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ORIGINAL PAPER

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

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Abstract

Backgrounds: There has been an increasing trend of copper (Cu)-based aquaculture industry in the world, and it is necessary to evaluate the effect of copper ion exposure on water pollution. This study was aim to determine the critical concentration of toxic copper in adult red sea bream.
Methods: Therefore, we investigated the effects of Cu toxicity on physiological stress and apoptosis exposed to various concentrations (10, 20, 30, and 40 μg/L). We measured physiological stress-related (corticotrophin-releasing hormone, adrenocorticotropic hormone, cortisol, and glucose), other toxic stress-related (metallothionein and Na^+/K^+-ATPase), and apoptosis-related (caspase-3 and hydrogen peroxide) parameters. In addition, we confirmed apoptosis.
Results: Physiological parameters were significantly increased from Cu concentration of 30 μg/L or more. However, no significant differences were observed after exposures at 10 and 20 μg/L. In addition, apoptotic cells were detected after exposure to 30 μg/L.
Conclusion: The results of this study indicate that high concentrations induce stress and apoptosis compared to normal conditions.

Keywords: Apoptosis, Copper, HPI axis, Physiological stress, Toxicity stress

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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[1]. 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 100-5000 μg/L have been reported to have antimicrobial and antioxidant functions against bacteria[1-3]. The concentration of copper in the Korean coastal area is 1-2 μg/L, which is below the Korean water quality standard[4]. However, recently there has been an increasing trend of copper-based aquaculture industry (such as copper alloy nets for fish cages) in the world, and it is necessary to evaluate the effect of copper ion exposure on water pollution and aquaculture[5]. And, 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[3,6]. To date, however, there have been no studies on the concentration range at which Cu acts as a toxic agent in marine fish.

When fish are exposed to environmental stresses such as toxicity, salinity, and adverse temperatures, they deploy various stress defense mechanism to maintain homeostasis[7,8]. A representative defense mechanism is the activity of the hypothalamus-pituitary-interrenal axis (HPI axis)[9], 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)[10]. 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[10,11]. Cortisol plays a role in directly increasing the plasma concentration of glucose, which is used as a metabolic energy source in damaged cells[12,13].

Metallothionein (MT) and Na^+/K^+-ATPase (NKA) are typically used as biomarkers for measuring the response of fish to heavy metal toxicity[14,15]. 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)[16,17]. It is known to be a substance that, in response to heavy metal exposure, binds to metals and chelates them[18,19]. According to Wu *et al*.[20], 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[21]. 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[22,23].

The apoptosis response is regulated by caspase, which a cysteine proteinase, and is characterized by morphological features such as DNA fragmentation and cell contraction[24]. 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[25,26]. Accordingly, the activity of capase-3 can be measured to determine the progression of apoptosis in tissues[27].

In fish, the amount of reactive oxygen species (ROS), including superoxide, hydrogen peroxide (H_2O_2), hydroxyl radical, and singlet oxygen[28,29], 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[30]. In particular, H_2O_2 has been reported to accelerate apoptosis by affecting cell viability by causing membrane damage and enzyme inactivation[27,29].

It is impossible to exclude the possibility that exposing copper may affect the physiological function of adult fish. Therefore, this study aim to determine the critical concentration of toxic copper in red sea bream, a major species of East Asian cultured species. To accomplish the purpose, the present study was conducted to investigate the effect of toxicity resulting from Cu exposure on physiological stress and cell death in the red sea bream, and to determine the concentration range over which Cu is toxic. To this end, we 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, we 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.

Materials & Methods

Experimental fish

The red seabream, *Pagrus major* (n = 150; length 18.5 ± 1.5 cm; mass 27.2 ± 0.8 g) used in the present study were obtained from the Hongjin Corparation (Tong-yeong, Korea), and were allowed to acclimate for 1 week in eight 300-L circulation filter tanks in the laboratory. And, we maintained the rearing water (pH 8, 33‰, and 20°C) used in all experimental groups during experiment. 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 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).

Cu treatment and sampling

The fish were divided into one control group (Cont.; non-Cu treated) and four experimental groups, which were maintained in separated eight 300L-circulation 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. In order to maintain the concentration of copper in each circulation tanks, it was measured and monitored with colorimeter (DR900, HACH, Cololado, USA). 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.

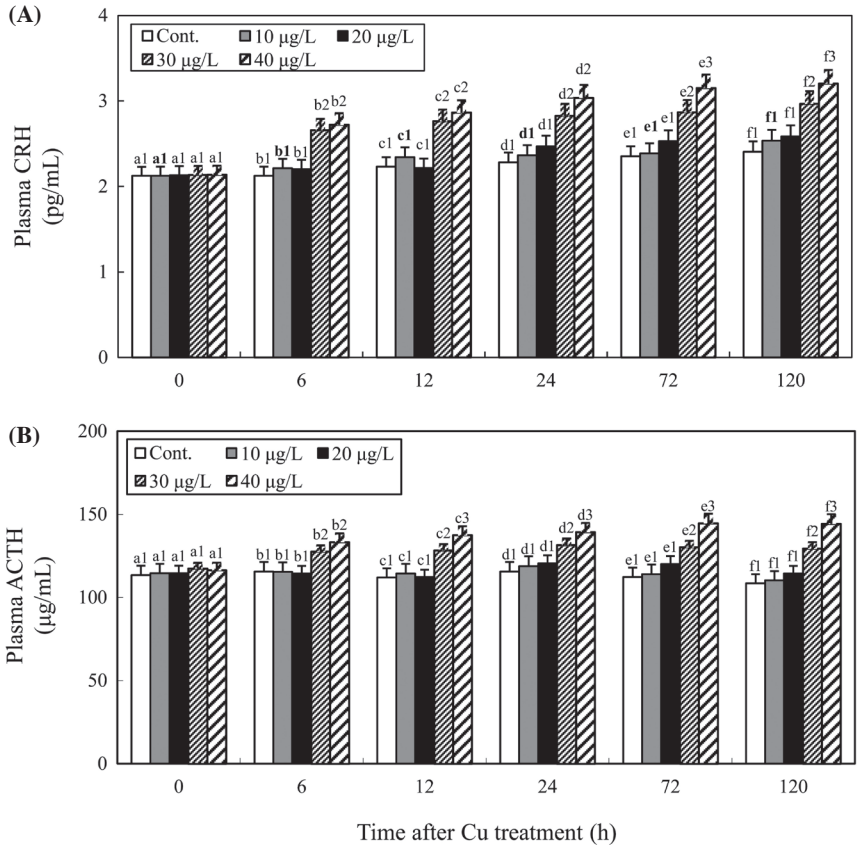

Figure 1. Changes in plasma corticotrophin-releasing hormone (CRH) (A) and corticotrophin-adrenocorticotropic hormone (ACTH) (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. Lower-case letters indicate significant ($P<0.05$) 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 ($n=5$).

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, MBS 012786: Mybiosource Inc., San Diego, CA, USA). An anti-antibody that was specific to the antibody of hormones was pre-coated onto a microplate. Fifty microliters of plasma were added per well, followed by 100 μL of HRP-conjugate, and then 50 μL antibody. Plate contents were mixed well and then incubate for 1 hour at 37°C. After the final wash, any remaining wash buffer was removed by aspirating or decanting. Fifty microliters of Substrate A and Substrate B were then added to each well, followed by incubation for 15 minutes at 37°C in the dark. Following incubation, 50 μL of stop solution was to each well. The optical density of each well was determined within 10 minutes, using a microplate reader set to 450 nm.

H_2O_2 concentrations (nmole peroxide/mL) were measured using the modified methods of Nouroozzadeh *et al*.[31] and a PeroxiDetect kit (Sigma-Aldrich, USA). Absorbance was read at 560 nm, and the concentration of H_2O_2 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).

TUNEL assay

To evaluate the affect of Cu toxicity on fish, we 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

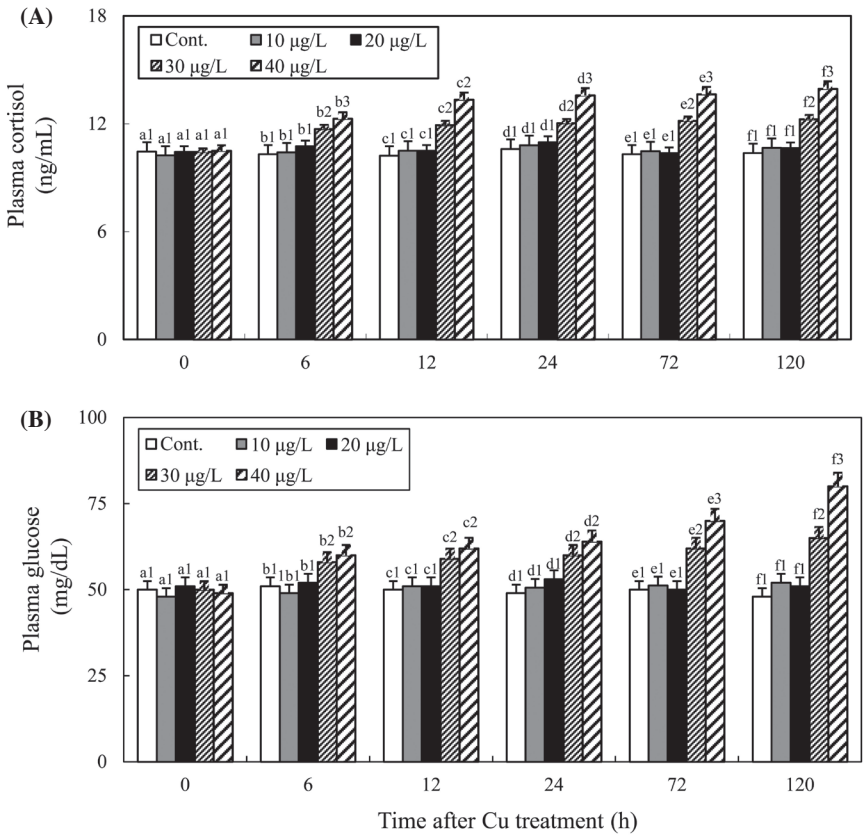

Figure 2. Changes in plasma cortisol (A) and glucose (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure periods at the same Cu concentrations.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

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.

Statistical analysis

All data were analysed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A 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 means ± standard error (SE).

Results

Changes in plasma levels of CRH and ACTH

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 Figure 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).

Changes in plasma concentrations of cortisol and glucose

The changes in cortisol and glucose concentrations in

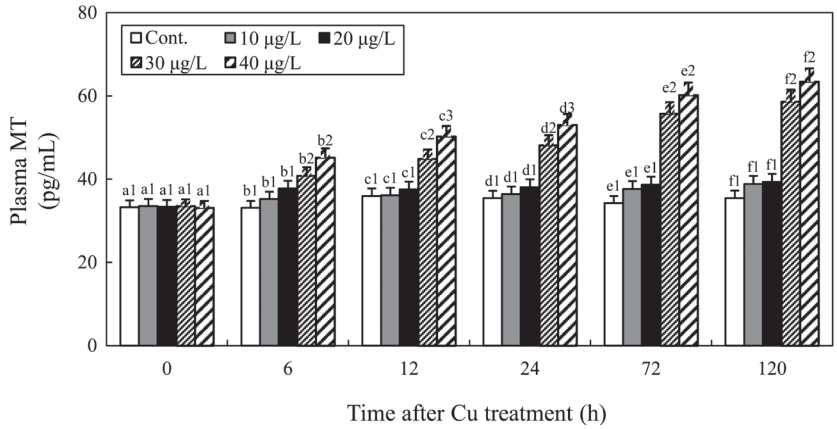

Figure 3. Changes in plasma metallothionein (MT) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure periods at the same Cu concentrations.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

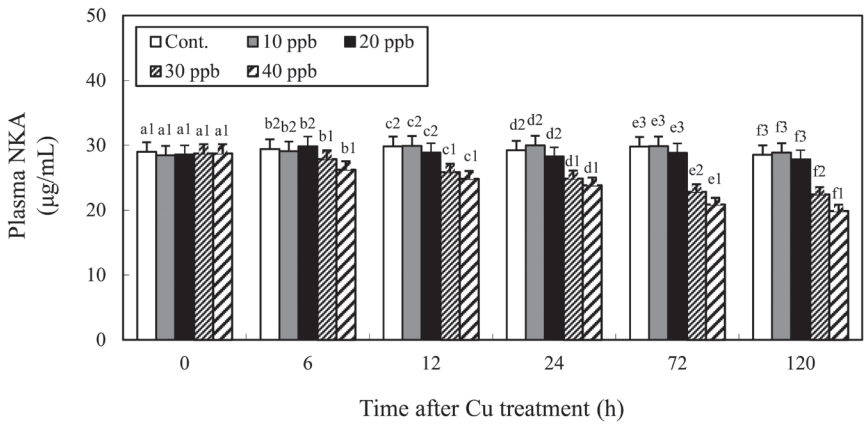

Figure 4. Changes in plasma Na^+/K^+-ATPase (NKA) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure periods at the same Cu concentrations.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

red seabream exposed to different concentrations of Cu (10, 20, 30, and 40 μg/L) are shown in Figure 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).

Changes in plasma concentrations 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 Figure 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).

Changes in plasma 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 Figure 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

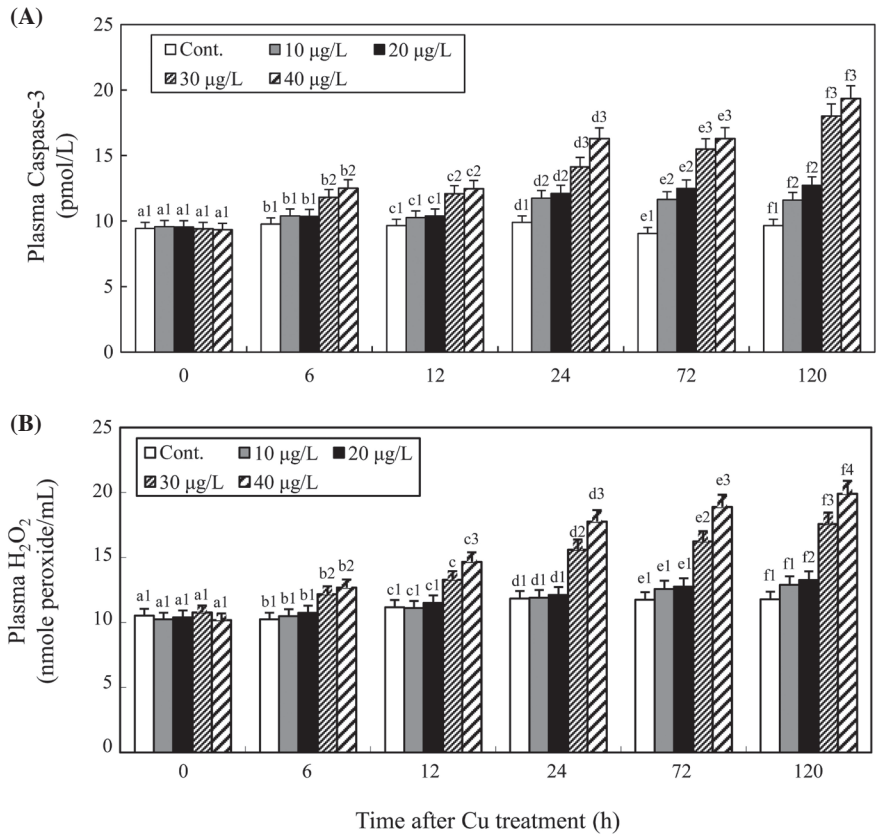

Figure 5. Changes in plasma caspase-3 (A) and H_2O_2 (B) levels during exposure to Cu [0 (Cont.), 10, 20, 30, and 40 μg/L] in red seabream, as measured using a microplate reader. The lower-case letters indicate significant ($P<0.05$) differences among the different exposure periods at the same Cu concentrations.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

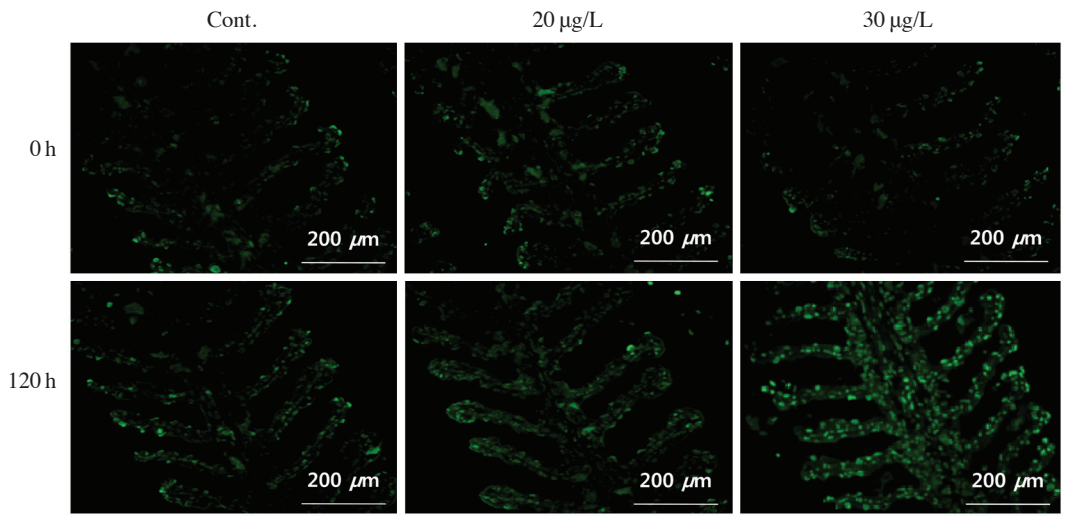

Figure 6. TUNEL detection of red seabream gill cell apoptosis under different concentrations of Cu [0 (Cont.), 20 and 30 μg/L] for 0 and 120 h. Cells were stained with acridine orange and visualized with a fluorescent microscope. Cells showing green fluorescence are apoptotic cells. Scale bars = 200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

(22.43 ± 1.6 and 19.86 ± 1.84 pg/mL, respectively) compared to the other groups.

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 Figure 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).

TUNEL assay

The results of the TUNEL assay used to investigate the occurrence of cell apoptosis are shown in Figure 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.

Discussion

In the present study, we 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. We examined the physiological responses of fish through molecular biology analysis.

We initially measured the changes in plasma CRH and ACTH concentrations, which are indicators of physiological stress due to Cu exposure. We 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, Donaldson *et al*.[32] reported that HPI axis activates by acting as a toxic substance when exposed to 10-7 M copper at Sockeye salmon, *Oncorhynchus nerka* to increase the concentration of corticosteroid, the final product of the HPI axis. And research on selenium, which is a trace element in the body like copper, Choi *et al*.[18] 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 sea bream. 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.

We 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 *et al*.[33], 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 sea bream 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. Thus, they were released in the order of CRH, ACTH, cortisol and glucose through the HPI axis by copper toxicity exposure. And, it is considered that the inducing stress finally is reduced.

The effect of toxic stress caused by Cu exposure on red sea bream 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[14]. 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. Also, it seems that the concentration of MT in the plasma gradually increases to maintain homeostasis in the fish body.

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. We 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*.[15] 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*.[14] reported that when *P. mesopotamicus* were exposed to 400 μg/L, the NKA concentration was significantly decreased compared to the control 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, we also analysed the effects of exposure to various concentrations of Cu on the change of plasma caspase-3 and H_2O_2 in red seabream. We found that the plasma levels of both caspase-3 and H_2O_2 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[34]. Although the relationship between Cu toxicity and apoptosis has yet to be conclusively determined, Luzio *et al*.[3] 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[3,27]. Therefore, the high-concentration environment of copper increased the caspase-3 concentration by acting as a stress in the body, and this caspase-3 induces apoptosis. In addition, it is considered that the longer the exposure period, the more the cell death increases and the adverse effect is exerted in the fish body.

de Souza Machado *et al*.[35] reported that when the guppy was exposed to 5, 9, 20 μg/L, the concentration of H_2O_2 in body increase, therefore the LPO level of the liver, gills and muscles increased. And, in higher the concentration, more higher LPO level was observed. And research on selenium, which is a trace element in the body like copper, Choi *et al*.[18] reported that when goldfish were exposed to selenium at various concentrations (2, 3, and 4 mg/L), H_2O_2 concentrations were significantly increased at 3 and 4 mg/L. If an organism is exposed to an oxidative stress environment, such as toxic exposure, superoxide dismutase (SOD), known as an enzyme that reduces oxidative stress, initially catalyzes the breakdown of ROS into water and hydrogen peroxide[36,37]. SOD generates a hydroxyl group through bonding with a metal component that binds with its own protein. Among these SODs, Cu/Zn-SOD is the most important antioxidant enzyme, which plays a role in reducing the free radicals produced by oxygen in all tissues[38]. Concentrations of Cu in excess of those required for normal functioning promote higher antioxidant activity in the body. It is considered that H_2O_2, which is a product of Cu/Zn-SOD activity, is increased. Similar to the findings of previous studies, exposure to Cu concentrations of 30 and 40 μg/L induced acute toxicity in red sea bream. The toxic stress response causes apoptosis by inducing H_2O_2, which is an oxygen free radical, and caspase-3.

Cu toxicity also induces morphological and cytochemical changes, which are known to be associated with the development of apoptosis in fish[39,40]. Therefore, in this study, a TUNEL assay was performed to investigate the effect of Cu exposure on the apoptosis of gill cells. We 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*.[3] 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, we suspect that high concentrations of Cu induced apoptosis in the gill tissue of red seabream.

Conclusion

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 acute 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.

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Conflict of Interest Tae Hwan Kim, Ji Yong Choi,

Min-Min Jung, Sung-Yong Oh & Cheol Young Choi 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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