Thesis for the Degree of Master of Science

Effects of Waterborne Copper on Toxicity and Oxidative Stress Responses in Red Seabream, *Pagrus major*

Tae Hwan Kim

Department of Marine BioScience and Environment

The Graduate School

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Advisor: Prof. Cheol Young Choi

by

Tae Hwan Kim

A dissertation submitted in partial fulfillment of the requirements for the degree of

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the Graduate School of Korea Maritime and Ocean University

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List of Abbreviations

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
cAMP	cyclic AMP
CRH	corticotropin-releasing hormone
Cu	copper
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GPX	glutathione peroxidase
H_2O_2	hydrogen peroxide
HPI	hypothalamic-pituitary-interrenal
IgM	immunoglobulin M
LPO	lipid peroxidation
MT	metallothionein
MTF-1	metal responsive element-binding transcription factor-1
NKA	Na^+/K^+-ATPase
$^1O_2$	singlet oxygen
O_2^-	superoxide
OH^-	hydroxyl radical
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
ROS	reactive oxygen species
SOD	superoxide dismutase
SE	standard error
TBS	tris-buffered saline
TUNEL	transferase dUTP nick end labeling

고농도의 구리 성분이 참돔, *Pagrus major*의 독성 및 산화 스트레스 반응에 미치는 영향

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요 약

본 연구는 다양한 농도의 구리에 참돔, *Pagrus major*을 노출시킨 후, 참돔 체내에서 독성 및 산화 스트레스를 유발시키는 구리의 농도 범위를 확인하기 위하여 수행되었다. 구리 농도에 따른 참돔의 생리학적 변화를 관찰하기 위하여 혈중 호르몬의 양적 변동 및 효소 활성을 포함한 분자내분비학적 분석 등을 통하여 비교·분석하였다.

1. 참돔의 독성 스트레스 및 세포사멸에 미치는 구리의 영향

본 연구는 독성 스트레스 및 세포사멸에 영향을 미치는 구리의 농도를 파악하기 위하여, 참돔을 다양한 구리 농도(10, 20, 30 및 40 μg/L) 실험구별로 각각 0, 6, 12, 24, 72 및 120 시간 동안 노출시킨 후, 생리학적 스트레스 반응[corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), cortisol 및 glucose] 및 독성 스트레스 지표[metallothionein (MT) 및 Na^+/K^+-ATPase (NKA)]의 변화를 분석하였다. 또한, 세포사멸 반응은 혈중 hydrogen peroxide (H_2O_2) 및 caspase-3의 활성 변화 그리고 terminal transferase dUTP nick end labeling (TUNEL) assay을 통하여 확인하였다.

그 결과, 30과 40 μg/L의 구리 농도 실험구에서는 노출 시간이 경과함에 따라

혈장 내 CRH, ACTH, cortisol, glucose, MT, H_2O_2 및 caspase-3 농도가 유의적으로 증가하는 경향이 관찰되었다. 그러나 혈장 내 NKA 농도는 오히려 감소하는 경향이 관찰되었다($P < 0.05$). 또한, TUNEL assay를 실시한 결과, 30 μg/L의 구리 농도 실험구에서 세포사멸이 가장 많이 관찰되었다.

따라서 참돔의 경우, 30 μg/L 이상의 구리 농도는 독성으로 작용하여 스트레스 및 세포사멸을 유발하는 것으로 판단되었다.

2. 참돔의 산화 스트레스 및 면역 반응에 미치는 구리의 영향

본 연구에서는 구리가 독성으로 작용하여 참돔의 산화스트레스와 면역 반응에 영향을 미치는 농도를 파악하기 위하여, 다양한 구리 농도별(10, 20, 30 및 40 μg/L) 실험구에 참돔을 각각 0, 6, 12, 24, 72 및 120 시간 동안 노출시킨 후, 항산화 효소인 superoxide dismutase (SOD)와 catalase (CAT) mRNA의 발현량 및 혈장 내 농도 변화 그리고 hydrogen peroxide (H_2O_2)와 lipid peroxidation (LPO)의 농도 변화를 분석하였다. 또한, 실험구별 면역 반응의 차이를 비교하기 위하여 immunoglobulin M (IgM), lysozyme, 및 melatonin의 농도 변화 및 단백질 발현 변화를 분석하였으며, 세포 내 핵 DNA 손상 정도를 시각적으로 확인하기 위하여 Comet assay를 실시하였다.

그 결과, 항산화 효소 mRNA의 발현 및 활성 그리고 H_2O_2과 LPO 농도는 30 μg/L 이상의 구리 농도에서 노출 시간이 경과함에 따라 유의적으로 증가하였다. 면역 관련 지표로서 사용되고 있는 IgM, lysozyme 및 melatonin의 농도 변화와 단백질 발현 변화는 30과 40 μg/L 구리 농도에서 노출 시간이 경과함에 따라 유의적으로 감소하였다. 또한, Comet assay 결과에서도 30 μg/L에서 120 시간 동안 노출하였을 경우, 핵 DNA의 손상 정도가 가장 심한 것으로 확인되었다.

따라서 참돔의 경우, 30 μg/L 이상의 구리 농도는 산화 스트레스로 작용하여 항산화 작용과 관련 호르몬의 분비를 유도하고 면역 관련 물질의 발현을 감소시키는 등 참돔의 생리학적 기능에 악영향을 미치는 것으로 판단되었다.

Effects of Waterborne Copper on Toxicity and Oxidative Stress Responses in Red Seabream, *Pagrus major*

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Abstract

This study was carried out to identify copper (Cu) concentrations that inducting toxic and oxidative stress at red seabream body in a variety of Cu environments. In order to observe the physiological changes, they were compared and analyzed by molecular biological experiment method.

1. Effects of waterborne copper on toxicity stress and apoptosis responses in red seabream, *Pagrus major*

The present study was conducted to investigate the effect of toxicity resulting from Cu exposure on physiological stress and cell death in the red seabream, and to determine the concentration range over which Cu is toxic. After exposure of red seabream to Cu at various concentrations (10, 20, 30, and 40 μg/L) for 0, 6, 12, 24, 72, and 120 hour (h). To this end, I analysed changes in the physiological stress response [corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone

(ACTH), cortisol, and glucose)] and toxic stress indices [(metallothionein (MT) and Na^+/K^+-ATPase (NKA)] in red seabream exposed to various concentrations of Cu. Furthermore, I measured the activity of hydrogen peroxide (H_2O_2) and caspase-3 in plasma to confirm the apoptosis response, and performed a terminal transferase dUTP nick end labeling (TUNEL) assay.

As a result, the concentration of CRH, ACTH, cortisol, MT, H_2O_2, and caspase-3 concentrations increased significantly following exposure times to Cu were observed in the experiment groups exposed to 30 and 40 μg/L. However, NKA in plasma was decreased ($P < 0.05$). TUNEL assay showed that cell death was the most frequent at 30 μg/L concentration. In conclusion, when exposed to Cu concentrations of 30 μg/L or more, they acted toxic in the fish body, indicating stress and apoptosis.

2. Effects of waterborne copper on oxidative stress and immune responses in red seabream, *Pagrus major*

This study was performed to determine the concentration range of Cu, which affects oxidative stress in the red seabream. I exposed red seabream to different concentrations of Cu (10, 20, 30, and 40 μg/L), and then investigated the changes in mRNA expressions and activities of anti-oxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] and measured changes in the levels of plasma oxidative stress indicators hydrogen peroxide (H_2O_2) and lipid peroxidation (LPO). I also analyzed lysozyme, immunoglobulin M (IgM), and melatonin levels to confirm changes to immune function caused by Cu exposure. In addition, I conducted comet assay to analyze the nuclear DNA damage in the red seabream liver cells caused by reactive oxygen species (ROS) arising from Cu exposure.

As a result, anti-oxidant enzyme mRNA expression and activity, H_2O_2 and LPO plasma levels were significantly increased following exposure time at Cu concentration above 30 μg/L. Protein expressions and plasma levels of the lysozyme, IgM, and melatonin, which used as immune-related index, were significantly decreased following

exposure time at 30 and 40 μg/L. Also, the comet assay also showed that the highest level of nuclear DNA damage occurred experiment group, which exposed to 30 μg/L for 120 h.

In red sea bream, Cu concentration above 30 μg/L was considered to have negative effect on the physiological function of red seabream such as induction of secretion of anti-oxidant activity hormone and decrease expression of substance related immune index.

Chapter 1.

Effects of waterborne copper on toxicity stress and apoptosis responses in red seabream, *Pagrus major*

1. Introduction

Recently, there has been increasing contamination of coastal environments due to the inflow of industrial wastewater and domestic sewage. Among the trace metals that enter the coastal environment, those that are particularly problematic include Ag, Cd, Cu, Hg, Pb, and Zn (Chen et al., 2012). Copper (Cu) is an essential trace element necessary for the maintenance of physiological functions, such as biological and physiological metabolic processes. In addition, trace amounts of Cu in the range 100～5,000 μg/L have been reported to have anti-microbial and anti-oxidant functions against bacteria (Ren et al., 2009; Chen et al., 2012; Luzio et al., 2013). It has been reported that high concentrations of Cu in water have a variety of physiological and behavioral affects, such as growth inhibition and ion control disorder, by acting as a toxin in aquatic organisms (Tellis et al., 2012; Luzio et al., 2013). To date, however, there have been no studies on the concentration range at which Cu acts as a toxic agent in fish.

When fish are exposed to environmental stresses such as toxicity, salinity, and adverse temperatures, they deploy various stress defense mechanism to maintain homeostasis (Jiang et al., 2015; Glover et al., 2016). A representative defense mechanism is the activity of the hypothalamus-pituitary-interrenal axis (HPI axis) (Gagnon et al., 2006), the first response of which is the release of corticotropin-releasing hormone (CRH) from the hypothalamus. The secreted CRH acts on the anterior pituitary gland, leading to secretion of adrenocorticotropic

hormone (ACTH) (Bonga, 1997). ACTH is derived from the pro-opiomelanocortin precursor protein and acts on the interrenal cells of the head kidney to synthesize and release the stress indicator cortisol (Bonga, 1997; Flik et al., 2006). Cortisol plays a role in directly increasing the plasma concentration of glucose, which is used as a metabolic energy source in damaged cells (Begg and Pankhrust, 2004; Small, 2004).

Metallothionein (MT) and Na^+/K^+-ATPase (NKA) are typically used as biomarkers for measuring the response of fish to heavy metal toxicity (Sampaio et al., 2012; Wu et al., 2015). MT, which is known to play a role in homeostasis and prevention of metal toxicity in the body, is induced by heavy metal ions through a metal responsive element-binding transcription factor-1 (MTF-1) (Carpenè et al., 2007; Takahashi, 2012). It is known to be a substance that, in response to heavy metal exposure, binds to metals and chelates them (Choi et al., 2015; Meena et al., 2015). According to Wu (2006) when tilapia, *Oreochromis mossambicus* were exposed to 100 μM Cu, MT protein concentration was significantly increased. In the case of aquatic organisms, the gill is the first organ to be affected by toxins, and the gill cells are the most vulnerable tissues in polluted environments (Mallatt and Stinson, 1990). NKA, which is present in the gills of fish, is an enzyme that has a pronounced influence on body homeostasis by controlling gas exchange, body osmolality, and ion concentration. NKA is used as an indicator of gill injury in environments exposed to heavy metals, such as Cu, as well as other toxic substances (McCormick et al., 2009; Schultz et al., 2012).

The apoptosis response is regulated by caspase, which a cysteine proteinase, and is characterized by morphological features such as DNA fragmentation and cell contraction (Alnemri, 1997). Caspase-3 is known to play an important role during the course of apoptosis, both biochemically and morphologically, particularly with regard to DNA damage and inflammatory responses (Kerr et al., 1972; Hacker, 2000). Accordingly, the activity of caspase-3 can be measured to determine the progression of apoptosis in tissues (Monteiro et al., 2009).

In fish, the amount of reactive oxygen species (ROS), including superoxide,

hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) (Roch, 1999; An et al., 2008), increases in a toxic stress environment. Excessive production of ROS due to exposure to toxic stress increases lipid peroxidation, oxidizes nucleic acids and proteins, and damages DNA (Monteiro et al., 2009). In particular, H_2O_2 has been reported to accelerate apoptosis by affecting cell viability by causing membrane damage and enzyme inactivation (Monteiro et al., 2009; Pandey, 2003).

The present study was conducted to investigate the effect of toxicity resulting from Cu exposure on physiological stress and cell death in the red seabream, and to determine the concentration range over which Cu is toxic. To this end, I analysed changes in the physiological stress response controlled by the HPI axis (CRH, ACTH, cortisol, and glucose) and toxic stress indices (MT and NKA) in red seabream exposed to various concentrations of Cu. Furthermore, I measured the activity of caspase-3 and H_2O_2 in the blood to confirm the apoptotic response of red seabream exposed to Cu, and performed a terminal transferase dUTP nick end labeling (TUNEL) assay to determine cell death rate in gill tissue.

2. Materials and methods

2.1. Experimental fish

The red seabream, *Pagrus major* (length 18.5 ± 1.5 cm; mass 127.2 ± 0.8 g; n = 150) used in the present study were obtained from the Korea Institute of Ocean Science & Technology (Tong-yeong, Korea), and were allowed to acclimate for 1 week in eight 300-L circulation filter tanks in the laboratory. The fish were reared using automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimatize to the conditions for 24 hour (h). The water temperature used for all experimental groups was maintained at 20°C. The photoperiod used was a 12-h light (L):12-h dark (D) cycle (lights on at 07:00 and lights off at 19:00) under the illumination of white fluorescent bulbs (27 W).

2.2. Cu treatment and sampling

The fish were divided into one control group (Cont.; non-Cu treated) and four experimental groups, which were maintained in separate tanks (n = 30, each group). Experimental groups were treated with waterborne copper (II) sulphate pentahydrate (Cu, $CuSO_4 \cdot 5H_2O$, 7758-99-8; Sigma-Aldrich, St. Louis, MO, USA) at one of four concentrations, 10, 20, 30, and 40 μg/L. Blood samples were collected from three different fish at each of following time points: 0, 6, 12, 24, 72, and 120 h. Fish were anaesthetized with 200 μg/L 2-phenoxyethanol (Daejung Chemicals & Metals Co., Ltd, Siheung, Gyeonggi, Korea) to minimize stress prior to blood and tissue collection. Blood was collected rapidly from the caudal vein using a 3-mL syringe coated with heparin. Plasma samples were separated from blood samples by centrifugation (4°C, 1,000 × g, for 10 min) and stored at -80°C until analysis.

2.3. Plasma parameter analysis

Plasma samples were separated by centrifugation (4°C, 1,000 × g, for 10 min). CRH, ATCH, cortisol, MT, NKA, and caspase-3 levels were analysed by

immunoassay using ELISA kits (CRH, MBS031034; ACTH, MBS019461; cortisol, MBS704055; NKA, MBS7203220; MT, MBS903939; caspase-3, MBS012786: Mybiosource Inc., San Diego, CA, USA). Absorbance was read at 450 nm, and the concentration was interpolated from a standard curve.

H_2O_2 levels were measured using a Peroxide Detection kit (Sigma). Absorbance was read at 560 nm, and the concentration was interpolated from a standard curve.

Plasma glucose levels were measured using a dry multiplayer analytic slide method in a biochemical auto analyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

2.4. TUNEL assay

To evaluate the affect of Cu toxicity on fish, I performed the TUNEL technique, using a commercially available in situ cell death detection kit (11 684795 910; Roche Co., Basel, Switzerland). To prevent apoptotic cells losing adherence to slides, the slides were coated with polylysine. After rearing fish for 120 h under exposure to 20 and 40 μg/L Cu, their livers were washed and fixed with 4% buffered paraformaldehyde and permeabilized with freshly prepared 0.1% Triton X-100, 0.1% sodium citrate solution. The livers were then incubated with TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. The slides were washed three times with phosphate-buffered saline 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, slides were dewaxed and fixed according to standard protocols, and were then treated as described above. Cells showing green fluorescence were apoptotic.

2.5. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by Tukey's post hoc test was used to compare differences in the data ($P < 0.05$). The values are expressed as the mean ± standard error (SE).

3. Results

3.1. Changes in plasma levels of CRH and ATCH

The changes in the plasma levels of CRH and ACTH in red seabream exposed to different concentrations of Cu (10, 20, 30, and 40 μg/L) are shown in Fig. 1A and 1B. Plasma CRH levels had increased significantly at 120 h after treatment with 30 and 40 μg/L Cu (2.9 ± 0.11 and 3.2 ± 0.11 pg/mL, respectively) compared to the Cont., 10, and 20 μg/L groups (2.406 ± 0.17, 2.536 ± 0.2, and 2.585 ± 0.22 pg/mL, respectively). In addition, ACTH levels had increased significantly after 120 h exposure to 30 and 40 μg/L Cu (approximately 1.32 and 1.19 fold, respectively).

3.2. Changes in plasma levels of cortisol and glucose

The changes in cortisol and glucose concentrations in red seabream exposed to different concentrations of Cu (10, 20, 30, and 40 μg/L) are shown in Fig. 2A and 2B. Significant increases in cortisol concentrations were observed in the 30 and 40 μg/L Cu experimental groups (12.3 ± 1.4 and 13.9 ± 1.1 ng/mL, respectively). Glucose levels were also significantly increased in fish exposed to 30 and 40 μg/L Cu. In particular, glucose levels in the 40 μg/L Cu treatment group were significantly higher (80.1 ± 4.3 mg/dL) than those observed in other groups (approximately 1.56 ~ 1.85 fold, respectively).

3.3. Changes in plasma levels of MT

The changes in MT levels following exposure of fish to different concentrations of Cu (10, 20, 30, and 40 μg/L) are shown in Fig. 3. The average initial plasma MT level was 33.05 ± 0.9 pg/mL; however, the plasma concentration of MT had increased significantly at 120 h in fish exposed to 30 and 40 μg/L Cu (58.60 ± 3.41 and 63.45 ± 4.1 pg/mL, respectively).

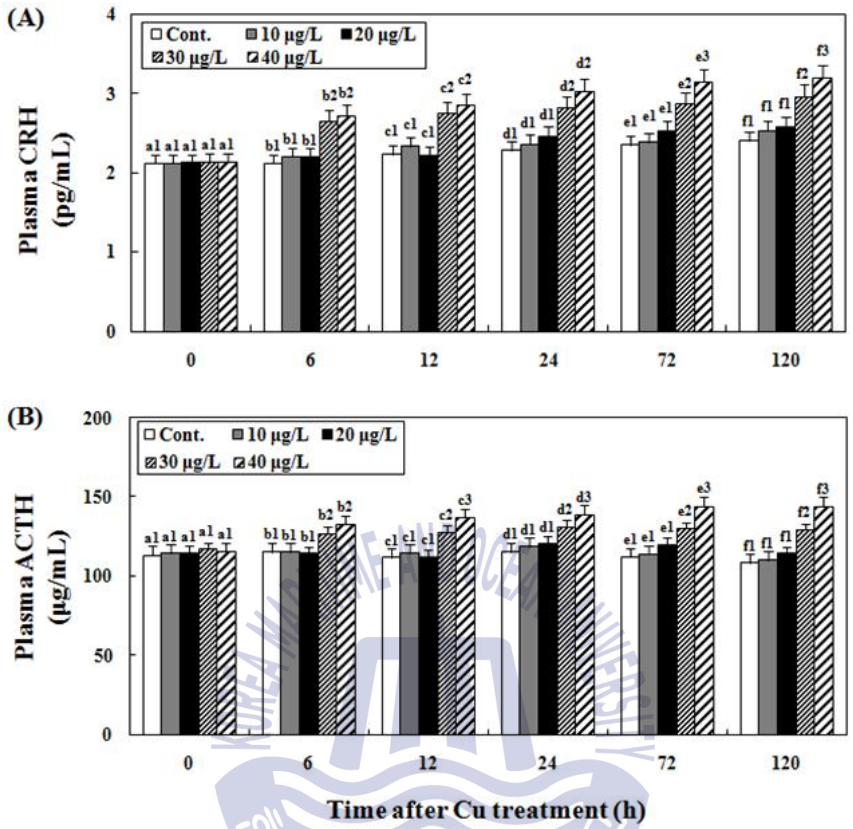

Fig. 1. Changes in plasma corticotrophin-releasing hormone (CRH) (A) and adrenocorticotropic hormone (ACTH) (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure periods at the same Cu concentrations. The numbers with letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($P < 0.05$). All values are the means ± SE ($n = 5$).

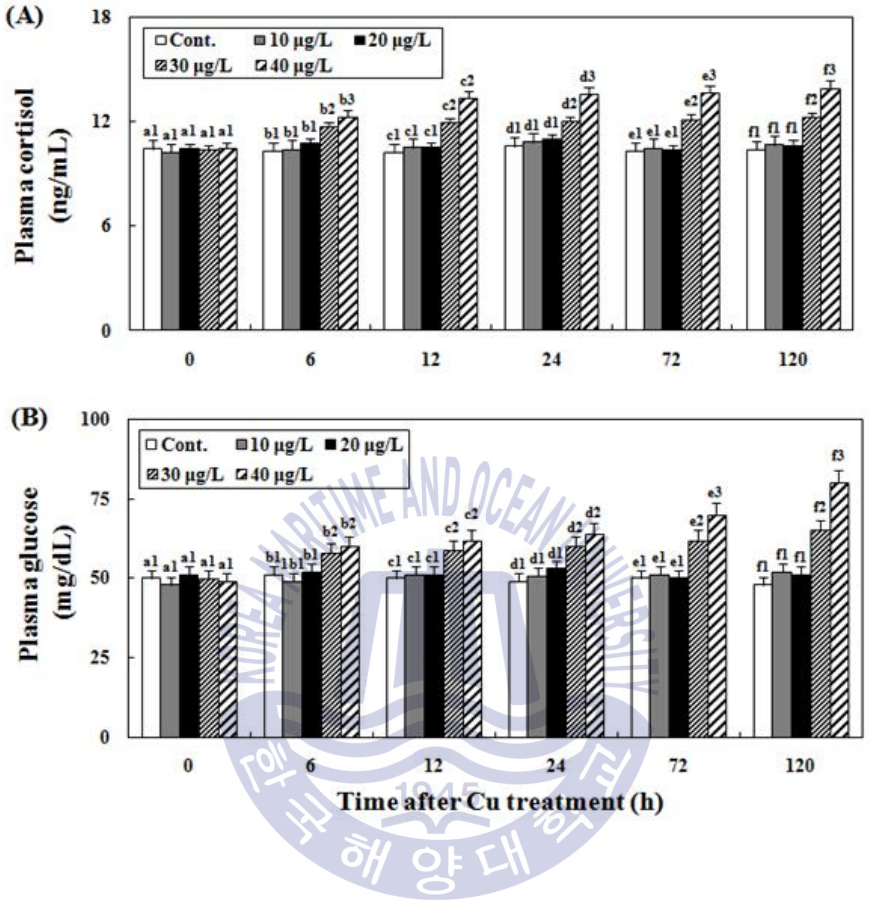

Fig. 2. Changes in plasma cortisol (A) and glucose (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure periods at the same Cu concentrations. The numbers with letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($P < 0.05$). All values are the means ± SE ($n = 5$).

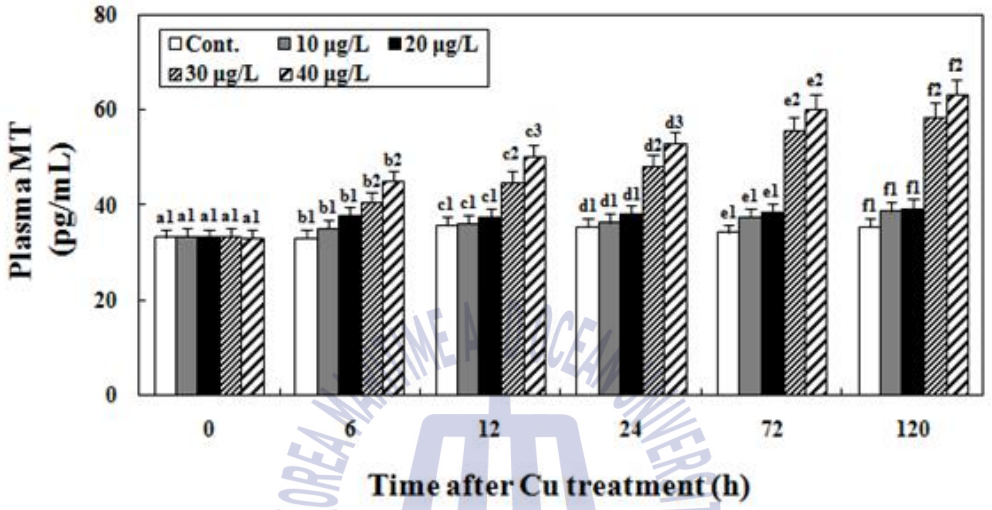

Fig. 3. Changes in plasma metallothionein (MT) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure periods at the same Cu concentrations. The numbers with letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($P < 0.05$). All values are the means ± SE ($n = 5$).

3.4. Changes in plasma levels of NKA concentrations

The variations in plasma NKA in red seabream exposed to different Cu concentrations (10, 20, 30, and 40 μg/L) are shown in Fig. 4. The average initial plasma NKA level was 28.56 ± 1.9 pg/mL; however, the NKA level in plasma had decreased significantly at 120 h following exposure to 30 and 40 μg/L Cu (22.43 ± 1.6 and 19.86 ± 1.84 pg/mL, respectively) compared to the other groups.

3.5. Change in plasma caspase-3 and H_2O_2 levels

The changes in plasma caspase-3 and H_2O_2 levels in fish exposed to different Cu concentrations (10, 20, 30, and 40 μg/L) are shown in Fig. 5A and 5B. The plasma concentration of caspase-3 in the 30 and 40 μg/L Cu groups increased significantly following exposure (18.0 ± 1.5 and 19.4 ± 1.7 pmol/L, respectively). Variations in the plasma H_2O_2 levels were similar to those observed for plasma caspase-3 levels. Levels of both plasma caspase-3 and H_2O_2 were highest at 120 h (19.36 ± 2.1 pmol/L and 18.88 ± 1.9 nmole peroxide/mL).

3.6. TUNEL assay

The results of the TUNEL assay used to investigate the occurrence of cell apoptosis are shown in Fig. 6. There were clear visible differences among the labeled cells in the Cont. and the experimental groups exposed to 20 and 30 μg/L Cu. The frequency of apoptotic cells increased after exposure to 30 μg/L Cu compared to the Cont. group.

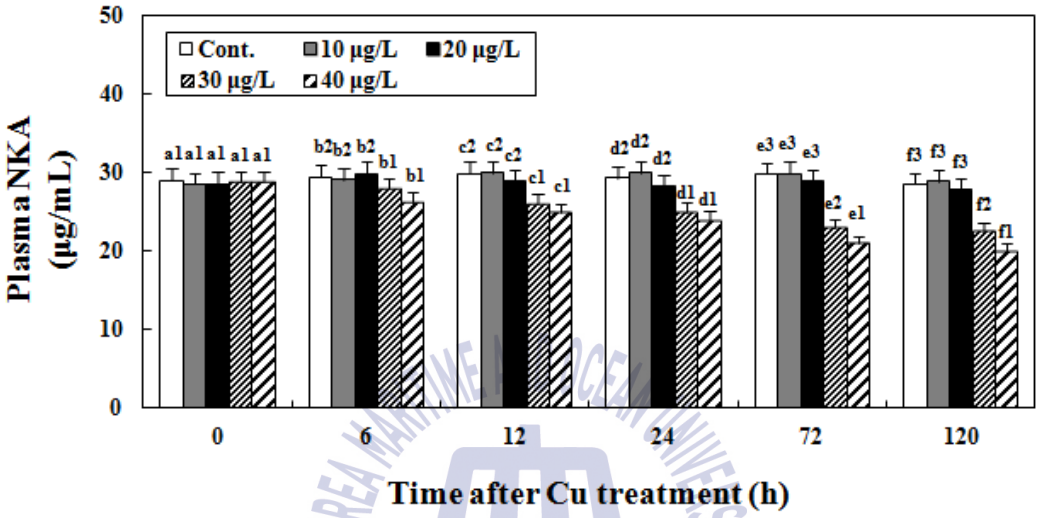

Fig. 4. Changes in plasma Na^+/K^+-ATPase (NKA) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure periods at the same Cu concentrations. The numbers with letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($P < 0.05$). All values are the means ± SE ($n = 5$).

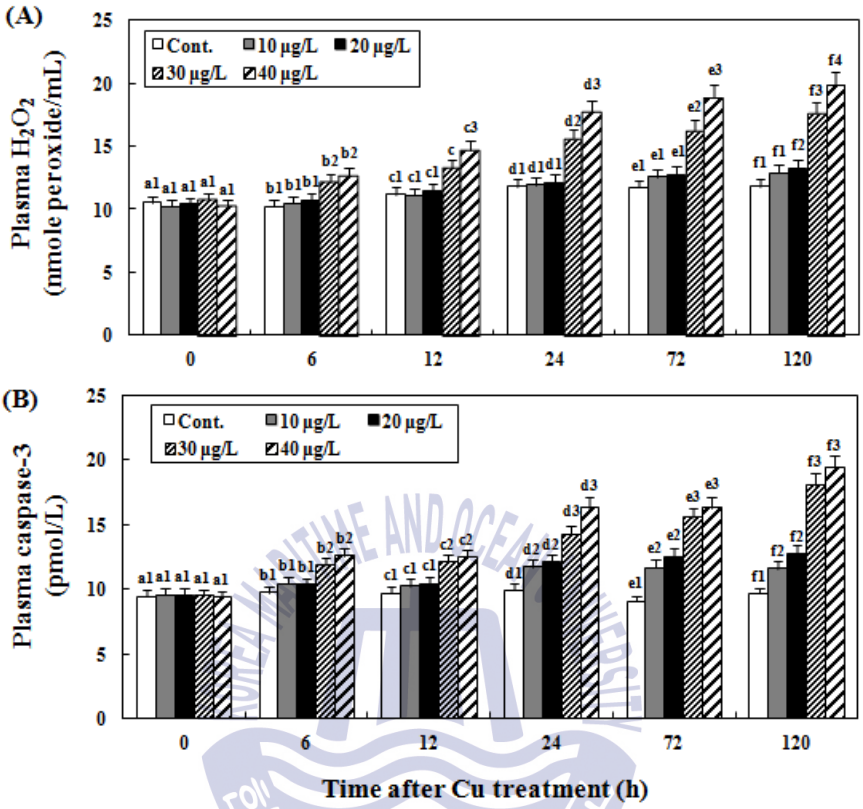

Fig. 5. Changes in plasma H_2O_2 (A) and caspase-3 (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure periods at the same Cu concentrations. The numbers with letters indicate significant differences among the different parameter values at the same Cu concentration and exposure period ($P < 0.05$). All values are the means ± SE ($n = 5$).

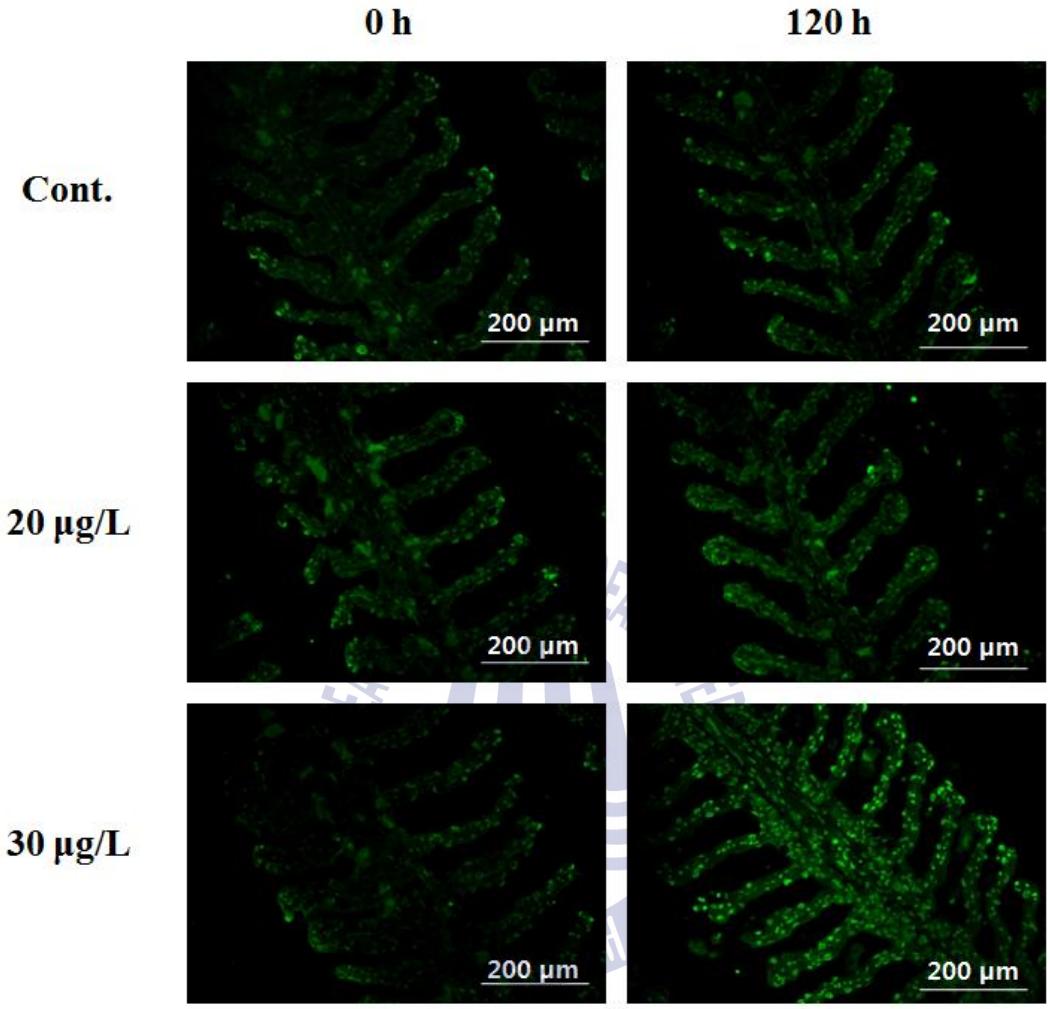

Fig. 6. TUNEL detection of red seabream gill cell apoptosis under different concentrations of Cu [0 (Cont.), 20 and 30 µg/L] for 0 and 120 h. Cells were stained with acridine orange and visualized with a fluorescent microscope. Cells showing green fluorescence are apoptotic cells. Scale bars = 200 µm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4. Discussion

In the present study, I exposed red seabream to different concentrations of Cu to examine the nature and degree of stress response and apoptosis. The red seabream were exposed to four Cu concentrations (10, 20, 30, and 40 μg/L), and fish in each experimental group was exposed for periods of 0, 6, 12, 24, 72, and 120 h. I examined the physiological responses of fish through molecular biology analysis.

I initially measured the changes in plasma CRH and ACTH concentrations, which are indicators of physiological stress due to Cu exposure. I accordingly observed that the CRH and ACTH concentrations in fish exposed to 30 and 40 μg/L Cu were significantly increased with an increase in exposure time. In a similar study, Choi et al. (2015) reported that when goldfish, *Carassius auratus* were exposed to various concentration of selenium, CRH and ACTH levels increased significantly at high concentrations of selenium (3 and 4 mg/L). Thus, consistent with previous studies, the findings of the present study indicate that high concentrations of Cu represent a stress to red seabream. Furthermore, as a defense mechanism against stress, the HPI axis was activated in the red seabream, as indicated by the increased blood levels of CRH and ACTH.

I analysed changes in cortisol and glucose concentrations in the plasma, secreted in response to CRH and ACTH. Plasma cortisol and glucose concentrations were significantly increased in the experimental group exposed to 40 μg/L Cu, and the concentrations tended to increase with an increase in exposure time. A similar finding was reported by Hedayati and Ghaffari (2013), who showed that when silver carp, *Hypophthalmichthys molitrix* were exposed to Cu of two concentrations (0.09 and 0.49 mg/L), plasma cortisol and glucose concentrations were significantly increased compared to the Cont. group. The results of the present study indicate that crotisol and glucose secretion mechanisms are activated as a defense against stress in red seabream at 30 μg/L, which is lower than the Cu concentration of 0.09 mg/L (=90 μg/L) shown to be toxic to silver carp.

The effect of toxic stress caused by Cu exposure on red seabream was confirmed by analysis of MT, a biomarker used to measure the level of heavy metal contamination. Increases in MT levels were observed in red seabream exposed to 30 and 40 μg/L Cu, and with an increase in the time exposed to Cu. A similar study reported that when the Neotropical pacu fish, *Piaractus mesopotamicus* was exposed to a Cu concentration of 400 μg/L, the MT level was significantly increased compared with the Cont. group (Sampaio et al., 2012). Therefore, in the present study, exposure to high concentrations of Cu (30 and 40 μg/L) acted as a toxic stress in the fish body, resulting in increased MT concentration as a defense mechanism against metal exposure.

In the present study, we also analysed the plasma concentrations of NKA, which plays a role in ion regulation, to investigate the effect of stress induced by Cu concentration on gill function. I observed no significant difference in NKA concentration between the 10 and 20 μg/L experimental groups and the Cont. group, but NKA was found to be significantly decreased in the 30 and 40 μg/L Cu experimental groups. A similar study by Wu et al. (2015) reported that when tilapia, *Oreochromis mossambicus* were exposed to a Cu environment (0.2, 1, and 2 mg/L), NKA activity was significantly decreased and that the level of damage tended to be higher in the 2 mg/L group. Similarly, Sampaio et al. (2012) reported that when *P. mesopotamicus* were exposed to 400 μg/L, the NKA concentration was significantly decreased compared to the Cont. group. Similar to the results of previous studies, the findings of the present study indicate that exposure to Cu at a concentration of at least 30 μg/L is toxic to the gills and induces stress. It is considered that Cu causes a decrease in the activity of NKA, which is an enzyme that plays a major role in maintaining homeostasis by regulating gas exchange, osmolality, and ion concentration.

In the present study, I also analysed the effects of exposure to various concentrations of Cu on the change of plasma H_2O_2 and caspase-3 in red seabream. I found that the plasma levels of both H_2O_2 and caspase-3 in red seabream exposed to high concentrations (30 and 40 μg/L) of Cu showed a tendency to

increase with exposure time. A similar response was observed in carp, *Cyprinus carpio* exposed to the heavy metal cadmium (2.5 and 10 μM), in which caspase-3 levels were significantly increased at a concentration of 10 μM (Olvera-Néstor et al., 2016). Although the relationship between Cu toxicity and apoptosis has yet to be conclusively determined, Luzio et al. (2013) confirmed the expression and activity of apoptosis-related genes in the gills of zebrafish, *Danio rerio* after exposure to 12.5 and 100 μg/L Cu. Exposure to Cu above a certain concentration produces reactive oxygen species, which activate p53, a gene related to apoptosis, leading to the production of tumor necrosis factor (TNF), and subsequently to the secretion of caspase-3 (Monteiro et al., 2009; Luzio et al., 2013).

Cu toxicity also induces morphological and cytochemical changes, which are known to be associated with the development of apoptosis in fish (Li et al., 1998; Mazon et al., 2002). Therefore, in this study, a TUNEL assay was performed to investigate the effect of Cu exposure on the apoptosis of gill cells. I observed that apoptosis was induced to the greatest extent in the gill cells of fish exposed to a Cu concentration of 30 μg/L for 120 h compared to the Cont. group. In a similar study, Luzio et al. (2013) exposed zebrafish to two Cu concentrations (12.5 and 100 μg/L) and then performed the TUNEL assay on gill tissues. They accordingly observed apoptosis in both experimental groups exposed to Cu, and that those fish exposed to the higher concentration of Cu showed a higher level of apoptosis. Therefore, in the present study, I suspect that high concentrations of Cu induced apoptosis in the gill tissue of red seabream.

In conclusion, when red seabream were exposed to Cu concentrations of at least 30 μg/L, stress was induced in the fish, and in order to cope with this stress, CRH, ACTH, cortisol activity, and glucose levels related to the HPI axis hormones were significantly increased. A high concentration of Cu (30 μg/L) not only reduced NKA activity but also increased H_2O_2 levels and induced caspase-3 activity, and was additionally considered to have caused cell death. The results of this study indicate that there may be a threshold concentration of Cu (30 μg/L) above which it becomes toxic to red seabream. However, to verify this assumption

it is necessary to study the toxicity associated with specific Cu concentrations in fish of different size and species.

Chapter 2.

Effects of waterborne copper on oxidative stress and immune responses in red seabream, *Pagrus major*

1. Introduction

Owing to recent industrial developments, a variety of oils, minerals, chemicals, and heavy metals have been introduced into the marine environment, and organisms living in these marine environments are easily exposed to these pollutants (Na, 2004). In particular, heavy metals accumulate in tissues of aquatic fish and can cause physiological, functional, morphological, and other disorders, or even be lethal (Lee, 1994). Among the heavy metals that pollute coasts and harm aquatic organisms, copper (Cu) is a trace element necessary for maintaining physiological functions related to cell function and metabolism control, and it has positive effects such as anti-microbial and anti-oxidant activities (Linder and Hazegh-Azam, 1996).

Recently, the anti-bacterial ability of Cu has been used in a newly developed application for inhibiting periphyton on aquaculture nets (Ji et al., 2012). However, it has been reported that a high concentration of Cu ions (Cu^{2+}) can be toxic to aquatic organisms, and can cause various physiological and behavioral changes such as growth inhibition and ion control disorder (Tellis et al., 2012; Luzio et al., 2013).

Changes in the water environment, such as contamination by heavy metals, including Cu, increase the reactive oxygen species (ROS) that cause oxidative stress in the fish body (Monteiro et al., 2009). There are a few kinds of ROS: superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) (Roch, 1999).

Exposure to a toxic stress environment leads to excessive production of ROS in the fish body, and induces physiological disorders such as reduced disease

resistance and decreased reproductive capacity, through denaturation and loss of function of nucleic acid and protein structures in the cell. In addition, it accelerates lipid peroxidation (LPO) damage in cell membranes and adversely affects cell survival (Oldham and Bowen, 1998; Pandey et al., 2003).

Thus, organisms must protect themselves against oxidative stress caused by ROS produced in the body, and so operate anti-oxidant systems to maintain body homeostasis (Bagnyukova et al., 2007). Superoxide dismutase (SOD) and catalase (CAT) are typical anti-oxidant enzymes involved in the anti-oxidant defense system that acts to reduce the ROS by responding quickly to the oxidative stress induced in the body (Mcfarland et al., 1999). This anti-oxidant defense system is located in the livers and kidneys of marine organisms (Basha and Rani, 2003; Hansen et al., 2006) and has the following anti-oxidant functions: as phase 1 enzymes, SOD and CAT directly scavenge ROS. SOD removes O_2^- through the process of dismutation to O_2 and H_2O_2, and then the H_2O_2 produced by SOD is sequentially reduced to H_2O and O_2 by CAT. CAT is an oxidoreductase that breaks two molecules of H_2O_2 down into two molecules of H_2O and one of O_2, thereby counteracting the toxicity of H_2O_2 (Kashiwagi et al., 1997). Recently, it was reported that exposure to high concentrations of Cu resulted in an increase of ROS, with a negative effect on the immune system as well as the anti-oxidant defense system in the body (Wang, 2008).

In general, the fish immune system plays a role in protecting against disease, and lysozyme and immunoglobulin, which are non-specific immune factors, are used as important indicators of immunity (Ingram, 1980; Ellis, 2001). Lysozyme is known to eliminate pathogens during bacterial invasion by damaging bacterial cell walls through phagocytosis (Saurabh and Sahoo, 2008; Shin et al., 2014). Immunoglobulin, whose secretion is controlled by the pituitary hormone, plays a major role in the humoral immune system; fish immunoglobulin has been categorized as immunoglobulin M (IgM) (Balm, 1997; Magnadóttir, 1998).

Recent studies have shown that melatonin (N-acetyl-5-methoxytryptamine) plays an important role in the immune system (Reiter et al., 1997; Gülçin et al., 2009).

Melatonin is a hormone released from the pituitary gland, and it is known to play an important role in biorhythm regulation in most vertebrate animals, including fish, as well as in the control of feeding, stress response, and maturation (Choi et al., 2016a; Falcón et al., 2007). Several recent studies have shown that melatonin is a powerful anti-oxidant, as it directly removes the ROS, increases anti-oxidant defense capacity, and has a positive effect on enhancing immunity (Tan et al., 2000; Reiter et al., 2000).

Therefore, this study was performed to determine the concentration range of Cu, which affects oxidative stress in the red seabream. I exposed red seabream to different concentrations of Cu (10, 20, 30, and 40 μg/L), and then investigated the changes in mRNA expressions and activities of anti-oxidant enzymes (SOD and CAT) and measured changes in the levels of plasma oxidative stress indicators H_2O_2 and LPO. I also analyzed lysozyme, IgM, and melatonin levels to confirm changes to immune function caused by Cu exposure. In addition, I conducted a comet assay to analyze the nuclear DNA damage in the red seabream liver cells caused by ROS arising from Cu exposure.

2. Materials and methods

2.1. Experimental fish and treatment

Red seabream, *Pagrus major* (length 18.5 ± 1.5 cm; mass 127.2 ± 0.8 g; n = 150) were supplied by the Korea Institute of Ocean Science & Technology (Tong-yeong, Korea). The fish were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimate for 1 week in thirty 300-L circulation filter tanks (rearing a tank per experimental group to minimize density stress, n = 5) in the laboratory. The water temperature was maintained at 20°C. No fish died during the Cu treatment. We supplied commercial pellet diet at twice a day (at 9:00 am and 4:00 pm) until the day prior to sampling, but not supplied during the experiment. During the experimental period, the salinity and photoperiod were maintained at 35 psu and 12-hour (h) light (L): 12-h dark (D) period, respectively. The fish in the control group were exposed to seawater with 0 μg/L dissolved Cu. The fish in the experimental groups were treated with waterborne Cu (II) sulphate pentahydrate (Cu, $CuSO_4 \cdot 5H_2O$, 7758-99-8; Sigma-Aldrich, St. Louis, MO, USA) at one of four concentrations (10, 20, 30, and 40 μg/L).

2.2. Sampling

Five fish from each group [control (Cont.; non-Cu treated), 10, 20, 30, and 40 μg/L Cu)] were randomly selected for tissue and blood collection and anesthetized with 200 μg/L 2-Phenoxyethanol (Daejung Chemicals & Metals Co., Ltd, Siheung, Gyeonggi, Korea) at 0, 6, 12, 24, 72, and 120 h. Liver samples were removed from the fish, immediately frozen in liquid nitrogen, and stored at -80°C until the total RNA was extracted for analysis. A blood sample was collected from the caudal vein using a 3-mL syringe coated with heparin. Plasma samples were separated from blood samples by centrifugation (4°C, 1,000 × g, for 10 min) and stored at -80°C until analysis.

2.3. Total RNA extraction, cDNA synthesis

Total RNA was extracted from the tissues using TRI Reagent (Molecular Research Center Inc., USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed in a total reaction volume of 20 μL by using an oligo-$(dT)_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The resulting cDNA was stored at 4°C until needed for quantitative real-time PCR (qPCR).

2.4. Quantitative real-time PCR (qPCR)

qPCR was conducted to determine the relative expressions of anti-oxidant enzymes SOD (GenBank accession no. AF329278) and CAT (AY734528) mRNA using total RNA extracted from the livers of red seabream. Primers for qPCR were designed with reference to the known sequences of red seabream, which are shown in Table 1. qPCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQTM SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR program was as follows: 95°C for 5 min, followed by 50 cycles of 95°C for 20 s and 55°C for 20 s. Amplification of a single product from PCR was confirmed by melt curve analysis, and representative samples were electrophoresed to verify that only a single product was present. As internal controls, experiments were duplicated with β-actin, and all data are expressed relative to the corresponding β-actin threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated as $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

2.5. Western blot analysis

The total protein isolated from the livers of red seabream was extracted using T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. A total of 30 μg protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used as a reference. Samples were electrophoresed at

180 V, and the gels were immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Subsequently, the membranes were blocked with 5% milk in Tris-buffered saline (TBS; pH 7.4) for 45 min, and then washed in TBS. The membranes were incubated with lysozyme (1:2000 dilution, NBP1-47443, Novus Biologicals, Littleton, CO, USA) and IgM (1:2000 dilution, sc-58332, Santa Cruz Biotechnology, Dallas, TX, USA) and melatonin (dilution 1:5000; C-57070; LSBio, Seattle, WA, USA) anti-bodies, followed by incubation with horseradish peroxidase conjugated anti-mouse IgG secondary anti-body (1:4000 dilution; Bio-Rad) for 60 min. β-tubulin (1:5000 dilution; ab6046; Abcam, UK) was used as the internal control. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) and 30 s of exposure with a Molecular Imager® from ChemiDoc™ XRS+ Systems (Bio-Rad). The membrane images were scanned using a high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0; Bio-Rad).

2.6. SOD and CAT activities analysis

Plasma samples were used for the analysis of SOD and CAT activities. SOD and CAT activities were determined using ELISA kits (SOD, CSB-E15929Fh; CAT, CSB-E15928Fh; Cusabio Biotech Co., Ltd., Wuhan, China). Each assay was performed in duplicate, and the enzyme units were recorded as pg/mL for both SOD and CAT.

2.7. Plasma H_2O_2 and LPO levels

H_2O_2 level (nmole peroxide/mL) was measured using the modified methods of Nouroozzadeh et al. (1994) and a PeroxiDetect kit (PD1-1KT, Sigma-Aldrich, USA). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve.

LPO level (nmole/mL) was quantified by measuring plasma malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of lipid

peroxidation of polyunsaturated fatty acids, according to the manufacturer's instructions (Lipid peroxide ELISA kit; MBS013426, MyBioSource Inc., San Diego, CA, USA). The absorbance was read at 450 nm using a plate reader.

2.8. Plasma lysozyme, IgM, and melatonin levels

Plasma levels of lysozyme, IgM, and melatonin were analyzed using an immunoassay ELISA kit (lysozyme, CBS-E17296Fh; IgM, CSB-E12045Fh, Cusabio Biotech Co., Ltd., Wuhan, China; melatonin, MBS013211, Mybiosourece Inc., San Diego, CA, USA). The absorbance was read at 450 nm using a plate reader.

2.9. Comet assay

The comet assay (single-cell gel electrophoresis) is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Red seabream liver cells (1×10^5 cells/mL) were examined using a CometAssay® Reagent kit for single cell gel electrophoresis assay (Trevigen Inc., Gaithersburg, MD, USA). At least 100 cells from each slide were analyzed. For the comet assay quantification analysis, we analyzed the tail length (distance of DNA migration from head) and % DNA in tail (percentage of DNA in tail; tail intensity/total intensity in tail) using Comet Assay IV image analysis software (version 4.3.2, Perceptive Instruments Ltd., Bury Saint Edmunds, UK).

2.10. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare differences in the data ($P < 0.05$). Values are expressed as mean ± standard error (SE). Tukey's post hoc test was used to assess statistically significant differences for the different temperatures and treatments.

3. Results

3.1. The expressions and activities of anti-oxidant enzymes SOD and CAT in the liver and plasma

In this study, I examined the expressions of mRNA and activities of anti-oxidant enzymes SOD and CAT in red seabream liver tissue and plasma exposed to different concentrations of Cu (Fig. 7 and 8). I found that the anti-oxidant enzyme mRNA expression and activities in the 30 and 40 μg/L Cu groups were significantly higher than those at low Cu concentrations (0, 10, and 20 μg/L Cu) groups. Additionally, the mRNA expressions and activities of SOD and CAT in the 30 and 40 μg/L Cu groups significantly increased with time (from 6 h to 120 h).

3.2. Plasma H_2O_2 and LPO levels

This study investigated the plasma H_2O_2 and LPO levels during exposure to water with different concentrations of dissolved Cu (Fig 9A and 9B). The levels of H_2O_2 in plasma were 10.4 ± 0.48 nmole peroxide/mL at the start of the experiment; they significantly increased after 12 h exposure to 30 and 40 μg/L Cu and continued to increase over time. They significantly peaked after 120 h exposure to 40 μg/L of Cu (18.88 nmole peroxide/mL). LPO levels were 2.3 ± 0.06 nmole/mL at the start of the experiment (Fig. 4), significantly increased after 12 h exposure to 30 and 40 μg/L Cu, and peaked at 120 h (approximately 1.65- and 1.77-fold compared to the control, 10, and 20 μg/L Cu, which were not different from each other).

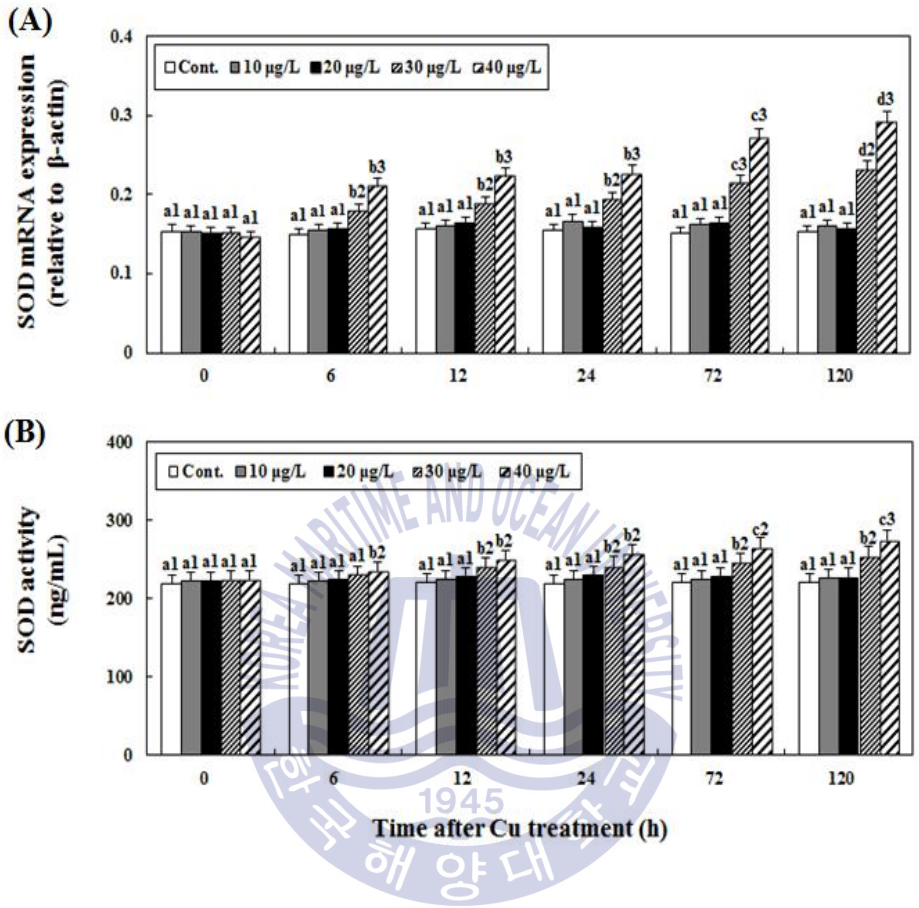

Fig. 7. Expression (A) and activity (B) of superoxide dismutase (SOD) in the liver and plasma of red seabream exposed to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L]. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure times at the same Cu concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu concentrations within an exposure period. All values are mean ± SE ($n = 5$).

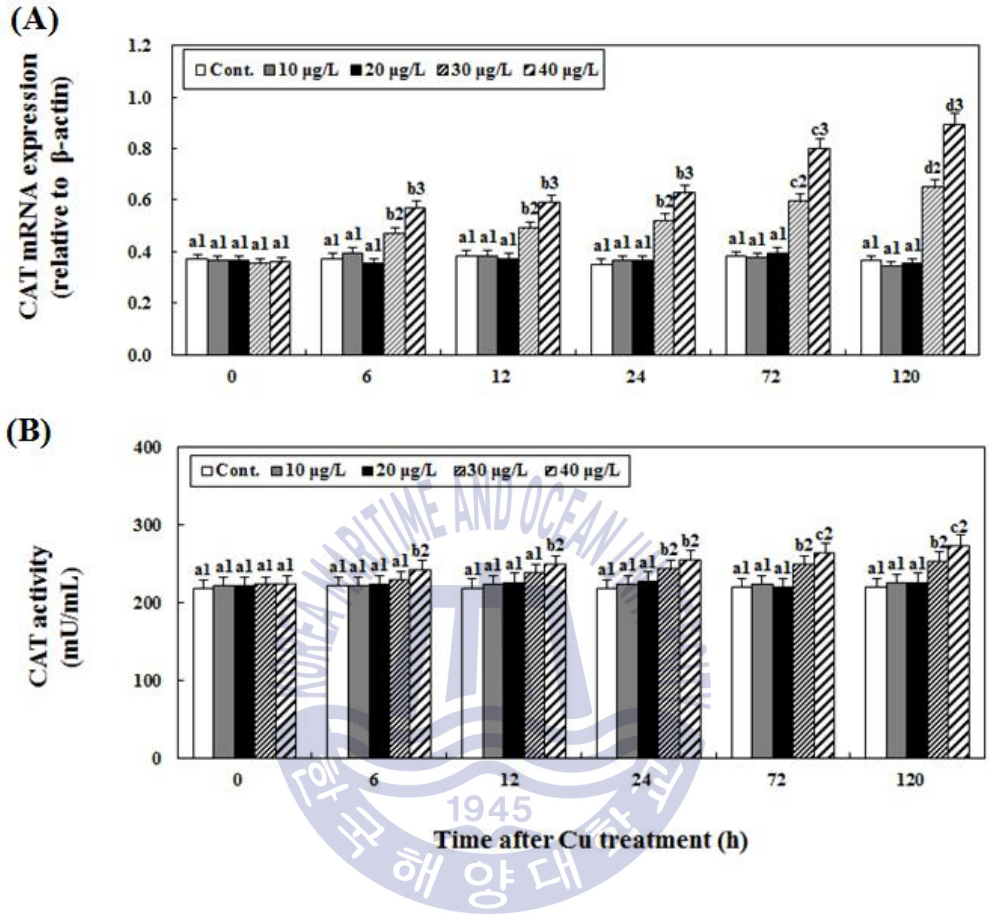

Fig. 8. Expression (A) and activity (B) of catalase (CAT) in the liver and plasma of red seabream exposed to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L]. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure times at the same Cu concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu concentrations within an exposure period. All values are mean ± SE ($n = 5$).

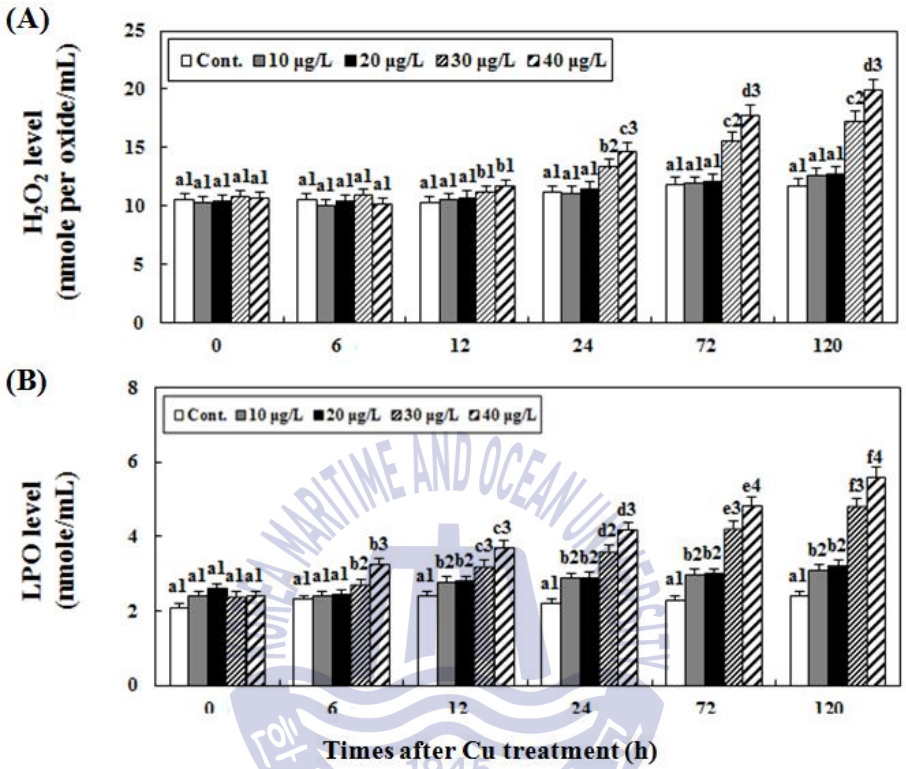

Fig. 9. Changes in plasma of H_2O_2 (A) and LPO (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure times at the same Cu concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu concentrations within an exposure period. All values are mean ± SE ($n = 5$).

3.3. Plasma lysozyme and IgM levels

The plasma lysozyme and IgM levels measured to evaluate the immune system disturbance following exposure to Cu are presented in Fig. 10. The levels of lysozyme in plasma were 21.94 ± 0.3 ng/mL at the start of the experiment; they significantly decreased after 12 h exposure to 30 and 40 μg/L Cu and continued to decrease over time. They significantly dropped after 120 h exposure to 30 and 40 μg/L Cu (10.82 and 8.14 ng/mL, respectively). IgM levels were 3.04 ± 0.1 μg/mL at the start of the experiment, significantly decreased after 12 h exposure to 30 and 40 μg/L of Cu, and dropped at 120 h (approximately 2.49 and 2.17 μg/mL, respectively).

3.4. Plasma Melatonin levels

I used an ELISA kit to examine melatonin expression in the liver following Cu exposure (Fig. 11). Western blot analysis revealed a protein with melatonin enzyme-specific immune reactivity, and a mass that corresponded to the predicted mass of red seabream melatonin (45 kDa). Initial plasma melatonin levels were 27.54 ± 0.3; pg/mL; however, they significantly decreased after exposure to 30 and 40 μg/L Cu (approximately 0.81- and 0.69- fold lower, respectively) compared to the control, 10, and 20 μg/L Cu treatment groups. The melatonin protein expression patterns resembled the expression patterns of melatonin levels in red seabream plasma. In particular, the melatonin levels after 120 h of exposure to 30 and 40 μg/L Cu were significantly decreased, reaching values of approximately 18.10 and 12.25 pg/mL, respectively.

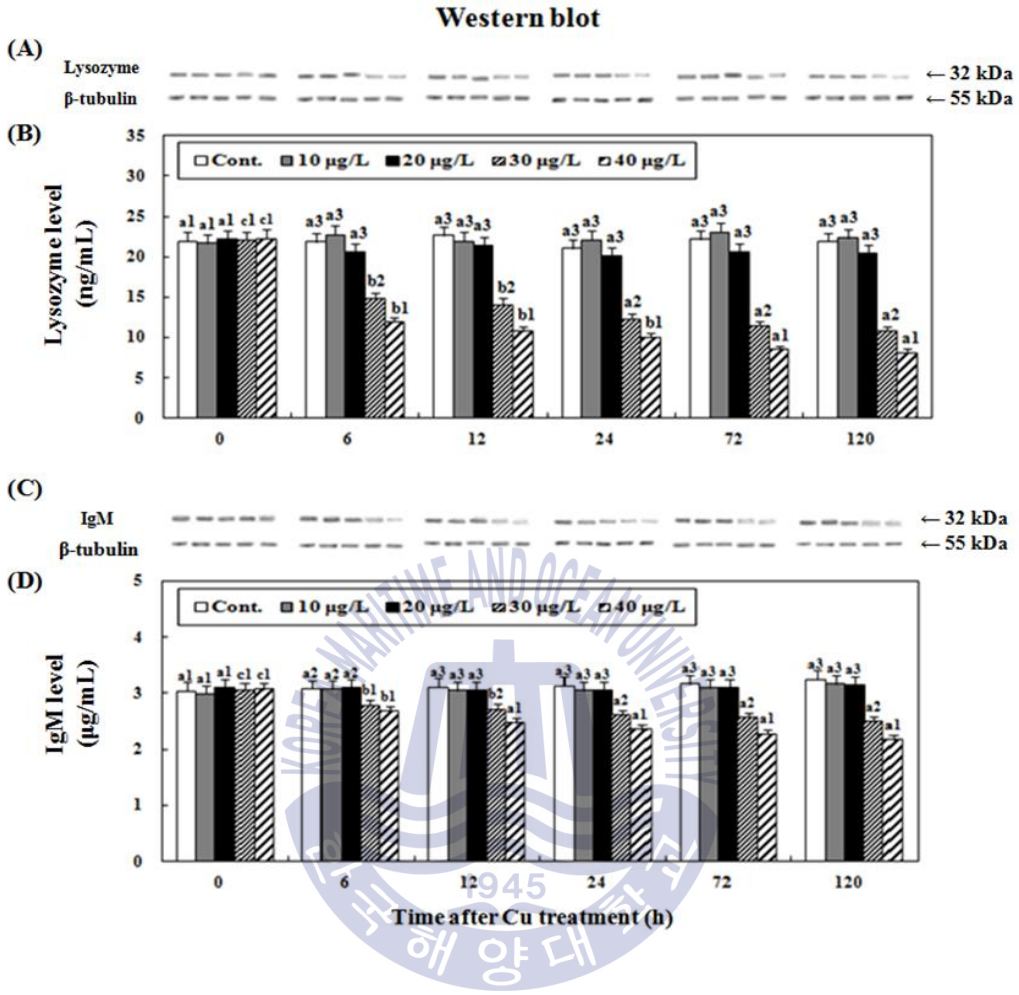

Fig. 10. Changes in plasma of lysozyme (A) and IgM (C) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. Western blots of the expression of immunity [lysozyme (B, 32 kDa), IgM (D, 32 kDa)] in the liver of rock bream, and the β-tubulin (55 kDa) was used as the internal control. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure times at the same Cu concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu concentrations within an exposure period. All values are mean ± SE ($n = 5$).

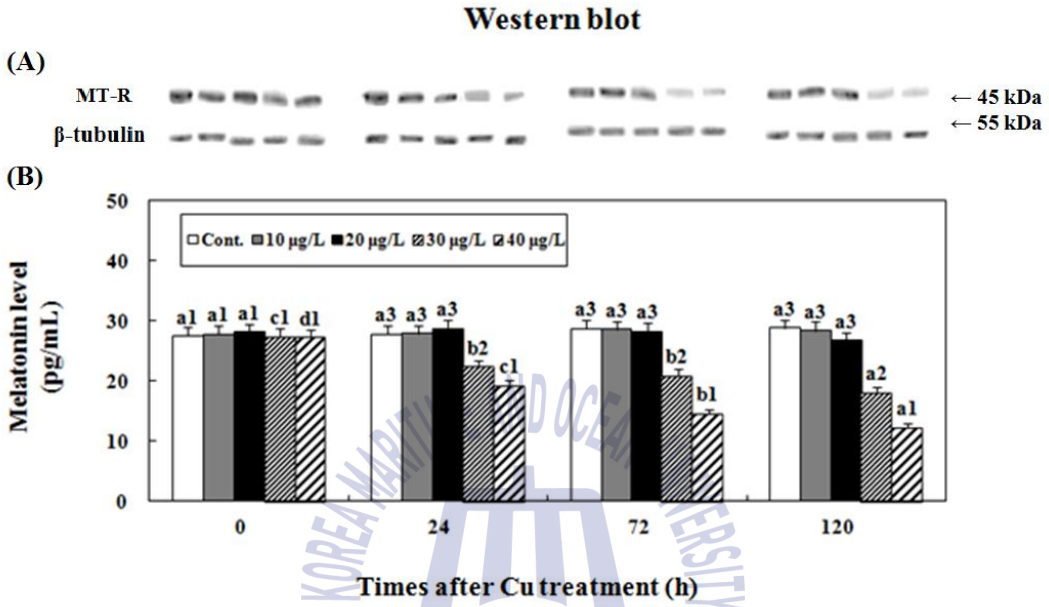

Fig. 11. Changes in plasma of melatonin level (B) during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. Western blots of the expression of melatonin (A, 45 kDa) in the liver of rock bream, and the β-tubulin (55 kDa) was used as the internal control. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure times at the same Cu concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu concentrations within an exposure period. All values are mean ± SE ($n = 5$).

3.5. Comet assay

Liver tissue DNA damage following a 120 h exposure to Cu was analyzed using at least 100 randomly selected cells from each individual. For the comet assay quantification analysis, I analyzed the tail length and % DNA in tail. The DNA content in the tail and tail length both significantly increased with higher Cu concentrations, as seen in the 30 μg/L Cu experimental groups (Fig. 6). In groups exposed to the high Cu concentrations (30 and 40 μg/L Cu), the % DNA in the tail and tail length were significantly higher compared to those exposed to Cont., 10, and 20 μg/L Cu.

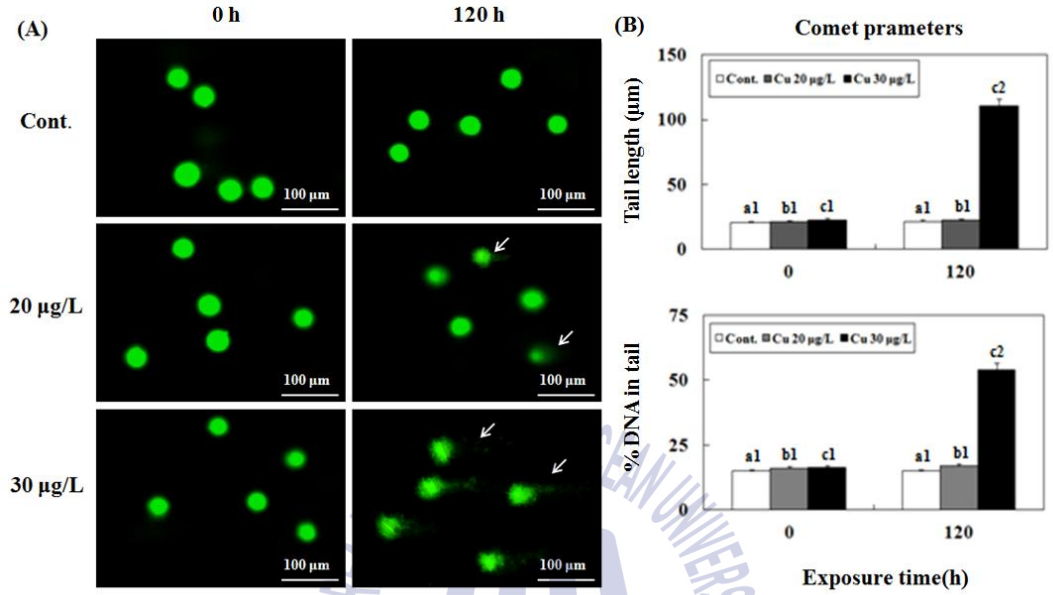

Fig. 11. Comet assay images (A) and comet assay parameters (B) tail length and percentage DNA in tail under different concentrations of Cu [0 (Cont.), 20, and 30 μg/L] for 0 and 5 day. White arrows (in A) indicate damaged nuclear DNA (DNA breaks) of liver cells which are stained with SYBR-green. Scale bars = 100 μm. The lowercase letters (in B) with different characters indicate a significant difference between different concentrations at the same time *(P* < 0.05). The numbers above the bars indicate a significant difference between different times within the same concentration *(P* < 0.05). All values are mean ± SE (*n* = 5).

4. Discussion

In order to determine the concentration of Cu that affects the anti-oxidant and immune functions of red seabream, I investigated the changes in several parameters related with oxidative stress and immunity on red seabream exposed to different Cu concentrations.

First, analysis of mRNA expressions and activities of SOD and CAT, which are anti-oxidant enzymes in red seabream, showed that mRNA expression and activity were significantly increased according to Cu concentration and exposure time in the 30 and 40 μg/L Cu exposure groups. There was no significant difference according to Cu concentration or exposure time for the Cont., 10, and 20 μg/L of Cu exposure groups. When organisms are exposed to an oxidative stress environment such as toxin exposure, SOD quickly reacts to decrease the ROS concentration through the process of transforming O_2^- into water and hydrogen peroxide (Sevcikova et al., 2011; Trivedi et al., 2012). In addition, SOD produces a hydroxyl group by binding to the metal component that binds to its own protein. Cu/Zn-SOD is the most important anti-oxidant enzyme in SOD, and plays a role in reducing ROS in all tissues in which oxygen exists (Umasuthan et al., 2012). This study corroborates the previous study (Jiang et al., 2014) which reported that when Jian carp, *Cyprinus carpio*, exposed to a high Cu concentration, 0.6 mg/L (= 600 μg/L), for 4 days, excessive ROS was produced in the brain, and the expressions of anti-oxidant enzyme (SOD, CAT, and GPX) mRNA were increased to enhance the anti-oxidant activity in the body. Compared with the Cu concentration of 600 μg/L that causes oxidative stress in Jian carp, in this study oxidative stress was induced at just 30 μg/L Cu in red seabream. As part of this defense mechanism, SOD and CAT mRNA expressions and activities were considered significantly increased. Additionally, plasma H_2O_2 and LPO are widely used as indicators of the degree of oxidative stress in the body (Halliwell and Gutteridge, 1984; Woo et al., 2006).

In this study, plasma H_2O_2 and LPO concentrations of red seabream exposed to

different Cu concentrations were measured; in groups exposed to 30 and 40 μg/L Cu, the levels of H_2O_2 and LPO significantly increased as the concentration of Cu and exposure time increased. However, in groups exposed to 10 and 20 μg/L Cu, there were no significant differences with Cu concentration and exposure time. H_2O_2 is one of the ROS and has high oxidizing power (Munter, 2001; Jin et al., 2013). Therefore, in the results of the present study, the concentration of toxic H_2O_2 in the muscle caused toxicity to exceed the anti-oxidant capacity of the body and lipid oxidation in the muscle begins (Jin et al., 2013).

Similarly, Choi et al. (2015), who measured H_2O_2 and LPO factors, reported that when goldfish were exposed to different concentrations of selenium, the levels of H_2O_2 and LPO were significantly increased at high concentrations of 3 and 4 mg/L, and suggested that selenium was toxic at these concentrations. Additionally, Pandey et al. (2001) also reported that as a result of exposing spotted snakehead, *Channa punctatus*, to 0.55 mg/L (= 550 μg/L) and 5.5 mg/L (= 5500 μg/L) concentrations of Cu, the levels of LPO were significantly increased in liver, kidney, and gills. Cu is a biologically essential nutrient, but it adversely affects organisms when it exists in high concentrations. Similar to selenium toxicity study results, this study found that high concentrations of Cu increased the levels of H_2O_2 and LPO by inducing a decrease in internal anti-oxidant function. The concentration of Cu that acts as toxic varies with fish species. This study suggests that 30 μg/L or higher concentrations of Cu have a toxic effect on red seabream.

In addition, plasma lysozyme, IgM, and melatonin levels were measured in order to investigate the change in immunity of red seabream caused by various Cu concentrations. Plasma levels of lysozyme, IgM, and melatonin were not significantly different according to Cu concentration and exposure time at 10 and 20 μg/L Cu, but in the 30 and 40 μg/L Cu experimental groups, the levels of immune-related hormones were decreased as the Cu concentration and exposure time increased. Lysozyme is one of the important bactericidal enzymes of innate immunity (Demers and Bayne, 1997). Lysozyme activity in fish blood is sensitive to environmental contaminants (Bols et al., 2001). Thus, when fish are exposed to toxic

substances, the susceptibility to the disease increases due to the decrease in innate immunity caused by toxic cellular damage to the blood in the body (Ghiasi et al., 2010). In a similar study, Lin and Shiau (2007) reported that grouper were fed an experimental diet containing Cu (20 mg/kg) or a commercial diet for 8 weeks. They found that lysozyme and IgM, which are immunity indices, were significantly decreased in the experimental (diet with Cu) group. Recent studies have also shown that in the case of rock bream, when the concentration of bisphenol A was more than 10 ng/L, ROS formation was induced and these ROS lowered plasma melatonin levels (Choi et al., 2016b). Similarly, in this study, ROS, which are induced by high concentration of Cu, seem to lower melatonin concentration. Therefore, similar to previous studies on organisms exposed to heavy metals such as Cu, ROS were induced in the body of red seabream that were exposed to high Cu concentrations and therefore ROS were considered to significantly reduce the immunity of red seabream.

Finally, I conducted a comet assay to measure the degree of nuclear DNA damage in the liver cells of red seabream exposed to high concentrations of Cu. Nuclear DNA in liver cells of red seabream exposed to Cont., 10, and 20 μg/L Cu was normal, but in liver cells of red seabream exposed to 30 and 40 μg/L Cu, tail length and % DNA in the tail were significantly increased. Additionally, Cont., 10, and 20 μg/L Cu experimental groups showed no differences according to the Cu exposure time, but the 30 and 40 μg/L Cu experimental groups confirmed that the degree of nuclear DNA damage was increased. Copper increased DNA single-strand breaks and micronucleus frequency (Bolognesi et al., 1999). Current research has suggested the relevance of free radicals and/or reactive oxygen species to induce DNA damage in copper (Regoli, 1998; Ueda et al., 1998). Therefore, I conclude that more than 30 μg/L of Cu concentration leads to damage of nuclear DNA in liver cells of red seabream by inducing oxidative stress.

In summary, our results suggest that Cu concentrations of more than 30 μg/L induce ROS in the body of red seabream and act as a factor to increase the oxidative stress. As part of a defense mechanism, mRNA expression of anti-oxidant

enzymes SOD and CAT, and activities significantly increase. Additionally, Cu concentrations higher than 30 μg/L increase lipid peroxidation (LPO) by inducing H_2O_2, but lower immunity of red seabream by decreasing lysozyme, IgM, and melatonin, and as the Cu concentration increases, the nuclear DNA damage increases.

The results of this study are expected to be used as an important guideline for establishing the environmental standard for Cu concentration that induces anti-oxidative reactions in aquatic organisms. In the future, studies on the improvement of anti-oxidative reactions and immunity of red seabream should be performed.

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