Cortisol signaling and stress-induced gene expressions in response to copper toxicity through heat stress during zebrafish embryogenesis

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Abstract

Climate change is leading to an increase in temperatures, which has a stressful impact on the aquatic environment. Cortisol signaling is involved in enhancing metabolic processes such as anti-oxidation, immune defense, and osmoregulation, under stress conditions in fish. The present study aimed at evaluating the effects of copper (Cu) toxicity along with an increase in temperature during zebrafish embryogenesis, based on the transcriptional responses of cortisol and stress-related genes. A decreased survival rate was observed following combined exposure to high temperature and Cu. Heart rates of zebrafish embryos significantly increased only during heat stress. An abnormal morphology was induced by exposure to a combination of Cu and heat stress. Furthermore, heat stress also triggered Cu-induced intracellular reactive oxygen species production with upregulation of superoxide dismutase (SOD) and glutathione s-transferase (GST) and cell death with modified expressions of p53 and B-cell lymphoma-2 (Bcl-2) in the zebrafish embryo. Finally, increased cortisol level and altered expressions of cortisol-signaling genes were observed following exposure to Cu and high temperature. These results highlight that the realistic exposure to combined stressors disturbs cortisol-related defense pathways as well as the stress-induced processes of anti-oxidation and cell death in fish.

Introduction

Water temperatures are expected to increase worldwide due to global climate change. The aquatic environments are undergoing unprecedented changes, including rising temperatures, alterations of chemical properties, and changing susceptibility of organisms\textsuperscript{1,2}. Cortisol, the key corticosteroid in fish, plays a pivotal role in stress response in physiological processes including growth, reproduction, metabolic pathway, immune defense, and osmoregulation\textsuperscript{3–5}. The stress response involves broad changes, from a molecular to an individual level, set to reduce potential toxic effects of the stressor and maintain homeostasis\textsuperscript{6}. Plasma cortisol levels are elevated during thermal stress induced by increasing temperature in juvenile Senegalese sole (\textit{Solea senegalensis}) and Atlantic cod (\textit{Gadus morhua L.})\textsuperscript{7,8}. Stressful conditions caused by elevated temperature induce cortisol binding to glucocorticoid receptor (GR) and serious alterations of the GR-complex in fish\textsuperscript{9,10}.

Copper (Cu), an essential trace metal, serves as a cofactor in metalloproteins induced in crucial cellular processes in aerobic metabolism. However, elevated Cu levels induce toxic effects in fish through the production of the reactive oxygen species (ROS) in redox-cycling\textsuperscript{11}. Cu exposure triggers alteration of the cortisol level in common carp, \textit{Cyprinus carpio} and in rainbow trout, \textit{Oncorhynchus mykiss}\textsuperscript{12,13}. The change in cortisol level may be a defense response to reduce the chronic stress of Cu exposure, and not due to endocrine disruption\textsuperscript{13}. Stressor combinations that reflect a realistic environment generally evoke complex responses in fish, but the underlying mechanisms remain largely unknown. Heat- and Cu-induced stress significantly increased expressions of DNA methyltransferases and heat-shock protein 70 (\textit{hsp70}) in zebrafish. However, the temperature-dependent elevation of Cu toxicity is not related to the energy metabolic pathway, although response to Cu showed an enhancement in hepatic aerobic
metabolism related to the energy-demanding process of metal detoxification in the killifish *Poecilia vivipara*14.

In order to determine whether thermal stress induced by increasing temperature can booster Cu toxicity in an aquatic environment, we investigated the physiological phenotypes, molecular responses of cortisol signaling, and stress-induced gene expressions to various Cu concentrations and heat stress exposures during zebrafish embryogenesis. This study aimed to verify the knowledge on the enhanced sensitivity of stress responsive indicators, including cortisol signaling mechanism, using transcriptional approaches in fish exposed to heat and Cu stressors.

## Results

### Biological responses to combined stressors.

Figure 1 shows that exposure to 0.1 mg L\(^{-1}\) of Cu at 26°C, decreased the survival rate to 70% at 2 days post fertilization (dpf), this survival rate was maintained till 7 dpf. Increasing the temperature (34°C) increased Cu toxicity at concentrations over 0.03 mg L\(^{-1}\), thus affecting the survival rate of the zebrafish embryos (10% at 3 dpf, Fig. 1B). However, heart rates were not different at combined exposures to Cu and heat compared to the control, although they significantly increased on exposure to elevated temperature (Fig. 1C). Following changes in the temperature, there was no difference in the size of yolk sac edema between the control and Cu-treated groups (Fig. 1D). The incidence of malformation (screened on the basis of curved body shape) increased on combined exposure to all Cu concentrations and heat stress (Fig. 1E).

### ROS production and cell death in live zebrafish embryos.

At 26°C, a significant increase in intracellular ROS production was observed at 0.007 and 0.01 mg L\(^{-1}\) Cu exposure in the live zebrafish embryos. Increasing the temperature significantly boosted the induction of intracellular ROS production at all Cu concentrations (Fig. 2). In addition, the expression of the antioxidant genes, superoxide dismutase (*SOD*) and glutathione s-transferase (*GST*), was significantly increased in response to Cu exposure at an elevated temperature. Cu-induced cell death caused by increasing the temperature was observed in live zebrafish by measuring the fluorescence intensity using acridine orange (Fig. 3). The mRNA of apoptosis-related genes, *p53* and B-cell lymphoma-2 (*Bcl-2*), were expressed at a relatively high concentration of Cu (0.01 mg L\(^{-1}\)). Thermal stress also boosted Cu-induced effects in the antioxidant defense mechanism and apoptotic process in zebrafish.

### Cortisol level and cortisol signaling expression.

The cortisol levels in 96 hours post fertilization (hpf) larvae following exposure to 0.003, 0.007, and 0.01 mg L\(^{-1}\) Cu at either 26°C or 34°C is shown in Fig. 4A. The cortisol levels were significantly increased on combined exposure to Cu and heat stress than on exposure to only Cu. The expression profiles of the 14 cortisol- and stress signaling-related genes are depicted in Fig. 4B. Elevated temperature at 34°C induced mRNA expression of *Fdx1b*, *GST*, and *SOD*. Combination of heat and Cu exposure significantly increased the transcriptional expression of *Fdx1b*, *GR*, *Cyp2k22*, *GST*, *Crhb*, *SOD*, *Foxi3a*, and *Cyp11c1*, whereas mRNA expression of *Hsd20b2*, *Hsd11b2*, *Cyp11a1*, *Cyp11b1*, and *Bcl-2* decreased on exposure to Cu and elevated temperature. Expression of *p53*...
was upregulated at 0.007 and 0.01 mg L$^{-1}$ Cu at 34°C. The most significant upregulation of GR and SOD was observed under exposure to 0.01 mg L$^{-1}$ Cu and heat stress ($P < 0.01$).

**Discussion**

In the present study, we investigated the correlation between sensitivity to Cu toxicity enhanced by increased temperature and the consequent disturbance in the cortisol signaling process during fish embryogenesis. Dorts et al., 2016$^{15}$ reported evidence of epigenetic modification in zebrafish upon exposure to heat and Cu during reprogramming of DNA methylation. Overall, in this study, the toxic stress of Cu induced by high temperature resulted in changes in the expression of cortisol signaling genes as well as antioxidant and apoptosis-related genes during zebrafish embryo development.

Fishes are frequently exposed to multiple stressors in combination introduced by intensive human activities or from natural sources that lead to the discharge of heavy metals into the aquatic environment$^{15,16}$. Thermal stress induced by an increase in temperature greatly alters the effects of Cu pollutants$^{11,17,18}$. The interaction of Cu and thermal stress modulated Cu-induced bioenergetic disturbances, including impairment of oxidative phosphorylation, by inhibiting the electron transport chain in rainbow trout, *Oncorhynchus mykiss*$^{11,19}$. Elevated temperatures accelerated the toxic effects of Cu through metal accumulation and elevated oxidative stress-related processes in the killifish *Poecilia vivipara*$^{20}$. In our study, heat stress boosted ROS production induced by Cu toxicity, also including the increased expression of antioxidant genes such as SOD and GST. Both stressors, high temperature and Cu, triggered cell death in zebrafish via the upregulation of the apoptosis-related gene $p53$ and downregulation of the anti-apoptotic gene Bcl-2. Several studies have reported Cu-induced apoptosis in fish$^{22-24}$. In the present study, we demonstrated that high temperature boosted Cu toxicity in apoptosis during zebrafish embryogenesis. Cell death in live zebrafish is likely to have been elicited by the production of ROS, similar to results observed for tilapia (*Oreochromis mossambicus*)$^{25}$.

Cortisol signaling regulates a broad range of metabolic and physiological process$^{26}$. Enhanced release of cortisol in fish is reflected as hormonal stress responses to exposure to heavy metals$^{27}$. Stressful conditions caused by elevated temperature induce cortisol binding to GR and serious alterations of the GR-complex in fish$^{10,28}$. Plasma cortisol levels were elevated by thermal stress induced by increasing temperature in juvenile Senegalese sole (*Solea senegalensis*) and Atlantic cod (*Gadus morhua L.*)$^{8,29}$. In the present study, Cu exposure induced the elevation of cortisol levels in zebrafish; this result is in agreement with those of previous studies on fish$^{12,13}$. The induction of cortisol levels is intensified by combined exposure to high temperature and Cu stressors. Increase in cortisol levels can be correlated to the hormonal defense responses to thermal stress and copper toxicity as a sensitive indicator of environmental stress in fish. Our results showed that the expression of genes related to the cortisol regulatory process is affected by exposure to high temperature and Cu singly or in combination. The excessive cortisol levels may also be due to the inhibition of cortisol catabolism and excretion, as evidenced by the downregulated expression of *Hsd11b2* and *Hsd20b2*. *Hsd11b2* plays a crucial role in
converting cortisol into its inactive form cortisone. *Hsd20b2*, together with *Hsd11b2*, represents a short pathway in zebrafish to rapidly inactivate and excrete cortisol\(^{10}\). *Cyp11b1*, the key enzyme in cortisol biosynthesis, is also downregulated as a result of Cu toxicity caused by increased temperature. Significant differences in expression have also been observed for *Cyp11a1*, involving steroid biosynthetic processes in zebrafish exposed to Cu and heat. Cortisol signaling in response to stress is mediated by the **GR** and **mineralocorticoid receptors (MR)** genes\(^{30}\). In our study, levels of **GR** mRNA in the zebrafish, activated by cortisol, increased after exposure to Cu and heat stress. The upregulation of **GR** correlated with the increase in cortisol levels reported during the early developmental stage of the channel catfish\(^{31}\).

Furthermore, combined heat and Cu stress significantly induced the transcriptional expression of cortisol signaling-related genes, such as *Cyp11c1*, involved in cortisol metabolic processes; *Cyp2k22*, involved in oxidation-reduction processes and the cortisol response; *Crhb*, involved in hormonal regulation of cortisol secretion; *Fdx1b*, which is an essential mitochondrial redox partner in cortisol biosynthesis; and *Foxi3a*, which is a key regulator in cortisol synthesis in the adrenal grand. *Crhb* is elevated in response to environmental stressors such as high temperature in the neuroendocrine system of medaka\(^{32}\). Our results indicated that the mRNA levels of *Crhb* increased on exposure to high temperature and Cu in zebrafish, while there was no significant difference in expression in response to Cu exposure alone. The increased expression of *Crhb* mRNA observed on exposure to heat and Cu is in agreement with results of other stress responses involved in the regulation of cortisol\(^{32,33}\). In general, cortisol signaling predominantly occurs through the genomic pathway, involving interaction with **GRs** and modulating the transcriptional functions of stress-response genes\(^{34}\). Cortisol signaling also facilitates rapid responses via non-genomic pathways without gene transcription. The induction of ROS production is suggested as part of the evidence for the action of cortisol through a nongenomic **GR**-mediated pathway\(^{35}\). In the present study, combined exposure to Cu and heat significantly upregulated the mRNA levels of antioxidant **SOD** and **GST** genes, while the expression of *p53* and *Bcl-2*, which control ROS production and apoptosis in zebrafish, was downregulated.

To conclude, our study is the first to report that cortisol signaling at the transcriptional level responds to enhanced Cu toxicity caused by increased temperature. Cu is a heavy metal commonly found in the environment. The consideration of an additional factor, such as an increase in temperature, is provided to predict a scenario similar to those caused by global warming. The increase in water temperature affected susceptibility to heavy metals\(^{36,37}\). Combined Cu and heat stress may disrupt the signaling pathways involved in cortisol synthesis and catabolism as well as the modulation of intracellular cortisol levels in zebrafish embryo. Finally, the alteration of cortisol signaling might exert negative effects on survival and development in fish by disrupting immune defense systems; these can be used to forecast the effects of global warming.

**Materials And Methods**

**Zebrafish maintenance and exposure conditions.** Adult zebrafish used in this study were obtained from the Seoul aquarium (Seoul, Korea) and reared at 26°C with a 14:10 h light-dark cycle in a 3-L acrylic tank.
using standard methods\textsuperscript{38}. Fish were fed TetraMin Flake supplemented with brine shrimp (Artemia salina) thrice daily. Male and female zebrafish (male: female ratio, 1:2) were placed together in spawning tanks (Esen Corp, Beijing) at night. The next morning, eggs were collected within 30 min of spawning and pooled from several spawning tanks. Zebrafish embryos were exposed to CuCl\textsubscript{2} (751944, Sigma-Aldrich, St. Louis, MA, USA) at nominal concentrations of 0.003, 0.007, 0.01, 0.03, 0.07, and 0.1 mg L\textsuperscript{−1} at either 26°C or 34°C. At 3–4 hpf, each well of a 12-well plate was filled with 900 µL embryo medium\textsuperscript{37}, and 15 embryos were placed in each well. The control group was maintained in only the embryo medium (non-treatment group) at either 26°C or 34°C. All methods for this animal study were approved and carried out in accordance with relevant guidelines and regulations by the Animal Care and Use Committee of Chonnam National University (Yeosu, South Korea). Each experiment was performed in triplicate. Zebrafish tissue samples were stored at −80°C.

**Observation of biological endpoints in zebrafish.** After Cu exposure at 26°C or 34°C, survival rates were observed daily until 7 dpf using 15 embryos from each of the treated conditions (six concentrations) and control well plate. The survival rate was determined individually daily. At 35 hpf, the heart rate was observed for 1 min in each group using a Nikon COOLPIX 8700 digital camera (Melville, NY) as described by Park et al., 2020\textsuperscript{36,37}. From 2 to 3 dpf, the frequency of malformation was determined by counting zebrafish observed with curved body shape under a stereomicroscope. At 3 dpf, yolk sac edema was determined by measuring the lateral area size of zebrafish larvae anesthetized in 0.03% MS-222 using the ISCapture V3.6 program (TUCSEN Photonics Co. Ltd.)\textsuperscript{37}.

**Intracellular ROS production and cell death in live zebrafish.** In live zebrafish embryos, intracellular ROS production was observed at 3 dpf using a fluorescent dye, DCFH-DA or 2,7-dichlorodihydrofluorescein diacetate, based on previously established protocols\textsuperscript{36}. Cu-induced cell death was detected in zebrafish embryos by staining with acridine orange, a nucleic acid-selective fluorescent cationic dye that is used to detect apoptosis\textsuperscript{36,37}. Each zebrafish was analyzed using a microscope equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan). The fluorescence intensity in the images of each zebrafish larva was determined using the ImageJ software.

**Whole-body cortisol measurements.** Zebrafish larvae were sacrificed through MS-222 overdose and 10 fish from each group were pooled and immediately frozen on dry ice for cortisol extraction. To extract cortisol, each sample was rinsed and homogenized in 1 ml of 1× PBS. The homogenized samples were stored overnight at −20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged and the supernatant was removed and assayed immediately. Cortisol levels of the samples were determined using cortisol ELISA kits (CSB-E08487fh, Cusabio, Houston, TX, USA) according to the manufacturer’s instructions.

**Quantitative real-time PCR (qPCR) and data analysis.** The transcription expression of a total of 14 genes were assessed using qPCR. Total RNA was isolated using RNAisoPlus (Takara, Japan) and treated with recombinant DNase I (Takara, Japan) according to the manufacturers’ protocol. The concentration and quality of the RNA was assessed using the Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific,
Waltham, MA USA) and agarose gel electrophoresis. Complementary DNA was synthesized from 1.5 µg total RNA using the SuperScript™ III RT kit (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed on an Exicycler™96 platform (Bioneer, Korea) using AccuPower® GreenStar™ qPCR PreMix (Bioneer, Korea) for SYBR Green-based detection. The PCR conditions used were as follows: 1 cycle at 95°C for 5 min and 40 cycles at 95°C for 10 s and 57–60°C depending on the gene for 40 s. This was followed by a melting curve analysis to ensure single PCR products. The relative level of mRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method\textsuperscript{39}. The primer information used for qPCR is presented in Supplementary Table 1.

The results are presented as the mean and the standard error of the mean. The mRNA levels of 14 specific transcripts in each sample were normalized to the combined mean of GAPDH and β-actin\textsuperscript{37}. Two-way analysis of variance (ANOVA) was used to analyze significant differences between Cu-exposed groups or heat stress and control using SPSS (16.0). Results were considered significant at $P<0.05$.

**Declarations**

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**Author Contributions**

K.P. and I.S.K. designed research; K.P. performed research and extracted samples; K.P. analyzed samples; K.P. and I.S.K. analyzed data; and K.P. and I.S.K. wrote and reviewed the paper.

**Competing financial interests:** The authors declare no competing financial interests.

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**Figures**
Figure 1

Biological and developmental responses to different concentrations of Cu, and the non-treated group (control) grown under temperature changes during zebrafish embryogenesis. Survival percentage (%) of zebrafish exposed to six concentrations (0.003, 0.007, 0.01, 0.03, 0.07, and 0.1 mg L⁻¹) at 26 °C (A) and 34 °C (B) up to 7 dpf. Heart rate (C) and yolk sac edema size (%) (D) in zebrafish exposed to different concentrations of Cu at 26 °C and 34 °C. Frequency of malformation (%) in zebrafish exposed to six concentrations of Cu at 26 °C and 34 °C for 3 dpf (E). Differences between exposed and control samples were considered significant at P < 0.05.

Figure 2

Cu-induced ROS production and relative expression levels of SOD and GST transcripts in zebrafish embryos at 26 °C (A) and 34 °C (B). ROS levels were measured through image analysis and fluorescence microscopy. Embryos were exposed to 0 (control), 0.003, 0.007, and 0.01 mg L⁻¹ Cu. Experiments were performed in triplicate, and the data are presented as mean ± SE. Significant effects of Cu concentrations at a given temperature are indicated by * (P < 0.05).
Figure 3

Cu-induced cell death and relative expression levels of p53 and Bcl-2 transcripts in zebrafish embryos at 26 °C (A) and 34 °C (B). Cell death levels were measured using image analysis and fluorescence microscopy. Embryos were exposed to 0 (control), 0.003, 0.007, and 0.01 mg L\(^{-1}\) Cu. Experiments were performed in triplicate, and the data are presented as mean ± SE. Significant effects of Cu concentrations at a given temperature are indicated by * (P < 0.05).
Figure 4

Cortisol levels in the whole body of zebrafish embryos and heat map based on the relative transcriptional expressions of 14 cortisol signaling and stress-induced genes on zebrafish exposed to different concentrations of Cu (0.003, 0.007, and 0.01 mg L−1), and the non-treated group (control) grown at 26 °C and 34 °C. Experiments were performed in triplicate, and the data are presented as mean ± SE. Significant effects of Cu concentrations at a given temperature are indicated by * (P < 0.05).

Supplementary Files

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