것으로 판단된다.

본 연구는 어류양식장에서 사육수를 살균하는 과정에 있어서 어류가 노출될 수 있는 유해한 환경요인 중 하나인 TRO가 넙치에 미치는 영향과 지구 오존층의 파괴로

인하여 조사량이 점차 증가하고 있는 UV 파장이 흰동가리에 미치는 영향을 확인하였다는 점에서 의미를 찾을 수 있을 것이다. 따라서 본 연구 결과는 TRO와 UV가 어류에게 스트레스 요인으로 영향을 미칠 수 있는 농도와 세기의 범위 등에 대한 중요한 정보를 생리학적 측면에서 제공하고 있을 뿐만 아니라 안정적인 어류 생산에 필요한 기초 데이터로도 활용될 수 있을 것으로 기대된다.

Chapter 1.

General Introduction

Fish are often exposed to diverse stress environments such as high or low water temperatures, changes in salinity and toxic substances. Notably, external stress factors can cause an increase in reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) (Roch, 1999), negatively affecting cell viability when present in excess by inducing cellular damage and aging (Nordberg and Arnér, 2001; Slaninova et al., 2009). Owing to these toxic oxidative effects of ROS, organisms have antioxidant defense mechanisms that convert ROS into a nontoxic substance through the actions of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (McFarland et al., 1999). Antioxidant enzymes are mainly expressed in the liver and kidney of fish and SOD primarily converts O_2^- into O_2 and H_2O_2 (Basha and Rani, 2003). Since H_2O_2 is also an ROS, it must be secondarily converted to non-toxic H_2O and O_2 by CAT (Kashiwagi et al., 1997; Hansen et al., 2006).

Overproduction of ROS in the body causes the denaturation of intracellular DNA and protein structures, reduces resistance to disease and induces physiological disturbances such as decreased reproductive capacity or the production of lipid peroxide (LPO) (Kim and Phyllis, 1998; Pandey et al., 2003). Cellular damage also promotes the synthesis of heat shock protein (HSP), a stress-related protein (Kregel, 2002). HSP plays an important role in external contaminant and abiotic stress responses, in addition to its role in high temperature stress and helps to prevent cellular damage and maintain homeostasis (Yamashita et al., 2010).

Additionally, oxidative stress and ROS in the body directly increase the level of liver damage. Typically, aspartate alanine aminotransferase (AlaAT) and aminotransferase (AspAT) are used as the indicators to measure liver damage levels

(Nemcsók et al., 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, the effects of environmental stress factors, such as TRO and UV, which induce oxidative stress in marine teleosts (olive flounder and yellowtail clownfish), were investigated by analyzing the changes in mRNA expression and activities of SOD and CAT. The plasma H_2O_2 and LPO levels were measured to determine the stress fluctuation levels under different conditions and plasma AlaAT and AspAT concentrations as liver damage indicators were assessed. Furthermore, environmental stress was evaluated by analyzing changes in HSP70 mRNA and activity. Finally, the DNA damage in the liver and gill tissues of fish, as a consequence of apoptotic activity, was measured by conducting a terminal transferase dUTP nick end labeling (TUNEL) and comet assays.

Chapter 2.

Effects of Total Residual Oxidants (TRO) On Oxidative Stress in Juvenile Olive Flounder *Paralichthys olivaceus*

1. Introduction

Ozone (O_3), an allotrope of oxygen with strong oxidizing capacity, has the ability to decompose organic matter and is used for water sterilization in various applications including aquaculture. The injection of ozone into water treatment systems to induce oxidation and remove microorganisms has been referred to as the 'ozone oxidation method' (Oh et al., 1999; Park et al., 2013). The ozone oxidation method is often applied in recirculating aquaculture systems that require purification and reuse of the process water (Schroeder et al., 2011). This method has been reported to be effective for the elimination of pathogenic microorganisms and viruses and for the removal of organic matter resulting from metabolic activities of aquatic organisms (Sharrer and Summerfelt, 2007; Wold et al., 2014). The removal of organic matter by ozone is not only attributable to ozone itself, but also to the effects of hypobromous acid (HOBr) and hypobromite ion (BrO^-), which are powerful oxidants formed by a reaction between ozone and Br^-. In this process, the oxidants that are created by ozone that function to destroy microorganisms are called total residual oxidants (TRO) (Buchan et al., 2005).

Studies on the effects of ozone-induced TRO on seawater sterilization and fish toxicity have been undertaken. Recently, studies on the optimal TRO concentration for direct application in aquaculture have become increasingly important (Jung et al., 2018).

Even when small amounts of TRO exist in water, sterilization occurs following the destruction of bacterial cell membranes through its powerful oxidative capacity. In addition, TRO and components of fish excreta, such as organic matter and colloid, cohere to bromoform ($CHBr_3$), which can be easily removed by skimmer and other filter equipment (Haag and Hoigné, 1984; Liltved et al., 2006). However, when organic matter that can react with TRO no longer exists in the water, TRO is difficult to be removed from the water and is known to be toxic to fish and other aquatic organisms (Hofmann, 2000). Direct exposure to TRO induces damage in red blood cells and tissues such as gills and may increase mortality rates in severe cases (Fukunaga et al., 1992a; Yoshimizu et al., 1995; Kim et al., 1999). While ozone is rapidly converted into secondary oxidants such as TRO in the seawater (Tango and Gagnon, 2003; Perrins et al., 2006), TRO is relatively stable and can, therefore, be used as an indicator of potential toxicity when ozone is used for water sterilization (Jones et al., 2006).

TRO induces oxidation-reduction reactions in the body and forms reactive oxygen species (ROS) that cause oxidative stress in cells and tissues (Fukunaga et al., 1992b). Representative ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) (Roch, 1999), which impart negative effects on cell viability when present in excess by inducing cellular damage, aging and apoptosis (Nordberg and Arnér, 2001; Slaninova et al., 2009). Because of these toxic oxidative effects of ROS, organisms have antioxidant defense mechanisms that convert ROS into a nontoxic substance through the actions of antioxidant enzymes such as SOD and CAT (McFarland et al., 1999). Antioxidant enzymes are mainly expressed in the liver and kidney of fish and SOD primarily converts O_2^- into O_2 and H_2O_2 (Basha and Rani, 2003). Since H_2O_2 is also an ROS, it must be secondarily converted to nontoxic H_2O and O_2 by CAT (Kashiwagi et al., 1997; Hansen et al., 2006).

Cellular damage caused by TRO promotes the synthesis of HSP, a stress-related protein (Kregel, 2002). HSP plays an important role in external contaminant and abiotic stress responses, in addition to its role in high temperature stress and helps to prevent cellular damage and maintain homeostasis (Yamashita et al., 2010). Despite the fact that TRO is an important byproduct in the ozone sterilization method, few studies have investigated the effects of TRO on fish antioxidant systems and stress responses.

This study was conducted to investigate the effects of TRO at different concentrations on the stress response of olive flounder *Paralichthys olivaceus*. Park et al. (2013) reported that TRO at 20 and 40 μg/L was effective for reducing organic matters and bacteria in a recirculating seawater system used for the culture of black seabream *Acanthopagrus schlegelii*. Based on this concentration, olive flounder were exposed to available concentrations of TRO (20, 40 and 60 μg/L) that could be applied for the purpose of water sterilization in aquaculture. In this study, I analyzed the expression of SOD and CAT mRNA in the gill and liver tissues of olive flounder maintained in seawater treated with ozone in order to investigate the induction of oxidative stress over time at different TRO concentrations. In addition, the concentration of H_2O_2 in plasma and HSP70 mRNA expression and activity in the gill and liver tissues were analyzed. The degree of apoptosis in the gill tissue was also analyzed by terminal transferase dUTP nick end labeling (TUNEL). The results of this study would provide an understanding of the safe concentrations of TRO that can be applied in aquaculture.

2. Materials and methods

2.1. Experimental fish and conditions

Juvenile olive flounder (total length 10.5 ± 0.4 cm; mass 9.2 ± 0.5 g) were purchased from a fish farm in a Pohang, Korea and were allowed to acclimate for two weeks in twelve 150-L recirculation filter tanks in the laboratory (Fig. 1). Experiment was performed in three 150-L recirculation filter tank (skimming filtration type) for each TRO concentration, with 25 fishes in each tank. The fish were exposed to a simulated natural photoperiod (27 W white flight light bulb as 12-h light:12-h dark photoperiod; lights on at 07:00 and off at 19:00) and water temperature was 20 ± 0.5°C. The fish were fed 0.6 ± 0.05 g of commercial feed twice daily (09:00 h and 17:00 h) and feed was withheld for 48 h prior to sampling.

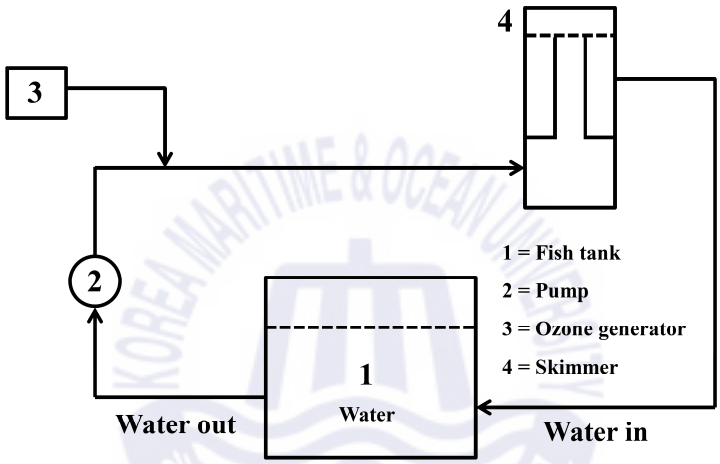

Fig. 1. Ozone recirculation system used in current study. Flow rate = 15 ± 0.5 L/min.

2.2. TRO treatment and sampling

To investigate the effects of TRO on oxidative stress in olive flounder, fish were maintained in water enriched with differing amounts of ozone to achieve TRO concentrations of 0, 20, 40 and 60 μg/L (TRO 0, TRO 20, TRO 40 and TRO 60 each). Ozone was generated from four microplasma ozone generators (MP1-1003; Ozonaid Co. Ltd., Jeju, Korea) intermittently to maintain TRO concentrations and TRO concentration was measured using the N,N-diethyl-p-phenylenediamine procedure and a spectrophotometer (DR3900; HACH, Loveland, CO, USA) five times daily at 06:00, 10:00, 14:00, 18:00 and 22:00. Concentrations of TRO measured during the experimental period are listed in Table 1. All fish were anesthetized using 20 μg/L of 2-phenoxyethanol (Sigma, St. Louis, MO, USA) and blood was collected from the caudal vein using a 1 mL syringe coated with heparin. Fish were decapitated prior to tissue collection. Plasma samples were separated by centrifugation (1,000 × *g* for 15 min at 4°C) and stored at -80°C until analysis. Tissues (gill and liver) were sampled from five different fish from each tank on days 0, 1, 3, 7 and 14. All tissues were collected and immediately stored at -80°C until total RNA was extracted.

2.3. 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. Reverse transcription of 2 μg of total RNA was performed in a 20-μL reaction mixture 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.

Table 1. TRO concentrations measured during the experimental period

Time (hour)	Concentration (μg/L)	1 day	3 day	7 day	14 day
06:00	TRO 20	21.9 ± 0.82	22.0 ± 1.00	21.7 ± 1.53	22.0 ± 1.00
	TRO 40	42.0 ± 1.00	41.3 ± 1.15	42.3 ± 0.58	42.0 ± 1.00
	TRO 60	62.3 ± 0.58	62.3 ± 0.58	62.3 ± 0.58	62.3 ± 1.15
10:00	TRO 20	21.7 ± 0.58	21.7 ± 0.58	20.3 ± 1.53	21.0 ± 2.00
	TRO 40	40.7 ± 1.53	41.0 ± 1.00	41.7 ± 0.58	41.3 ± 2.08
	TRO 60	61.7 ± 0.58	61.3 ± 1.15	61.3 ± 1.53	60.3 ± 1.15
14:00	TRO 20	21.3 ± 0.58	21.3 ± 1.15	20 ± 2.65	19.7 ± 1.53
	TRO 40	42.0 ± 1.00	40.3 ± 0.58	41.0 ± 1.00	41.0 ± 1.73
	TRO 60	58.7 ± 1.00	60.7 ± 2.31	60.3 ± 2.52	61.0 ± 2.65
18:00	TRO 20	21.7 ± 1.15	21.3 ± 2.31	21.3 ± 0.58	21.3 ± 1.15
	TRO 40	40.3 ± 0.58	42.0 ± 1.73	41.3 ± 1.15	41.0 ± 1.00
	TRO 60	60.3 ± 0.58	61.3 ± 1.53	62.0 ± 1.00	62.7 ± 0.58
22:00	TRO 20	21.0 ± 1.73	20.7 ± 2.52	20.0 ± 1.73	20.0 ± 0.58
	TRO 40	40.0 ± 2.65	39.7 ± 2.89	39.7 ± 2.08	41.0 ± 1.00
	TRO 60	60.0 ± 1.00	61.3 ± 2.08	61.0 ± 2.00	62.7 ± 0.58

Data presented as mean ± SD.

2.4. Real-time quantitative polymerase chain reaction (qPCR) analysis

The qPCR analysis was conducted to determine the relative expression of SOD, CAT and HSP70 mRNA using the cDNA synthesized from total RNA extracted from the gill and liver of olive flounder. The qPCR primers were designed using known olive flounder sequences (Table 2). I conducted the 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) following the manufacturer's instructions. The β-actin gene served as an internal control and was also amplified in each sample. All data were expressed relative to the corresponding β-actin threshold cycle (ΔCt) number. 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 values (ΔΔCt) for each sample and the internal controls (β-actin) were calculated using the 2-ΔΔCt method: [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$]. After the PCRs were completed, the qPCR data from three replicate samples were analyzed using the Bio-Rad software to estimate transcript copy numbers in each sample.

Table 2. Olive flounder primers used for qPCR amplification

Genes (**Accession no.**)	Primer	DNA sequences
SOD (**EF681883**)	Forward	5′-CGT TGG AGA CCT GGG GAA TGT G-3′
	Reverse	5′-ATC GTC AGC CTT CTC GTG GAT C-3′
CAT (**GQ229479**)	Forward	5′-CCA AAC TAC TAT CCC AAC AGC-3′
	Reverse	5′-CCA CAT CTG GAG ACA CCT T-3′
HSP70 (**AF053059**)	Forward	5′-GCA AAC AGG TTG AGC AG-3′
	Reverse	5′-ATC GTG TCC CTC TTC AGC-3′
β-actin (**FJ975145**)	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
	Reverse	5′-TGA TCT CCT TCT GCA TCC TG-3′

2.5. HSP70 enzyme-linked immunosorbent assay (ELISA)

HSP70 activities in gills and liver were analyzed using a commercial ELISA kit (MBS007829; Mybiosource Inc., San Diego, CA, USA). Five hundred micrograms of tissues was homogenized with 500 μL phosphate buffered saline and homogenates are centrifuged (1,500 × *g* for 15 min at 20°C) according to the manufacturer's instructions. The absorbance at 450 nm was measured using a spectrophotometer.

2.6. Plasma parameter analysis

Plasma was separated from heparinized blood samples by centrifugation (1,500 × *g* for 15 min at 4°C). Plasma samples were assayed immediately and stored at -80°C. Plasma H_2O_2 levels were measured using a Peroxide Detect™ Kit (Sigma).

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

To evaluate the apoptotic response in fish gill cells exposed to TRO, I performed the TUNEL assay using a commercial in situ cell death detection kit (catalog number 11 684 795 910; Roche, Basel, Switzerland). Olive flounder gill tissue was washed, fixed with 4% buffered paraformaldehyde and permeabilized with freshly prepared 0.1% Triton X-100 and 0.1% sodium citrate solution. For the paraffin-embedded tissue sections, the slides were dewaxed and fixed according to standard protocols and then treated as described above. The gill tissue was then incubated with the TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. The slides were washed three times with phosphate-buffered saline and the incorporated biotin-dUTP was detected under an optical microscope (Eclipse Ci; Nikon, Tokyo, Japan).

2.8. Statistical analysis

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

3. Results

3.1. Survival rate

Survival rate decreased with time and TRO concentration in all groups during the 14-day exposure period (Fig. 2). Survival rate in the TRO 40 and 60 groups were decreased relative to the survival rate in the TRO 0 and 20 groups. Survival rate in TRO 40 group decreased rapidly at day 7 and all flounder had died by day 14. Likewise, survival rate in TRO 60 group decreased rapidly at day 3 and all flounder had died by day 7.

3.2. Changes in SOD and CAT mRNA expression

SOD and CAT mRNA expression in gills and liver significantly increased over time in all groups exposed to TRO (Fig. 3). In the gill tissue, SOD and CAT mRNA expression in TRO 20, 40 and 60 groups were significantly increased in a dose-dependent manner by day 3. However, gill SOD and CAT transcript abundances in TRO 20 group were decreased to control levels at days 7 and 14, respectively. In the liver, SOD and CAT mRNA expression also increased in a dose-dependent manner by days 1 and 3, respectively. No difference in CAT mRNA expression in the liver was observed between days 7 and 14 in TRO 20 group.

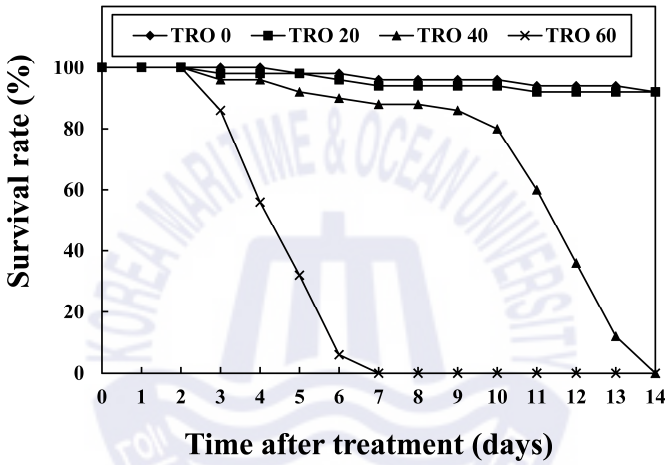

Fig. 2. Survival rate of olive flounder in control group (TRO 0) and experimental groups (TRO 20, 40 and 60) for 14 days.

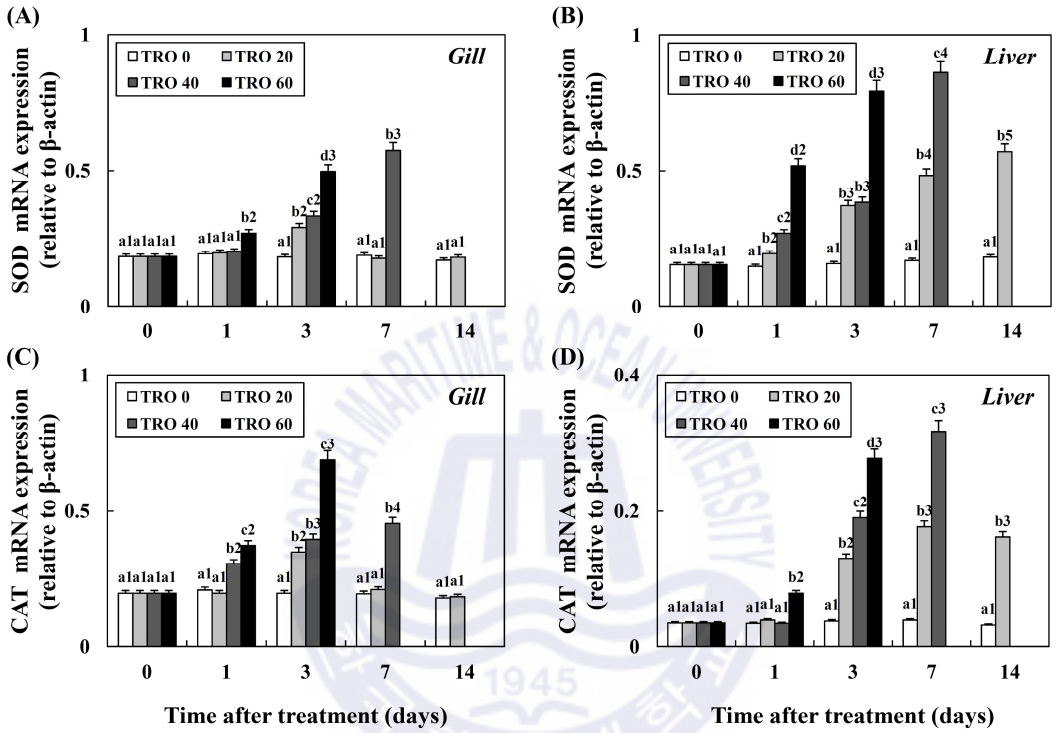

Fig. 3. Expression of SOD mRNA in gill (A) and in liver (B), CAT mRNA in gill (C) and in liver (D) of olive flounder in control group (TRO 0) and experimental groups (TRO 20, 40 and 60) for 14 days. Different numbers indicate significant differences among the different exposure periods at the same TRO concentration ($P < 0.05$). The lowercase letters indicate significant differences among the different TRO concentrations at the same exposure periods ($P < 0.05$). All the values are means ± SE ($n = 5$).

3.3. Changes in HSP70 mRNA and activity

HSP70 mRNA expression and activity in gills and liver were significantly increased over time in all groups exposed to TRO (Fig. 4). Significant dose-dependent increases were also observed in HSP70 mRNA expression and protein levels in both gills and liver between the control and TRO treatment groups; however, mRNA expression and protein level at day 14 were decreased in comparison with those at day 7 in both tissues. In addition, HSP70 activity in the gills of TRO 0 and 20 groups were not different at day 14.

3.4. Changes in plasma H_2O_2 level

Plasma H_2O_2 levels were significantly increased over time within all groups exposed to TRO (Fig. 5). Significant dose-dependent increases in plasma H_2O_2 were also observed between the controls and each treatment group by day 3. At day 14, no significant difference in plasma H_2O_2 was observed between the TRO 0 and 20 groups.

3.5. TUNEL assay

The TUNEL assay was used for the detection of apoptotic cells (Fig. 6). I observed an increase in the number of labeled cells between the control group and the experimental groups (TRO 40 at day 7 and TRO 60 at day 3). However, there was no visible difference in the number of apoptotic cells between the control group and the TRO 20 group at days 3, 7 and 14. Also, there were hypertrophy and deformation of gill tissues upon exposure to TRO 40 for 7 days and TRO 60 for 3 days.

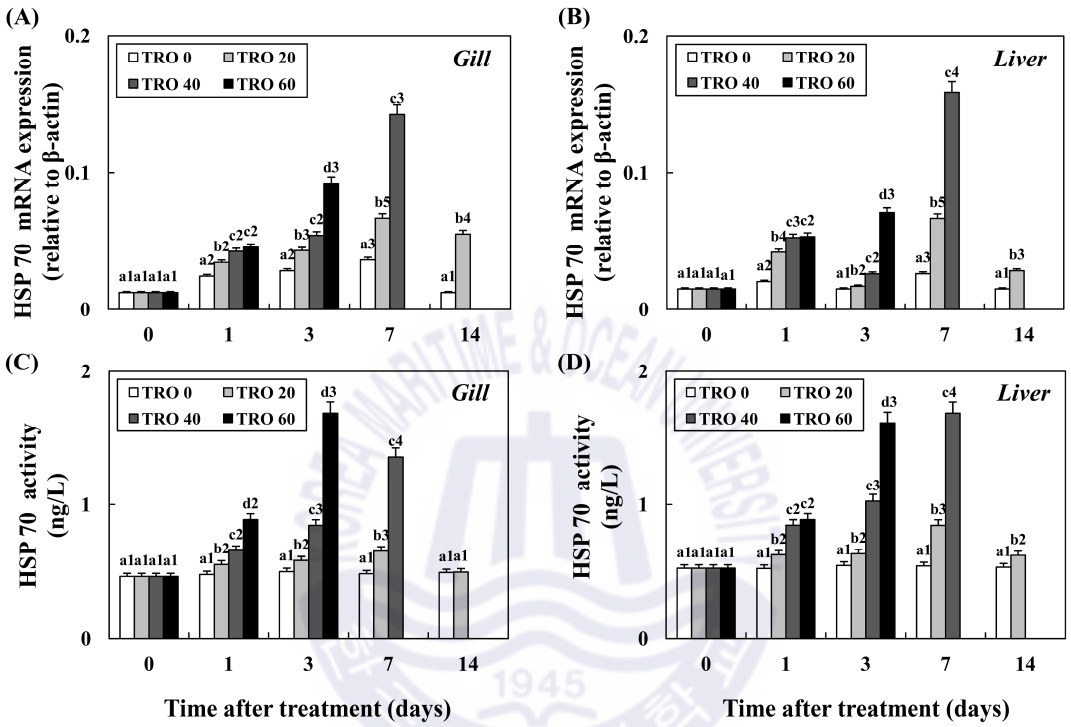

Fig. 4. Expression of HSP70 mRNA in gill (A) and in liver (B), HSP70 activity in gill (C) and in liver (D) of olive flounder in control group (TRO 0) and experimental groups (TRO 20, 40 and 60) for 14 days. Different numbers indicate significant differences among the different exposure periods at the same TRO concentration *(P* < 0.05). The lowercase letters indicate significant differences among the different TRO concentrations at the same exposure periods (*P* < 0.05). All the values are means ± SE (*n* = 5).

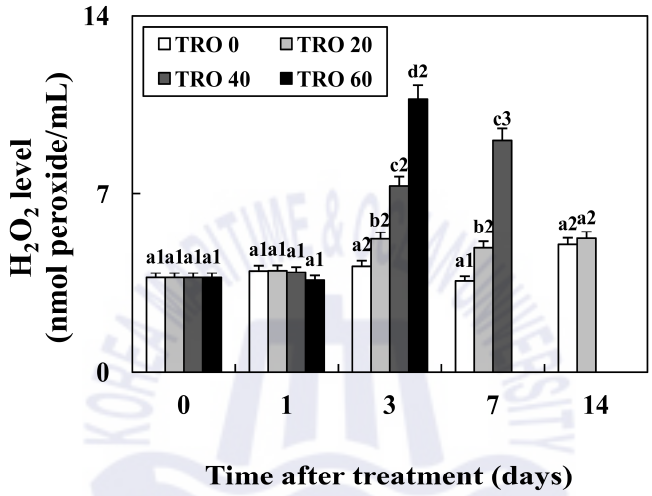

Fig. 5. Plasma H_2O_2 in olive flounder in control group (TRO 0) and experimental groups (TRO 20, 40 and 60) for 14 days. Different numbers indicate significant differences among the different exposure periods at the same TRO concentration ($P < 0.05$). The lowercase letters indicate significant differences among the different TRO concentrations at the same exposure periods ($P < 0.05$). All the values are means ± SE ($n = 5$).

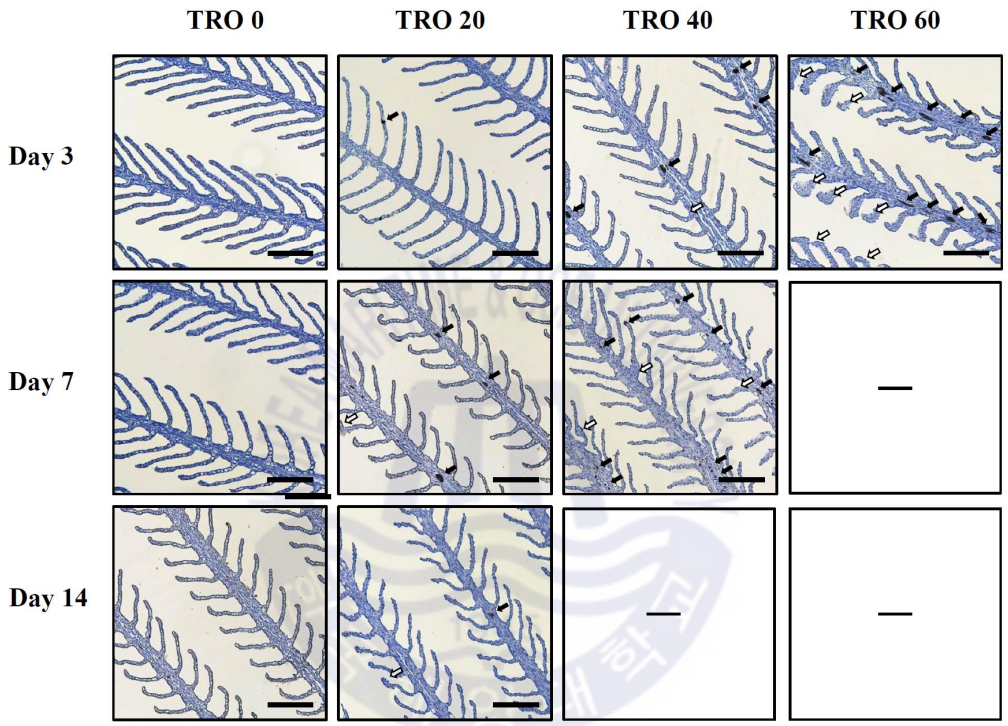

Fig. 6. TUNEL detection images following control group (TRO 0) and experimental groups (TRO 20, 40 and 60) for 14 days in gill of olive flounder Dark-colored cells indicated with black arrows are apoptotic and blank box means no survival observed. White arrows mean gill deformation. Scale bars = 100 μm.

4. Discussion

In this study, I examined changes in the stress response and antioxidant capacity of olive flounder after exposure to TRO, an indicator of toxicity used in ozone-mediated water sterilization in aquaculture. Three concentrations of TRO (20, 40 and 60 μg/L) were used. Olive flounder survival in the cont. and TRO 20 groups was 92% at day 14; however, in the TRO 40 and 60 groups, all individuals died by day 14 and day 7, respectively. Similarly, Kim et al. (1999) investigated the survival rate of olive flounder exposed to seawater treated with different concentrations of TRO (0-47 μg/L) for 96 h, reporting a gradual decrease in survival with increases in TRO and 0% survival at 47 μg/L TRO. Based on this and findings of previous studies, it appears that concentrations of TRO up to 20 μg/L can be safely used for olive flounder culture without causing significant increases in fish mortality.

In this experiment, expression of mRNA for antioxidant enzymes SOD and CAT in the gill and liver tissues of olive flounder was significantly increased in relation to TRO concentration and exposure time. Interestingly, SOD and CAT mRNA expression in the gill decreased to control levels at days 7 and 14, respectively, in the TRO 20 group. A previous study reported that short-term exposure to 5.2 μg/L of ozone-treated freshwater in rainbow trout *Oncorhynchus mykiss* culture significantly increased the activities of SOD and CAT in gills and liver, but that enzyme activities were decreased to control levels after 48 h (Ritola et al., 2002). It has also been reported that TRO in freshwater caused by ozone damages the gill tissue of rainbow trout (Wedemeyer et al., 1979). Similar to previous studies, TRO caused damage to the gill and liver tissues and induced oxidative stress in olive flounder in this study, as indicated by the increased expression of mRNA for the antioxidant defense enzymes, SOD and CAT. The return of SOD and CAT transcript abundance in the gill and liver to control levels after 3 days suggests an

adaptation to TRO exposure in olive flounder over time.

In this study, HSP70 mRNA expression and activity in gills and liver of olive flounder were significantly increased by TRO in relation to exposure time and concentration. However, in the TRO 20 group, HSP70 mRNA expression and activity in gills and liver on day 14 were decreased relative to those on day 7 and HSP70 activity in the gill was not different from that in the controls. Similarly, Reiser et al. (2011) exposed turbot *Psetta maxima* to 0.15 mg/L ozone-treated seawater and reported significantly increased HSP70 mRNA expression on days 1 and 7 that recovered to control levels by day 21. The transient increase in HSP70 in the TRO 20 group suggests adaptation to TRO over time in olive flounder, similar to that observed for SOD and CAT.

Plasma H_2O_2 concentration in olive flounder was significantly increased with increasing TRO concentration and exposure time within TRO exposure groups in the current study. Fukunaga et al. (1999) exposed rainbow trout to 20 mg/L ozone-treated freshwater for a short period and observed a significant increase in red blood cell H_2O_2 levels. These authors also reported that ozone exposure resulted in membrane damage that induced the production of H_2O_2. Therefore, it is suggested that exposure of olive flounder to TRO generated by ozone injection in seawater causes oxidative stress and cellular damage through the generation of ROS including H_2O_2. Meanwhile, H_2O_2 concentrations on day 14 were significantly increased in the TRO 20 groups relative to the control group at other sampling times, but it is assumed that dissolved ozone temporarily increases the H_2O_2 in water (Glaze, 1986).

Increases in apoptotic (TUNEL-positive) cells and hypertrophy and deformation of gill tissues were observed after exposure to TRO 40 for 7 days and TRO 60 for 3 days; these concentration and exposure time combinations also coincided with the highest rates of mortality. In a previous study, Paller and Heidinger (1980)

reported that the gill tissue of bluegill *Lepomis macrochirus* was damaged by exposure to ozone-treated freshwater at 90 μg/L for 6 h. Jung et al. (2018) exposed olive flounder to 25 μg/L TRO-treated seawater for 26 days, reporting hypertrophy and necrosis in the epithelial cells of gills. In general, it is known that cellular damage caused by external stressors can induce apoptosis (Häcker, 2000). Therefore, in this study, it is suggested that apoptosis was induced by the damage inflicted to gill tissues as a result of direct exposure to TRO.

In summary, the results of this study suggest that TRO 20 does not significantly affect the survival of olive flounder over 14 days. At higher concentrations (TRO 40 and 60), TRO not only causes oxidative stress, but also induces deformation and apoptosis of gill tissue, which both negatively affect survival. TRO 20 may be suitable for water treatment in recirculating aquaculture systems containing olive flounder. Further studies on the concentrations of TRO that cause physiological stress in other important aquaculture fish species and long-term exposure on diverse concentrations of TRO are needed.

Chapter 3.

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

1. Introduction

Recently, use of chlorofluorocarbon known as 'freon gas' is reducing the stratospheric ozone level and destroying the ozone layer. Because of this, the ultraviolet (UV) radiation reaching the surface of the sea between latitudes 30-50° is increasing (Zagarese and Williamson, 2001). UV are classified as UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (100-290 nm) depending on its wavelength (Pfeifer, 2012). The depth of penetration into the surface of the sea is different depending on the type of UV radiation. Especially, UV-A can penetrate up to 23 m depth while UV-B can penetrate up to 7-12 m (Lesser et al., 2001). UV penetrated into seawater have much higher energy per photon than other wavelengths of light, so they affect on aquatic organisms negatively from the first producers to upper predators such as fish in the ecosystem. Also, they are known to damaging in vivo molecules or inducing oxidative stress (Zagarese and Williamson, 2001). It means, UV can penetrate to the water depth of the coral reef in coastal area. Yellowtail clownfish *Amphiprion clarkii* that I used in this study can be directly affected by UV-A because it forms habitats at 5-20 m depths with coral reefs (Moyer, 1976). Yellowtail clownfish are subtropical fish species inhabiting the Pacific and Indian Oceans and the Japanese coastal area (Allen, 1975; Foutin et al., 1992). Also, they are known to inhabiting in the coastal area of JeJu Island in Korea (Choi et al., 2013).

UV can act as a stress factor to fish and result oxidation-reduction reaction which is known to induce reactive oxygen species (ROS) that cause oxidative stress in cells and tissues (Pourzand and Tyrrell, 1999). There are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) as a representative ROS (Roch, 1999).

Overproducted ROS in the body causes denaturation of intracellular DNA and protein structure, reduces resistance to disease and induces physiological disturbances such as decreased reproductive capacity or production of lipid peroxide (LPO) (Kim and Phyllis, 1998; Pandey et al., 2003).

Therefore, organisms including fishes activate an antioxidant mechanism to protect themselves from oxidative stress caused by ROS generated in the body. These mechanisms are mediated by antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Mcfarland et al., 1999). These enzymes are especially work in the liver and kidney in 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 are converted to non-toxic H_2O and O_2 by CAT which is known to have an antioxidant action to remove ROS (Kashiwagi et al., 1997; Basha and Rani, 2003; Hansen et al., 2006).

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

In this study, therefore, I investigated the effect of UV wavelength which penetrating the depth of yellowtail clownfish habitat on oxidative stress induction in fish. Lesser et al. (2000) reported that the UV intensity reaching 8 m depth was 0.260 W/m^2. Based on this, I exposed two intensities of UV (0.2 and 0.4 W/m^2)

which are expected to affect yellowtail clownfish habitat on 5-20 m depth.

In this study, I measured the expression and activity of SOD and CAT mRNA in order to investigate the oxidative stress induction of yellowtail clownfish according to the exposure time and UV wavelength. Also, to investigate the levels of oxidative stress in the body, I measured plasma concentrations of H_2O_2 and LPO. In order to investigate the effects of DNA damage and apoptosis in liver tissues, comet assay and terminal transferase dUTP nick end labeling (TUNEL) assay were used. Also, I investigated AlaAT and AspAT in order to determine the liver damage level caused by stress.

2. Materials and methods

2.1. Experimental fish and conditions

Yellowtail clownfish (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 allowed to acclimate 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 near the water surface of the tanks was approximately 0.96 W/m^2. The water temperature and photoperiod were 27 ± 1°C and a 12-h light : 12-h dark period (lights on 07:00 h and light off 19:00 h) respectively. For the experimental groups, the fish were exposed to UV radiation (380 nm), maintained at an intensity of approximately 0.2 and 0.4 W/m^2 (Fig. 7). The UV radiations were set 20 cm above the surface of water and intensities were determined in the middle of water as using a spectrometer (MR16; Rainbow Light Technology Co. Ltd., Taoyuan, Taiwan) and Photo-Radiometer (HD 2102.1; Delta OMH CO., Caselle di Selvazzano, Italy). I sampled three fishes from each groups at day 0, day 1, day 3, day 7 and day 14. The dose of each UV radiation on day 0, day 1, day 3, day 7 and day 14 were 8.64 kJ/m^2, 25.92 kJ/m^2, 60.48 kJ/m^2, 120.96 kJ/m^2 at intensity of 0.2 W/m^2 and 17.28 kJ/m^2, 51.84 kJ/m^2, 120.96 kJ/m^2, 241.62 kJ/m^2 at intensity of 0.4 W/m^2. Control groups were sampled on 0 day only. All the experiment was repeated three times. All 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 (1,000 × *g* for 15 min at 4°C) and stored at -80°C until analysis. The liver tissues were collected from fish, immediately frozen in liquid nitrogen and stored at -80°C until total RNA extraction.

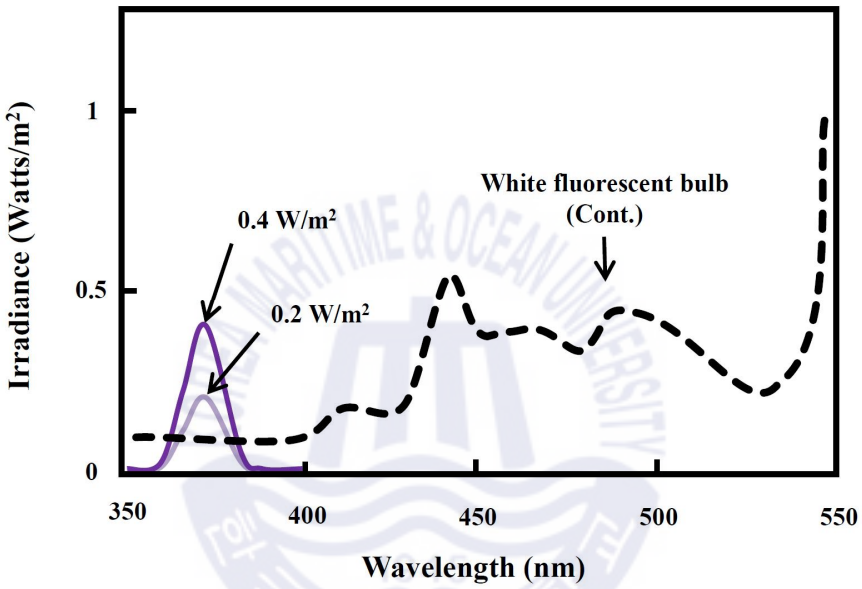

Fig. 7. Spectral profiles of UV radiation (380 nm) and white fluorescent bulb (Cont.) used in the present study. Two different intensities were used (0.2 and 0.4 W/m^2) for each UV treatment.

2.2. 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 instruction. Then, 2 μg of total RNA were 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℃.

2.3. qPCR analysis

The qPCR analysis was conducted to determine the relative expression levels of SOD and CAT using the total RNA extracted from the liver of yellowtail clownfish. The qPCR primers were designed using known yellowtail clownfish sequences (Table 3). I conducted the qPCR amplification using a Bio-Rad iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and the iQ SYBR green supermix (Bio-Rad, USA), following the manufacturer's instructions. As a control, the β-actin gene was also amplified for each sample and all data were expressed as the difference with the corresponding calculated β-actin threshold cycle (Ct) levels. The Ct values of the PCR products formed the basis for all analyses. The Ct levels were defined as the PCR cycle in which the fluorescence signal crossed a threshold during the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) per sample and the internal control (β-actin) were calculated as follows: [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$]. After the PCRs were completed, the qPCR data from three replicate samples were analyzed using Bio-Rad to estimate the transcript copy numbers of each sample.

Table 3. Yellowtail clownfish 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′

2.4. Plasma parameter analysis

Plasma was separated from whole blood through centrifugation (1,500 × *g* for 15 min at 4°C). Collect plasma using heparin as an anticoagulant. Assay immediately store samples at -20°C. The levels of SOD, CAT and LPO were analyzed using an immunoassay from an ELISA kit (SOD, CSB-E15929fh; CAT, CSB-E15928fh; Cusabio Biotech Co., Ltd., China; LPO, MFBS285269). H_2O_2 levels were measured using a peroxide detect kit (Sigma, USA). AlaAT and AspAT levels were analyzed by a dry multiplayer analytic slide method using a biochemical automatic analyzer (Fuji Dri-Chem 4000, Fujifilm, Japan).

2.5. Comet assay

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells (Bajpayee et al., 2005). Liver cells (1 × 105 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. Cells were immobilized in agarose gels on CometAssay comet slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Next, slides were electrophoresed at 18 V for 30 min. The samples were stained with SYBR Gold (Trevigen) for 30 min in the dark and then read using a fluorescence microscope (excitation filter 465-495 nm; Eclipse Ci, Nikon, Japan). At least 100 cells from each slide were analyzed. For quantification of comet assay results, I analyzed the 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).

2.6. TUNEL assay

To evaluate the apoptotic response of the fish liver cells to UV radiation, I performed the TUNEL assay using a commercially available in situ cell death detection kit (catalog number, 11 684 795 910, Roche, Switzerland). Polylysine coated slides were used to prevent the loss of adherence of the apoptotic cells to the slides. The fish liver tissue was washed and fixed with 4% buffered paraformaldehyde and was permeabilized with freshly prepared 0.1% Triton X-100 and 0.1% sodium citrate solution. This liver tissue was then incubated with the TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. The slides were washed three times with phosphate-buffered saline (PBS) and the incorporated biotin-dUTP was detected under a fluorescence microscope (excitation filter 465-495 nm; Eclipse Ci, Nikon, Japan). For the paraffin-embedded tissue sections, the slides were dewaxed and fixed according to standard protocols and then treated as described above. The green fluorescent cells indicated apoptosis.

2.7. Statistical analysis

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

3. Results

3.1. Changes in SOD and CAT mRNA expression and activity

SOD and CAT mRNA expression and activity were significantly increased over time in all groups exposed to UV radiation (Fig. 8). Groups with higher UV intensity (0.4 W/m^2) had higher mRNA expression and levels of plasma than the lower groups (0.2 W/m^2). However, there was no significant difference in mRNA expressions and plasma levels in the control group.

3.2. Changes in plasma H_2O_2, LPO, AlaAT and AspAT level

H_2O_2, LPO, AlaAT and AspAT plasma levels were significantly increased over time in all groups exposed to UV radiation (Fig. 9). Groups with higher UV intensity (0.4 W/m^2) had higher mRNA expression and levels of plasma than the lower groups (0.2 W/m^2). However, there was no significant difference in mRNA expressions and plasma levels in the control group.

3.3. Comet assay

Liver tissue DNA damage following 14 days exposure to UV radiation was analyzed using 100 randomly selected cells. The DNA content in the tail and tail moment both in the experimental groups (0.4 W/m^2) were increased significantly as the exposure time increasing compared with control group (Fig. 10). However, there was no significant difference in the control group over time.

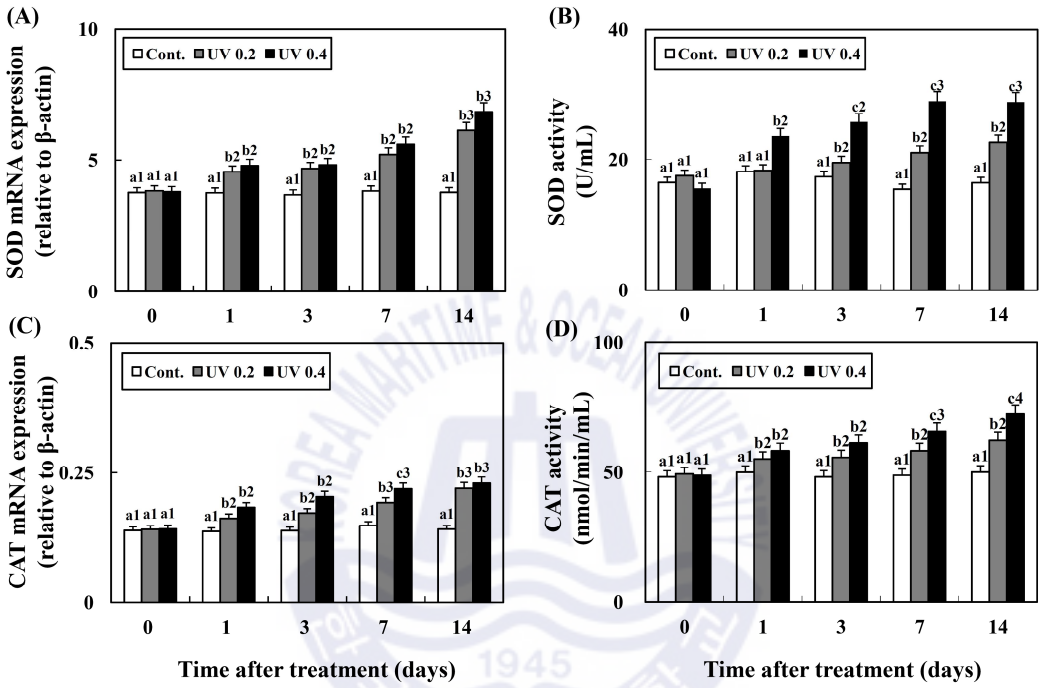

Fig. 8. Change in SOD mRNA expressions (A), SOD activity (B), CAT mRNA expression (C) and CAT activity (D) in yellowtail clownfish exposed to UV radiation at different light intensities (0.2 and 0.4 W/m^2) and white fluorescent bulb (Cont.). Different numbers indicate significant differences among the different exposure periods at the same UV intensity ($P < 0.05$). The lowercase letters indicate significant differences among the different UV intensities at the same exposure periods ($P < 0.05$). All values are means ± SE ($n = 9$).

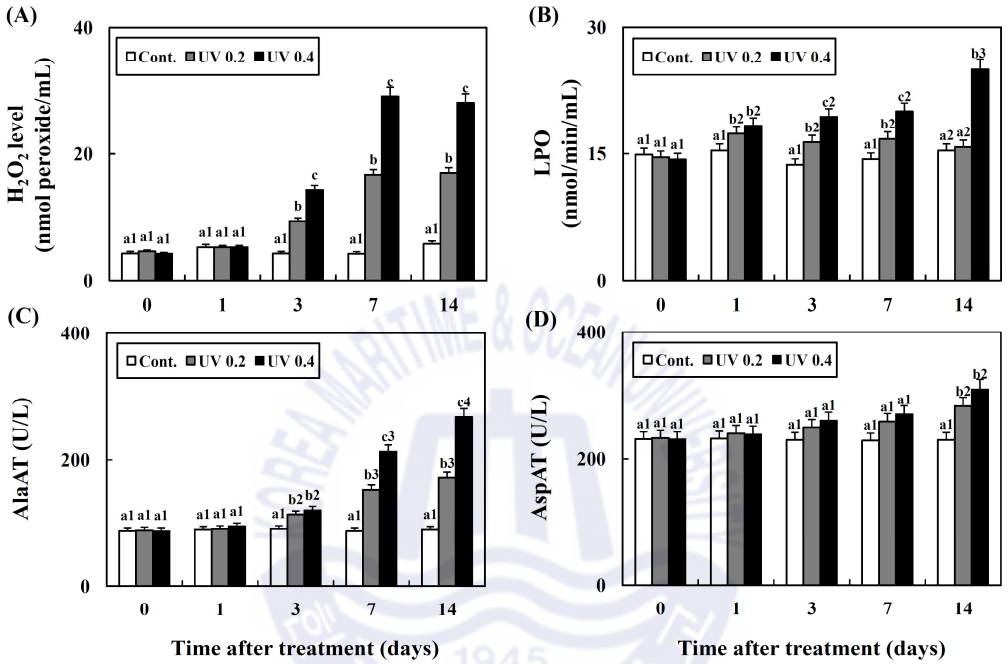

Fig. 9. Change in plasma H_2O_2 (A), LPO (B), plasma AlaAT (C) and plasma AspAT (D) levels in yellowtail clownfish exposed to UV radiation at different light intensities (0.2 and 0.4 W/m^2) and white fluorescent bulb (Cont.). Different numbers indicate significant differences among the different exposure periods at the same UV intensity ($P < 0.05$). The lowercase letters indicate significant differences among the different UV intensities at the same exposure periods ($P < 0.05$). All values are means ± SE ($n = 9$).

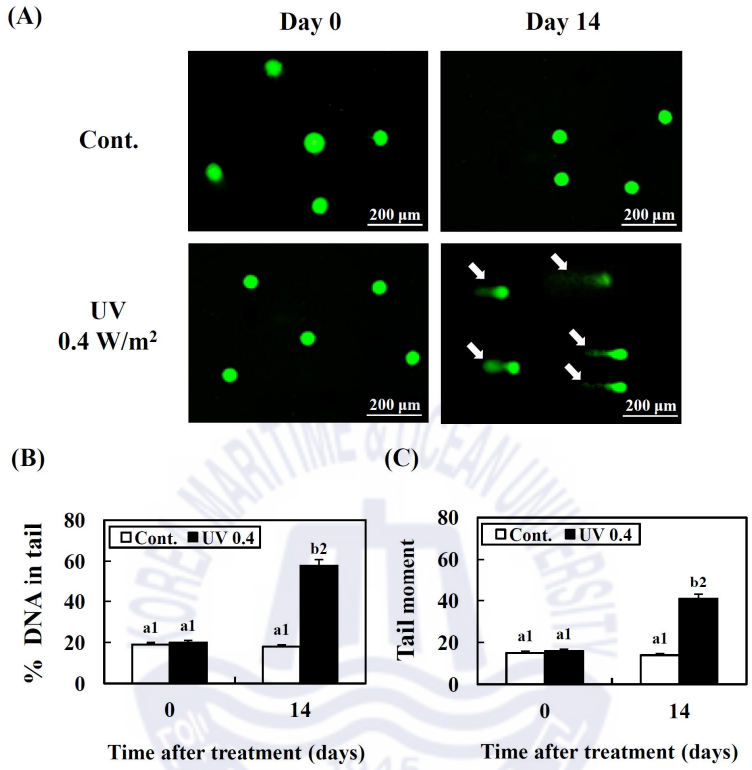

Fig. 10. Comet assay images following initial conditions (at day 0) and UV radiation (0.4 W/m^2 at day 14) in liver cells of yellowtail clownfish. White arrows in (A) indicate damaged nuclear DNA (DNA breaks) in liver cells, which have been stained with SYBR Gold. Scale bars = 200 μm. Different numbers indicate significant differences among the different exposure periods at the same UV intensity ($P < 0.05$). The lowercase letters indicate significant differences among the different UV intensities at the same exposure periods ($P < 0.05$). All values are mean ± SE ($n = 9$).

3.4. TUNEL assay

The TUNEL assay was used to investigate the presence of apoptotic cells (Fig. 11). There were significant visible differences among the labeled cells in the TUNEL assay 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 control group.

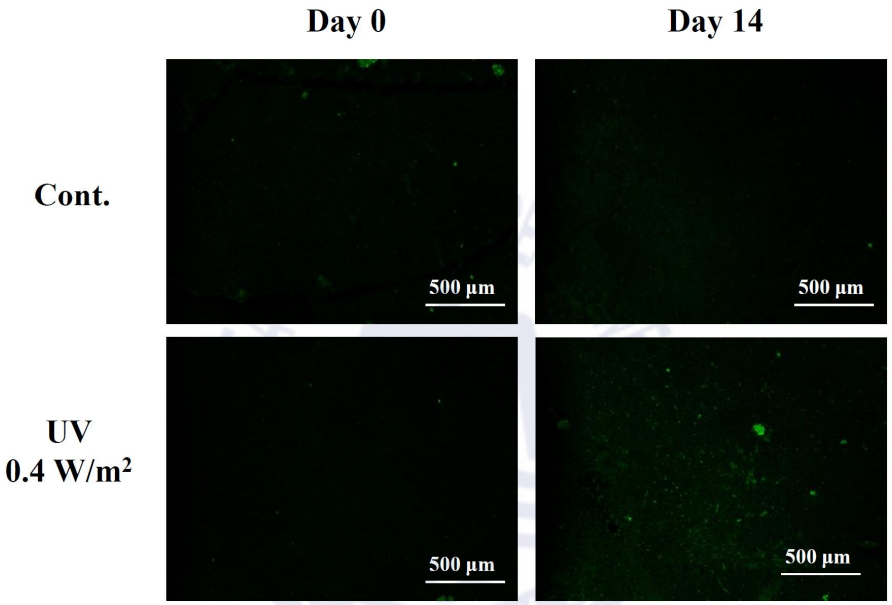

Fig. 11. TUNEL detection images following initial conditions (at day 0) and UV radiation (0.4 W/m^2 at day 14) in liver cells of yellowtail clownfish. Cells were stained with acridine orange and visualized with a fluorescent microscope. Cells showing green fluorescence are apoptotic cells. Scale bars = 500 μm.

4. Discussion

This study investigated the effects of exposure to UV 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.

After UV irradiation, I analyzed mRNA expression and activity of SOD and CAT in yellowtail clownfish. The expression of antioxidant genes and the activities of antioxidant enzymes in plasma were significantly increased. In each experimental group, the expression of SOD and CAT mRNA was not significantly different, but the activity was significantly higher in the UV 0.4 group than UV 0.2 group appears. Similar to this study, Lesser et al. (2001) examined the effect of UV on the oxidative stress of atlantic cod *Gadus morhua* larvae after 10 days exposure at different wavelengths of UV. As a result, they reported that SOD activity was significantly higher in the experimental group which exposed to UV. And as UV became shorter, SOD activity of the larvae was increased. Also, Charron et al. (2000) investigated the effects of UV on the oxidative stress of fish body after exposure to zebrafish *Brachydanio rerio* for 25 hours. As a result, the amount of SOD and CAT mRNA expression in the skin and muscle was significantly higher than that in the group not exposed to UV. Overall, similar to those of previous studies, our results show that oxidative stress in yellowtail clownfish was induced by UV and antioxidant gene expression and antioxidant enzymes activity in plasma were significantly increased to relieve oxidative stress.

After UV irradiation, the concentrations of H_2O_2, LPO, AlaAT and AspAT in the plasma of yellowtail clownfish were lower in UV 0.2 group than those of the UV 0.4 group. But there is no significant difference in AspAT concentration. Similar to this study, Zigman and Raffertyt (1994) analyzed the H_2O_2 concentration in the lens of dogfish *Mustelus canis* after exposure to UV. As a result, the decomposition of H_2O_2 is reduced in the experimental group exposed to UV and

induced the oxidative stress. Gouveia et al. (2015) reported that the oxidative stress of silverside *Odontesthes argentinensis* increased the ROS and LPO after exposure to UV. Also they reported that the intensity of UV increased, antioxidant capacity in silverside was decreased. Overall, similar to those of previous studies, our results show that UV exposure induced oxidative stress in yellowtail clownfish, significantly increased H_2O_2, LPO and AlaAT levels in the plasma and significantly decreased antioxidant capacity. Also, According to those results, the liver function is degraded.

After UV irradiation, a comet assay was performed to determine the degree of DNA damage in liver tissue of yellowtail clownfish. As a result, the DNA tail length and tail moment values were significantly increased and DNA damage in the nucleus of the liver tissue was observed in the experimental group that irradiated with UV 0.4 W/m^2 compared to the control group. Sandrini et al. (2009) reported that the expression of DDB2 and XPC mRNA, which are genes affecting DNA damage, were increased in zebrafish *Danio rerio* by exposure to UV. Dietrich et al. (2005) collected sperm from rainbow trout and exposed it to UV for comet assay. As a result, they reported that the longer the exposure time to UV, the higher the tail length and tail moment values of the DNA. Overall, similar to those of previous studies, our results show that the expression of genes affecting DNA damage was increased due to UV exposure, resulting DNA damage in liver tissue of yellowtail clownfish.

After UV irradiation, a TUNEL method was performed to observe the cytotoxicity of yellowtail clownfish liver tissue. As a result, it was found that TUNEL-positive cells were distributed in a large amount in UV 0.4. Yabu et al. (2001) observed apoptosis by exposure to UV through TUNEL analysis of zebrafish embryos. As a result, they reported that TUNEL-positive cells were observed in a large amount in the whole tissues exposed to UV and it induced cell death. Therefore, in this study, it is considered that UV acts as a stress factor in yellowtail clownfish

and induces apoptosis.

The results of this study suggest that UV irradiation causes oxidative stress by acting as a stress factor in yellowtail clownfish, which not only causes DNA damage in liver cell, but also induces apoptosis. In addition, UV at 0.2 W/m^2 intensity, which is lower than 0.4 W/m^2 intensity, has a negative effect on antioxidant defense mechanisms. Overall, UV, even at a low intensity of 0.2 W/m^2 seems to be negatively affecting the physiological mechanism in fish.

Therefore, further studies are required to investigate the discursive wavelengths and intensities of UV that can cause physiological changes in various fish species and the results of this study can be used as basic research data.

Chapter 4.

General Discussion

During their lifetime, fish may be exposed to various environmental stress factors. Recently, due to the rapid change in technology and environment, fish have been exposed to new environmental stress factors such as oxidative stress and possess a defense mechanism for protection against these stress factors.

Due to the destruction of the ozone layer, organisms are being increasingly exposed to high amounts of UV. TRO is being examined as a new sterilizing substance to be used in aquaculture. In this study, the mechanism underlying environmental factor-induced stress in the fish body was investigated.

I. To examine changes in stress response and antioxidant capacity of the olive flounder to TRO treatment, the survival rate and mRNA expressions of the antioxidant enzymes SOD and CAT were investigated. Furthermore, the mRNA expression and activity of the stress indicator HSP70 were evaluated. Additionally, the plasma H_2O_2 concentration was measured and cell damage was assessed using a TUNEL assay. The results demonstrated that the survival rate and expression of SOD and CAT were significantly higher after exposure to high TRO concentrations (TRO 40 and TRO). Similarly, mRNA expression and activity of HSP70 and plasma H_2O_2 concentration were also significantly higher and TUNEL-positive cells were observed after exposure to high TRO concentrations (TRO 40 and TRO 60). However, following exposure to lower TRO concentrations (TRO 20), minimal to no differences were observed within the control group for all investigated factors. These results supported previous evidence indicating that the TRO 40 and TRO 60 concentrations are harmful to marine fish species including the olive flounder (Kim et al., 1999; Jung et al., 2018). Moreover, the present results

indicated that the TRO 20 concentration failed to induce oxidative stress and apoptosis in the olive flounder. Furthermore, these findings suggested that low concentrations of TRO may be suitable for water treatment in recirculating aquaculture systems containing olive flounder. Additional studies evaluating the concentrations of TRO that induce physiological stress in other important aquaculture fish species and long-term exposure following exposure to varying concentrations of TRO are needed.

II. To evaluate the effects on UV irradiation in yellowtail clownfish, the mRNA expression and the activities of the antioxidant enzymes SOD and CAT were measured using stress indicator. The plasma concentrations of H_2O_2, LPO, AlaAT and AspAT were also investigated. Cell damage was assessed using comet and TUNEL assays. The results indicated significantly higher expression and activity of SOD and CAT after UV irradiation. Similarly, plasma concentrations of H_2O_2, LPO, AlaAT and AspAT were higher after UV irradiation. DNA damage in cells and cell death measured using comet and TUNEL assays were both increased following UV irradiation. Moreover, higher UV irradiation levels worsened the stress factors more than lower UV levels. These results demonstrated that UV is a stress factor in yellowtail clownfish and that it not only induces DNA damage in liver cells but also apoptosis. Moreover, both high and low intensity UV negatively affected the physiology of the yellowtail clownfish. Further studies are required to investigate the physiological changes induced 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 performing such studies.

In conclusion, the present study demonstrated that external environmental stress factors (TRO and UV) can lead to oxidative stress in marine fish (olive flounder

and yellowtail clownfish) due to the production of ROS. The antioxidant enzymes, SOD and CAT, were increased according to the concentration or intensity of and exposure time to stress factors. This tendency was the similar to that observed with the other stress factors, HSP70, H_2O_2, LPO, AlaAT and AspAT. DNA damage and cell death were also increased in accordance with the concentration or intensity of and exposure time to stress factors. In summary, this study indicates that the changes induced by external environmental factors can negatively affect fish species.

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