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ORIGINAL PAPER

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

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Abstract

Backgrounds: Copper (Cu) plays an essential role in many enzymatic processes including cellular respiration. In this study, we investigated the oxidative stress and immunity of red seabream, *Pagrus major*, exposed to different concentrations of Cu^{2+} (10, 20, 30, and 40 μg/L) for 120 h.
Methods: We measured the expressions of mRNA, activities of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), the levels of plasma hydrogen peroxide (H_2O_2) and lipid peroxide (LPO), and immune parameters lysozyme, immunoglobulin M (IgM), and melatonin.
Results: The mRNA expressions, activities of antioxidant enzymes, and the levels of plasma H_2O_2 and LPO were significantly higher after exposure to 30 and 40 μg/L of Cu^{2+} than after exposure to lower concentrations (0, 10, and 20 μg/L). However, the levels of plasma lysozyme, IgM, and melatonin were significantly lower after exposure to 30 and 40 μg/L of Cu^{2+}.
Conclusion: These results indicate that Cu^{2+} concentrations of 30 and 40 μg/L can induce an acute toxic oxidative stress and decrease the immune response in red seabream in aquarium conditions.

Keywords: Antioxidant activity, Copper, Immune response, Oxidative stress, Red seabream

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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[1]. In particular, heavy metals accumulate in tissues of aquatic fish and can cause physiological, functional, morphological, and other disorders, or even be lethal[2]. 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 antimicrobial and antioxidant activities[3,4].

Recently, the antibacterial ability of Cu has been used in a newly developed application for inhibiting periphyton on aquaculture nets[5]. 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[6,7].

Changes in the water environment, such as contamination by heavy metals, including Cu^{2+}, increase the reactive oxygen species (ROS) that cause oxidative stress in the fish body[8]. There are a few kinds of ROS: superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$)[9].

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 peroxide (LPO) damage in cell membranes and adversely affects cell survival[10,11].

Thus, organisms must protect themselves against oxidative stress caused by ROS produced in the body, and so operate antioxidant systems to maintain body homeostasis[12]. Superoxide dismutase (SOD) and catalase (CAT) are typical antioxidant enzymes involved in the antioxidant defense system that acts to reduce the ROS by responding quickly to the oxidative stress induced in the body[13]. This antioxidant defense system is located in the livers and kidneys of marine organisms[14,15] and has the following antioxidant 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[16]. Recently, it was reported that exposure to high concentrations of Cu^{2+} resulted in an increase of ROS, with a negative effect on the immune system as well as the antioxidant defense system in the body[17].

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[18,19]. Lysozyme is known to eliminate pathogens during bacterial invasion by damaging bacterial cell walls through phagocytosis[20,21]. 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)[22,23].

Recent studies have shown that melatonin (N-acetyl-5-methoxytryptamine) plays an important role in the immune system[24,25]. 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[26,27]. Several recent studies have shown that melatonin is a powerful antioxidant, as it directly removes the ROS, increases antioxidant defense capacity, and has a positive effect on enhancing immunity[28,29].

Therefore, this study was performed to determine the concentration range of Cu^{2+} that affects oxidative stress in the red seabream. We exposed red seabream to different concentrations of Cu^{2+} (10, 20, 30, and 40 μg/L), and then investigated the changes in mRNA expressions and activities of antioxidant enzymes (SOD and CAT) and measured changes in the levels of plasma oxidative stress indicators H_2O_2 and LPO. We also analyzed lysozyme, IgM, and melatonin levels to confirm changes to immune function caused by Cu^{2+} exposure. In addition, we conducted a comet assay to analyze the nuclear DNA damage in the red seabream liver cells caused by ROS arising from Cu^{2+} exposure.

Materials & Methods

Experimental fish and treatment

Red seabream (length 18.5 ± 1.5 cm; mass 127.2 ± 0.8 g; $n = 150$) were supplied by the Hongjin Fishery & Aquaculture Corporation (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 (five fish per tank to minimize density stress) in the laboratory. The water temperature was maintained at 20°C. No fish died during the Cu^{2+} treatment. We supplied a commercial pellet diet twice a day (at 9:00 am and 4:00 pm) until the day prior to the experiment. No food was provided during the experiment. During the experimental period, the salinity and photoperiod were maintained at 35 psu and 12 h light : 12 h dark period, respectively. To select Cu^{2+} concentrations for this study, we conducted a preliminary toxicity study using exposure to a wide range of concentrations (2.5, 5, 10, 20, 30, 40, 50, 100, 500, or 1000 μg/L) of copper, which were chosen partially on the basis of various acute copper toxicity-related papers[30-32] and partially arbitrarily. There, were found that 30 μg/L Cu^{2+} was toxic to red seabream (data not shown), so we used concentrations just above and below that point for this study. The fish in the control group were exposed to seawater with 0 μg/L dissolved Cu^{2+}. The fish in the experimental groups were treated with waterborne Cu (II) sulfate pentahydrate (Cu^{2+}, $CuSO_4 \cdot 5H_2O$, 7758-99-8; Sigma-Aldrich, St. Louis, MO, USA) at one of four concentrations (10, 20, 30, or 40 μg/L).

Sampling

Five fish from each group (control, 10, 20, 30, and 40 μg/L Cu^{2+}) 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.

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).

Quantitative real-time PCR (qPCR)

qPCR was conducted to determine the relative expressions of antioxidant 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 iQ™ 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 $\Delta\Delta Ct = 2\text{^} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

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) antibodies, followed by incubation with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (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).

Table 1. Primers used for qPCR amplification

Genes (Accession no.)	Primer	DNA Sequences
SOD (AF329278)	Forward	5′-ATG GTG CAG AAA GCA GTG TG-3′
	Reverse	5′-GGC AGA TGC CAA TGA CTC CA-3′
CAT (AY734528)	Forward	5′-CCT CCT CCA CTG TCA GAT TC-3′
	Reverse	5′-CTA CGG CTC TCA CAC CTT C-3′
β-actin (JN226150)	Forward	5′-TGT TCG AGA CCT TCA ACA CC-3′
	Reverse	5′-ATC TCC TTC TGC ATC CTG TC-3′

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.

Plasma H_2O_2 and LPO levels

H_2O_2 level (nmole peroxide/mL) was measured using the modified methods of Nourooz-Zadeh *et al*.[33] 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; MBS 013426, MyBioSource Inc., San Diego, CA, USA). The absorbance was read at 450 nm using a plate reader.

Plasma lysozyme, IgM, and melatonin levels

Plasma levels of lysozyme, IgM, and melatonin were analyzed using an immunoassay ELISA kit (lysozyme, CBS-E17296Fh, Cusabio Biotech Co., Ltd., Wuhan, China; IgM, CSB-E12045Fh, Cusabio; melatonin,

MBS013211, MyBioSource Inc.). The absorbance was read at 450 nm using a plate reader.

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).

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). Two-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.

Results

The expressions and activities of antioxidant enzymes SOD and CAT in the liver and plasma

In this study, we examined the expressions of mRNA and activities of antioxidant enzymes SOD and CAT in red seabream liver tissue and plasma in fish exposed to different concentrations of Cu^{2+} (Figures 1 and 2). We found that the antioxidant enzyme mRNA expression and activities in the 30 and 40 μg/L Cu^{2+} groups were significantly higher than those at low Cu^{2+} concentrations (0, 10, and 20 μg/L Cu^{2+}) groups. Additionally, the mRNA expressions and activities of SOD and CAT in the 30 and 40 μg/L Cu^{2+} groups significantly increased with time (from 6 h to 120 h).

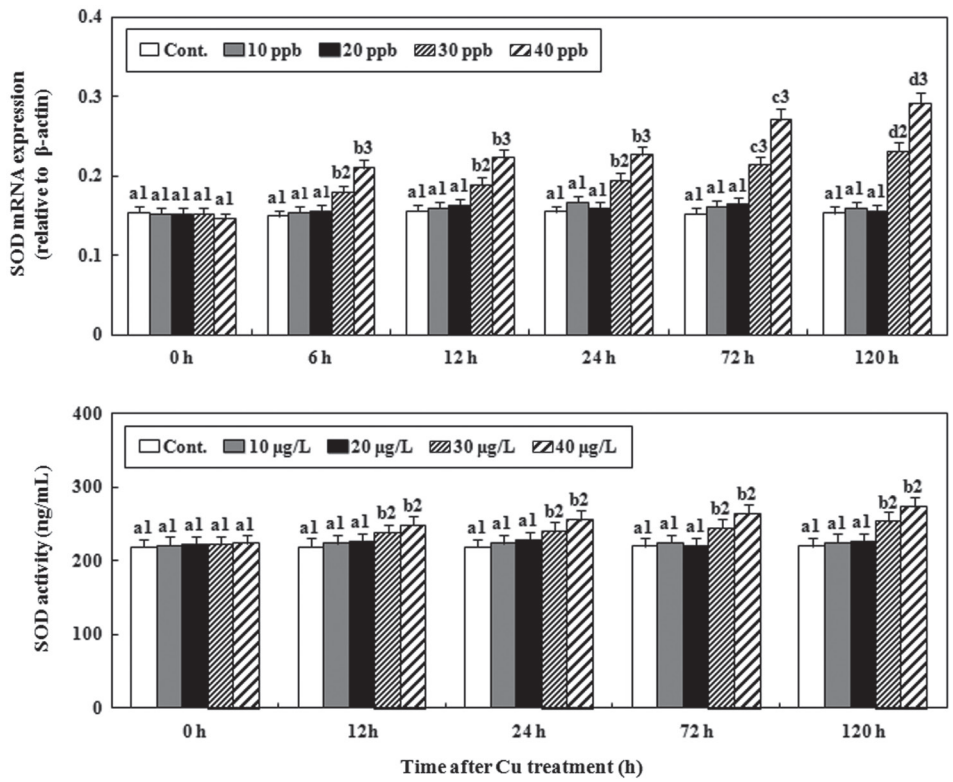

Figure 1. Expression and activity of superoxide dismutase (SOD) in the liver and plasma of red seabream exposed to Cu^{2+} [0 (Cont.), 10, 20, 30, and 40 μg/L]. The lower-case letters indicate significant ($P < 0.05$) differences among the different exposure times at the same Cu^{2+} concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu^{2+} concentrations within an exposure period. All values are mean ± SE ($n = 5$).

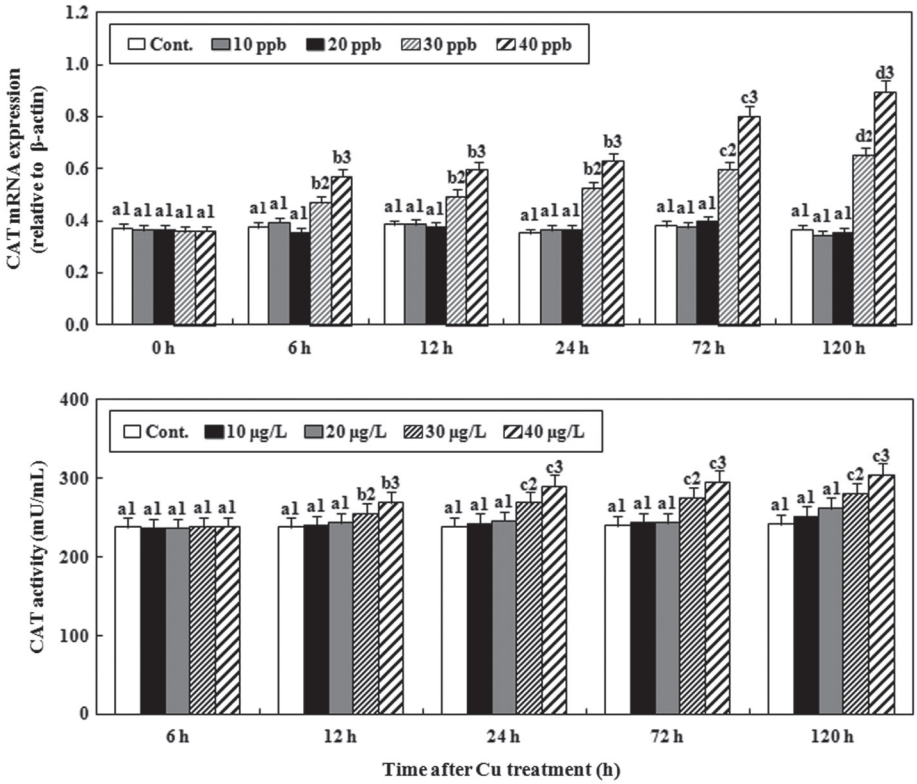

Figure 2. Expression and activity of catalase (CAT) in the liver and plasma of red seabream exposed to Cu^{2+} [0 (Cont.), 10, 20, 30, and 40 μg/L]. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure times at the same Cu^{2+} concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu^{2+} concentrations within an exposure period. All values are mean ± SE ($n=5$).

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^{2+} (Figure 3). 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^{2+} and continued to increase over time. They significantly peaked after 120 h exposure to 40 μg/L of Cu^{2+} (18.88 nmole peroxide/mL). LPO levels were 2.3 ± 0.06 nmole/mL at the start of the experiment (Figure 4), significantly increased after 12 h exposure to 30 and 40 μg/L Cu^{2+}, and peaked at 120 h (approximately 1.65- and 1.77-fold compared to the control, 10, and 20 μg/L Cu^{2+}, which were not different from each other).

Plasma lysozyme and IgM levels

The plasma lysozyme and IgM levels measured to evaluate the immune system disturbance following exposure to Cu^{2+} are presented in Figure 4. 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^{2+} and continued to decrease over time. They significantly dropped after 120 h exposure to 30 and 40 μg/L Cu^{2+} (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^{2+}, and dropped at 120 h (approximately 2.49 and 2.17 μg/mL, respectively).

Melatonin levels

Western blot analysis of melatonin expression in the liver following Cu^{2+} exposure revealed a protein with melatonin enzyme-specific immune reactivity, and a mass that corresponded to the predicted mass of red seabream melatonin (45 kDa) (Figure 5). Initial plasma melatonin levels were 27.54 ± 0.3; pg/mL; however, they significantly decreased after exposure to 30 and 40 μg/L Cu^{2+} (approximately 0.81- and 0.69- fold lower, respectively) compared to the control, 10, and 20 μg/L Cu^{2+} treatment groups. The melatonin protein expression patterns resembled the expression patterns of melatonin levels in red seabream plasma. In particular,

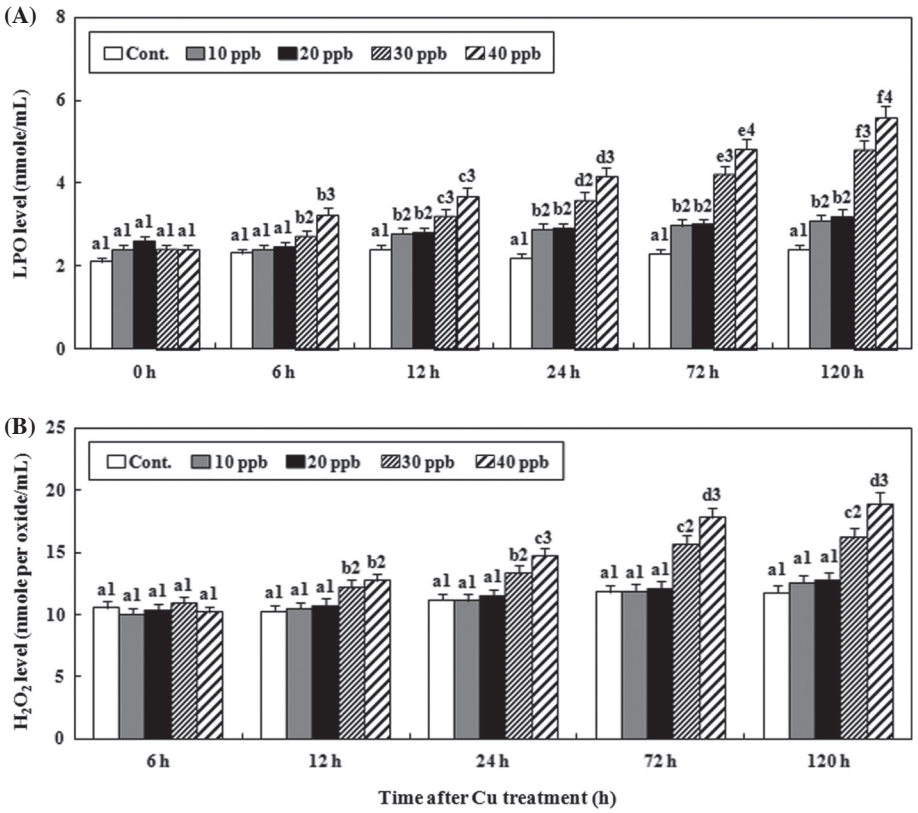

Figure 3. Changes in plasma H_2O_2 (A) and LPO (B) levels during exposure to Cu^{2+} [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure times at the same Cu^{2+} concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu^{2+} concentrations within an exposure period. All values are mean ± SE ($n=5$).

the melatonin levels after 120 h of exposure to 30 and 40 μg/L Cu^{2+} were significantly decreased, reaching values of approximately 18.10 and 12.25 pg/mL, respectively.

Comet assay

Comet analysis showed that the DNA content in the tail and tail length both significantly increased with higher Cu^{2+} concentrations, as seen in the 30 μg/L Cu^{2+} experimental groups (Figure 6). In groups exposed to the high Cu^{2+} concentrations (30 and 40 μg/L Cu^{2+}), the % DNA in the tail and tail length were significantly higher compared to those exposed to 0, 10, and 20 μg/L Cu^{2+}.

Discussion

To determine the concentration of Cu^{2+} that affects the antioxidant and immune functions of red seabream, we investigated the changes in several parameters related to oxidative stress and immunity in red seabream exposed to different Cu concentrations.

First, analysis of mRNA expressions and activities of SOD and CAT, which are antioxidant enzymes in red seabream, showed that mRNA expression and activity were significantly increased according to Cu^{2+} concentration and exposure time in the 30 and 40 μg/L Cu^{2+} exposure groups. There was no significant difference according to Cu^{2+} concentration or exposure time for the control, 10, and 20 μg/L of Cu^{2+} 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[34,35]. 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 antioxidant enzyme in SOD, and plays a role in reducing ROS in all tissues in which oxygen exists[36]. This study corroborates the previous study[37] that reported that when Jian carp, *Cyprinus carpio*, were exposed to a high Cu^{2+} concentration, 0.6 mg/L (= 600 μg/L), for 4 days, excessive ROS was produced in the brain, and

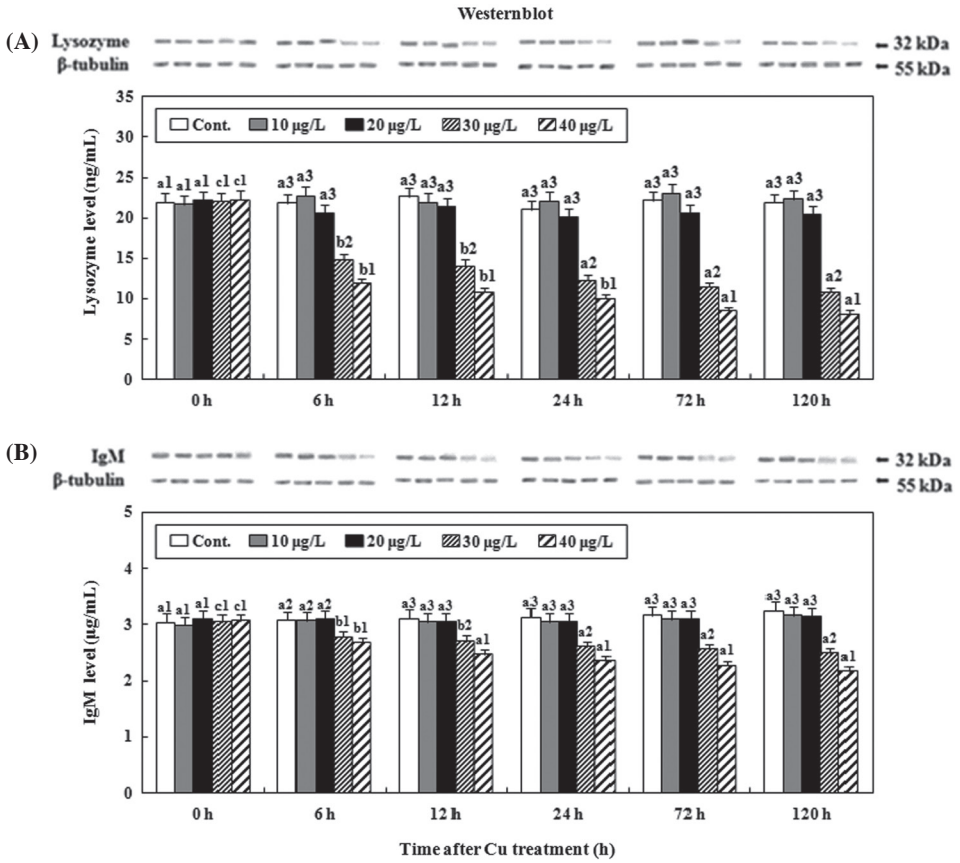

Figure 4. Changes in plasma lysozyme (A) and IgM (B) levels during exposure to Cu^{2+} [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. Western blots of the expression of immunity [lysozyme (32 kDa), IgM (32 kDa)] in the liver of rock bream, and β-tubulin (55 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^{2+} concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu^{2+} concentrations within an exposure period. All values are mean ± SE ($n=5$).

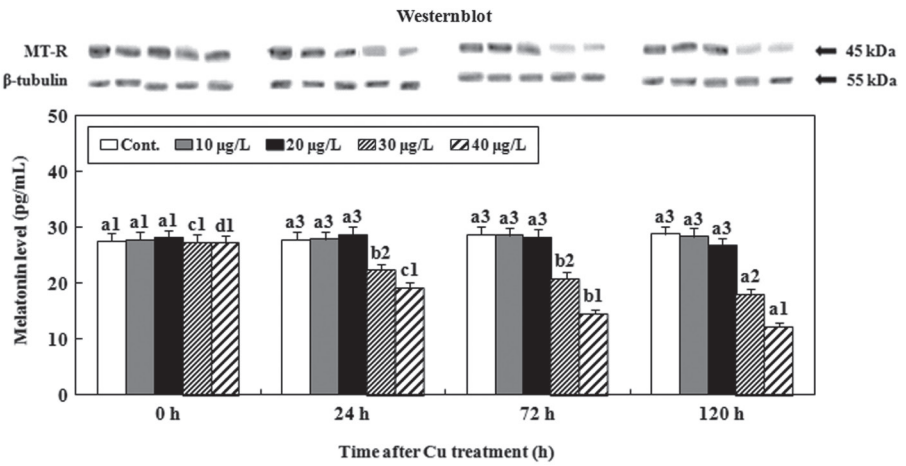

Figure 5. Change in plasma of melatonin level during exposure to Cu^{2+} [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. Western blots of the expression of melatonin (45 kDa) in the liver of rock bream, and the β-tubulin (55 kDa) was used as the internal control. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure times at the same Cu^{2+} concentrations. The numbers above the bars indicate significant differences among the fish exposed to different Cu^{2+} concentrations within an exposure period. All values are mean ± SE ($n=5$).

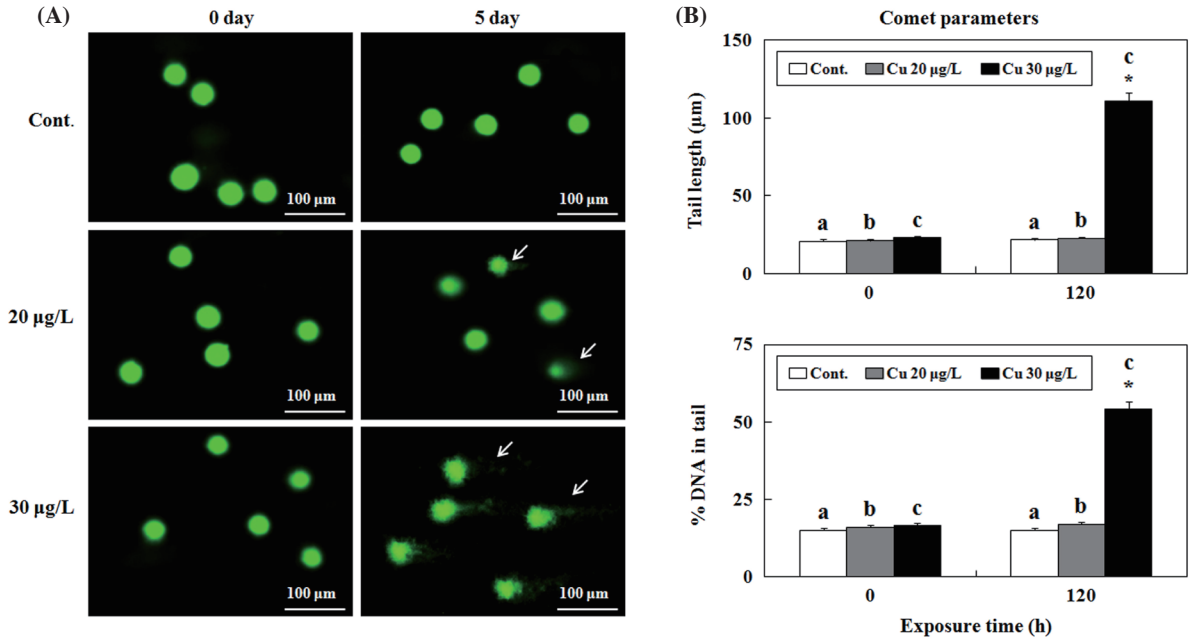

Figure 6. Comet assay images (A) and comet assay parameters (B) tail length and percentage DNA in tail under different concentrations of Cu^{2+} [0 (Cont.), 20, and 30 μg/L] for 0 and 120 hours. White arrows (in A) indicate damaged nuclear DNA (DNA breaks) of liver cells which are stained with SYBR-green. 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 ($n=5$).

the expressions of antioxidant enzyme (SOD, CAT, and GPX) mRNA were increased to enhance the antioxidant activity in the body. Compared with the Cu^{2+} 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^{2+} 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[38,39]. In this study, plasma H_2O_2 and LPO concentrations of red seabream exposed to different Cu^{2+} concentrations were measured; in groups exposed to 30 and 40 μg/L Cu^{2+}, the levels of H_2O_2 and LPO significantly increased as the concentration of Cu^{2+} and exposure time increased. However, in groups exposed to 10 and 20 μg/L Cu^{2+}, there were no significant differences with Cu^{2+} concentration and exposure time. H_2O_2 is a ROS and has high oxidizing power[40,41]. In the present study, the concentration of toxic H_2O_2 in the muscle exceeded the antioxidant capacity of the body, and lipid oxidation in the muscle began[42].

Similarly, Choi *et al.*[43], 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.*[44] 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^{2+}, 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^{2+} increased the levels of H_2O_2 and LPO by inducing a decrease in internal antioxidant function. The concentration of Cu^{2+} that acts as toxic varies with fish species. This study suggests that 30 μg/L or higher concentrations of Cu^{2+} 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^{2+} concentrations. Plasma levels of lysozyme, IgM, and melatonin were not significantly different according to Cu^{2+} concentration and exposure time at 10 and 20 μg/L Cu^{2+}, but in the 30 and 40 μg/L Cu^{2+} experimental groups, the levels of immune-related hormones were decreased as the Cu^{2+} concentration and exposure time increased. Lysozyme is an important bactericidal

enzyme involved in innate immunity[45]. Lysozyme activity in fish blood is sensitive to environmental contaminants[46]. Thus, when fish are exposed to toxic substances, susceptibility to disease increases due to the decrease in innate immunity caused by toxic cellular damage in the blood[47]. In a similar study, Lin *et al*.[48] reported that groupers 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 (BPA) was more than 10 ng/L, ROS formation was induced and these ROS lowered plasma melatonin levels[49]. Similarly, in this study, ROS, which are induced by high concentrations of Cu^{2+}, 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^{2+} concentrations and therefore ROS were considered to significantly reduce the immunity of red seabream.

Finally, we 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^{2+}. Nuclear DNA in liver cells of red seabream exposed to control (0), 10, and 20 μg/L Cu^{2+} was normal, but in liver cells of red seabream exposed to 30 and 40 μg/L Cu^{2+}, tail length and percentage of DNA in the tail were significantly increased. Additionally, control, 10, and 20 μg/L Cu^{2+} experimental groups showed no differences according to the Cu^{2+} exposure time, but the 30 and 40 μg/L Cu^{2+} experimental groups confirmed that the degree of nuclear DNA damage was increased. Copper increased DNA single-strand breaks and micronucleus frequency[50]. Current research has suggested the relevance of free radicals, reactive oxygen species, or both, in inducing DNA damage after copper exposure[51,52]. Therefore, we conclude that more than 30 μg/L of Cu^{2+} concentration leads to damage of nuclear DNA in liver cells of red seabream by inducing oxidative stress.

Conclusion

In summary, our results suggest that Cu^{2+} 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 antioxidant enzymes SOD and CAT, and their activities, significantly increase. Additionally, Cu^{2+} 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^{2+} 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^{2+} concentration that induces antioxidative reactions in aquatic organisms. In the future, studies on the improvement of antioxidative reactions and immunity of red seabream should be performed.

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Conflict of Interest Cheol Young Choi, Jong Ryeol Choe, Yoon Sub Shin, Tae Hwan Kim, Ji Yong Choi & Bong-Seok Kim declares that they have no conflict of interest.

Human and animal rights All housing and handling of animals and the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of National Institute of Fisheries Science (IACUC approval No. 563). The procedures were carried out in accordance with the Animal Care and Use Guidelines of National Institute of Fisheries Science.

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