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Exposure to Copper (II) Chloride Induces Behavioral and Endocrine Changes in Zebrafish

Jiwon Sung[1], Jeongwon Lee[2] and Seungheon Lee[1,2,3]*

[1]*Department of Marine Life Sciences, Jeju National University, Jeju 63243, Korea*
[2]*Department of Aquatic Life Medicine, Jeju National University, Jeju 63243, Korea*
[3]*Marine Research Institute, Jeju National University, Jeju 63243, Korea*

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The aim of this study was to investigate the effect of copper (II) chloride ($CuCl_2$) on zebrafish. Zebrafish were exposed to various $CuCl_2$ concentrations and subjected to different exposure times to determine the median lethal concentration (LC_{50}) values. To evaluate stress responses, we measured whole-body cortisol levels and behavioral parameters using the open field test (OFT) or the novel tank test (NTT). The zebrafish were exposed to $CuCl_2$ solution at concentrations of 1.5 - 150 μg/l or a vehicle for 1 hr before behavioral tests or sample collection for whole-body cortisol. The LC_{50} values were 30.3, 25.3, and 14.8 μg/l at 24, 48, and 96 hr, respectively. The NTT showed that mobility, velocity, and distance covered were significantly lower in zebrafish exposed to $CuCl_2$ than in the control group ($p<0.05$), while the turn angle was significantly higher in zebrafish exposed to a $CuCl_2$ concentration of 150 μg/l than in the control group ($p<0.05$). The OFT also showed that mobility, velocity, and distance covered were significantly lower and the turn angle and meandering were significantly higher in zebrafish exposed to all concentrations of $CuCl_2$ than in the control group ($p<0.05$). The whole-body cortisol levels were significantly higher in zebrafish exposed to $CuCl_2$ than in the control group ($p<0.05$). These results suggest that exposure to lethal $CuCl_2$ concentrations induces an intense toxic and stress response in zebrafish, causing behavioral changes and increasing whole-body cortisol levels.

Key words : Behavioral test, $CuCl_2$, heavy metals, Toxicity Zebrafish

Introduction

Organisms in the aquatic environment are exposed to contamination of various organisms or minerals [4]. Globally, aquatic ecosystems are continuously polluted from toxic factors by commercial or industrial activities, such as aquaculture [22], and chronic or acute exposures of anthropogenic pollutants might lead to negative consequences to aquatic organisms [3].

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***Corresponding author**
Tel : +82-64-754-3476, Fax : +82-64-756-3493
E-mail : slee76@jejunu.ac.kr

Heavy metals generally refer to metals with high molecular weight, high density and high atomic number. Some heavy metals are used as essential nutrients such as iron, cobalt, zinc and copper, and partial heavy metals such as ruthenium, silver and indium are relatively harmless. However, most heavy metals are known to be highly toxic. Interestingly, many studies demonstrated that excessive exposures to heavy metals induced some adverse actions to ecosystems as well as organisms [46].

Copper, which is an essential micronutrient, exists in the form of Cu^{+} or Cu^{2+} in vivo as a structural cofactor of a variety of catalytic and structural enzymes which have a part of energy metabolism, mitochondrial respiration, and antioxidant defense [34]. The crucial role of copper is through the direct interaction of amino acid residues and polypeptide chains with activation of structural change catalysis and protein-protein interactions [16, 29].

Copper is a metal commonly used in industry and in life, such as copper pipes. Copper pipes are strong enough to withstand thermal deformation because they have good stretch ability, are cheap, and have excellent corrosion resist-

ance, so they are frequently used to transport water, which transports water to homes and industrial sites. Copper pipes with these various advantages can also cause problems. Even if the quality of raw water or treated water meets environmental standards, secondary water contamination due to internal corrosion of water treatment pipe or network during transportation may occur [1, 23].

Nowadays, concerns are growing about copper exposure in various environments, whereas the related research is insufficient. Therefore, we performed analysis of median lethal concentration, whole-body cortisol and behavioral pharmacological to investigate toxicity to copper.

Materials and Methods

Animals

We used 5-6 month-old wild-type zebrafish purchased from World-fish aquarium (Jeju, Republic of Korea). All fish were acclimated for at least two weeks in the experimental room and maintained in constant temperature (26±1℃) tanks with aerated water. Fish were kept on a 14-10 hr light/dark cycle (lights on from 07:00-21:00 hr) and fed two times a day with TetraMin (Tetra, Germany) commercial flakes. During experiment, zebrafish were not fed. Animal treatment and maintenance were conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, 8th edition).

Measurement of Median lethal concentration (LC_{50})

The estimates of median lethal concentration of $CuCl_2$ were conducted according to the OECD Guidelines for the Testing of Chemicals Test No. 203: Fish, Acute Toxicity Test [27]. Zebrafish were randomly selected and were put into the 1-liter beaker filled with vehicle or $CuCl_2$ solutions (1 g fish/300 ml). We conducted pilot studies to determine a valid concentration or exposure time. The concentrations increased in geometric ratio of 1.4. Vehicle or $CuCl_2$ solutions were replaced with fresh ones every 24 hr. Mortality after 24, 48 and 96 hr exposure were recorded and median lethal concentrations of $CuCl_2$ (LC_{50}) were computed by probit calculations to get regression line of each time.

Novel tank test (NTT)

To assess the effect of $CuCl_2$ exposure on zebrafish behavior, NTT was performed according to the method of Robinson *et al* [25] between 11:00 and 15:00 hr. Zebrafish were randomly selected and were placed in a 300 ml vehicle or $CuCl_2$ solution for 1 hr before behavioral test. The zebrafish (n= 8-10 in each group) were put to the tank (15 cm height × 28 cm top × 23 cm bottom × 7 cm width) maximally filled with water and divided into two equal virtual horizontal portions. Zebrafish behaviours were recorded with subsequent automated analysis of generated traces by Ethovision XT 8.5 software (Noldus IT, Wageningen, Netherlands) from the side view for 6 min to calculate the distance moved, velocity, mobile duration, turn angle and duration in top.

Open Field test (OFT)

To assess the effect of $CuCl_2$ exposure on zebrafish behavior, OFT was performed according to the method of Cachat *et al* [8] between 11:00 and 15:00 hr. Zebrafish were randomly selected and were placed in a 300 ml vehicle or $CuCl_2$ solution for 1 hr before behavioral test. The zebrafish (n= 8-10 in each group) were put to the white plastic cylinder (22 cm bottom × 24 cm top × 20.5 cm height) filled with 4litter of water and divided into two portions. Zebrafish behaviours were recorded with subsequent automated analysis of generated traces by Ethovision XT 8.5 software (Noldus IT, Wageningen, Netherlands) from the side view for 6 min to calculate the distance moved, velocity, mobile duration, turn angle and meandering movement.

Zebrafish adult whole-body cortisol extraction

Whole-body cortisol was measured using the method of Barcellos et al [18]. Zebrafish were sacrificed by tricaine (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 150 mg/l to obtain the body fluid. After the skin moistness of the zebrafish was dried, it was put into a prepared cryo tube with 2 ml of 0.1 M phosphate buffered saline (PBS) for homogenization. The 5 ml of diethyl ether was put into a cryotube, then voltexing for 1 minute 3 times. After which, Samples were centrifuged (Hanil, Korea) at 4,000 g for 15 min and were rapidly cooled for 45 seconds by liquid nitrogen to move supernatant to the test tube. The test tubes containing a sample were evaporated by vacuum evaporator (CVE-2000, EYELA, Japan) to remove diethyl ether from the sample. After the evaporation of diethyl ether, 1 ml of 0.1 M PBS was added to the test tube, and the content was moved to a 1.7 ml tube. The tube was then stored at -20℃ until it was submitted for cortisol measurement.

Cortisol assay

Whole-body cortisol level was measured using a cortisol assay kit (R&D system, Minneapolis, MN, USA). To analyze the ELISA plate, the absorbance was measured at 450 nm using a microplate reader (Molecular Device, San Jose, CA USA). The absorbance value was converted to cortisol concentrations based on 4-parameter sigmoid minus. Whole-body cortisol was expressed the ratio of concentration to weight of each fish.

Statistical analysis

Values are expressed as the means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Statistical significance was set at $p<0.05$.

Results

$CuCl_2$ time-dependently increases mortality of zebrafish

Mortality after 24, 48 and 96 hr exposure were recorded and median lethal concentrations of $CuCl_2$ (LC_{50}) were computed by Biostat LE program to get regression line of each time. The LC_{50} values of $CuCl_2$ were 30.3, 25.3 or 14.8 μg/l in the 24 hr, 48 hr or 96 hr, respectively (Fig. 1).

$CuCl_2$ induces anxiety-like behaviors on novel tank test in zebrafish

As a result of measuring the distance moved for 6 min in the NTT, the distance moved values were respectively 1,282.9±105.5, 1,236.2±109.8, 840.9±98.7 or 530.8±89.3 cm at the concentrations of control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 15 and 150 μg/l, the distance moved values significantly decreased compared with control group, but not significantly decreased at the concentration of 1.5 μg/l of $CuCl_2$ compared with control group ($p<0.05$; Fig. 2A).

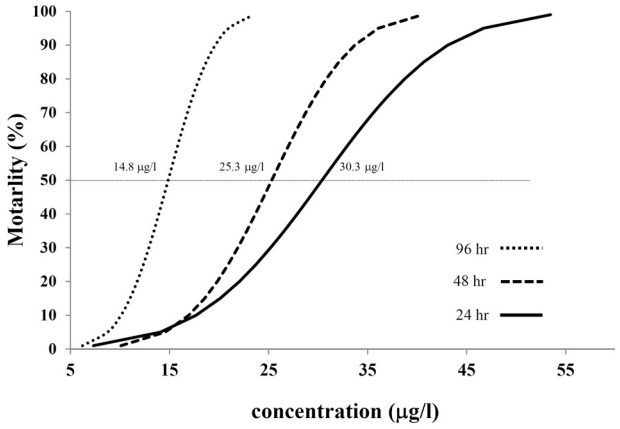

Fig. 1. Probit analysis for the median lethal concentration value of $CuCl_2$ in zebrafish. The graph shows the probit values of observed and expected percentage of mortality.

As a result of measuring the velocity for 6 min in the NTT, the velocity values were respectively 3.6±0.3, 3.4±0.3, 2.3±0.3 or 1.5±0.3 cm/s at the concentrations of control group, 1.5, 15 or 150 μg/l. In the $CuCl_2$-treated groups at the concentrations of 15 and 150 μg/l, the velocity values significantly decreased compared with control group, but not significantly increased at the concentration of 1.5 μg/l of $CuCl_2$ compared with control group ($p<0.05$; Fig. 2B).

As a result of measuring the mobile duration for 6 min in the NTT, the mobile duration values were respectively 36.6±3.0, 24.5±4.4, 8.7±2.5 or 2.8±1.1 seconds at the concentrations of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 1.5-150 μg/l, the mobile duration values significantly decreased compared with control group ($p<0.05$; Fig. 2C).

As a result of measuring the turn angle for 6 min in the NTT, the turn angle values were respectively 36.5±6.6, 36.7±5.1, 53.7±4.7 or 84.9±12.2 deg at the concentration of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentration of 150 μg/l, the turn angle values significantly increased compared with control group, but not significantly increased at the concentrations of 1.5 and 15 μg/l of $CuCl_2$ compared with control group ($p<0.05$; Fig. 2D).

As a result of measuring the duration at the top for 6 min in the NTT, the duration at the top values were respectively 38.8±20.6, 24.0±7.6, 10.9±7.0 or 65.9±33.7 seconds at the concentrations of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 1.5-150 μg/l, the duration at the top values did not significantly change compared with control group (Fig. 2E).

$CuCl_2$ induces anxiety-like behaviors on Open Field test (OFT) in zebrafish

As a result of measuring the distance moved for 6 min in the OFT, the distance moved values were respectively 1,694.1±87.6, 1,022.7±212.0, 1,028.4±98.8 or 526.4±72.7 cm at the concentrations of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 1.5-150 μg/l, the distance moved values significantly de-

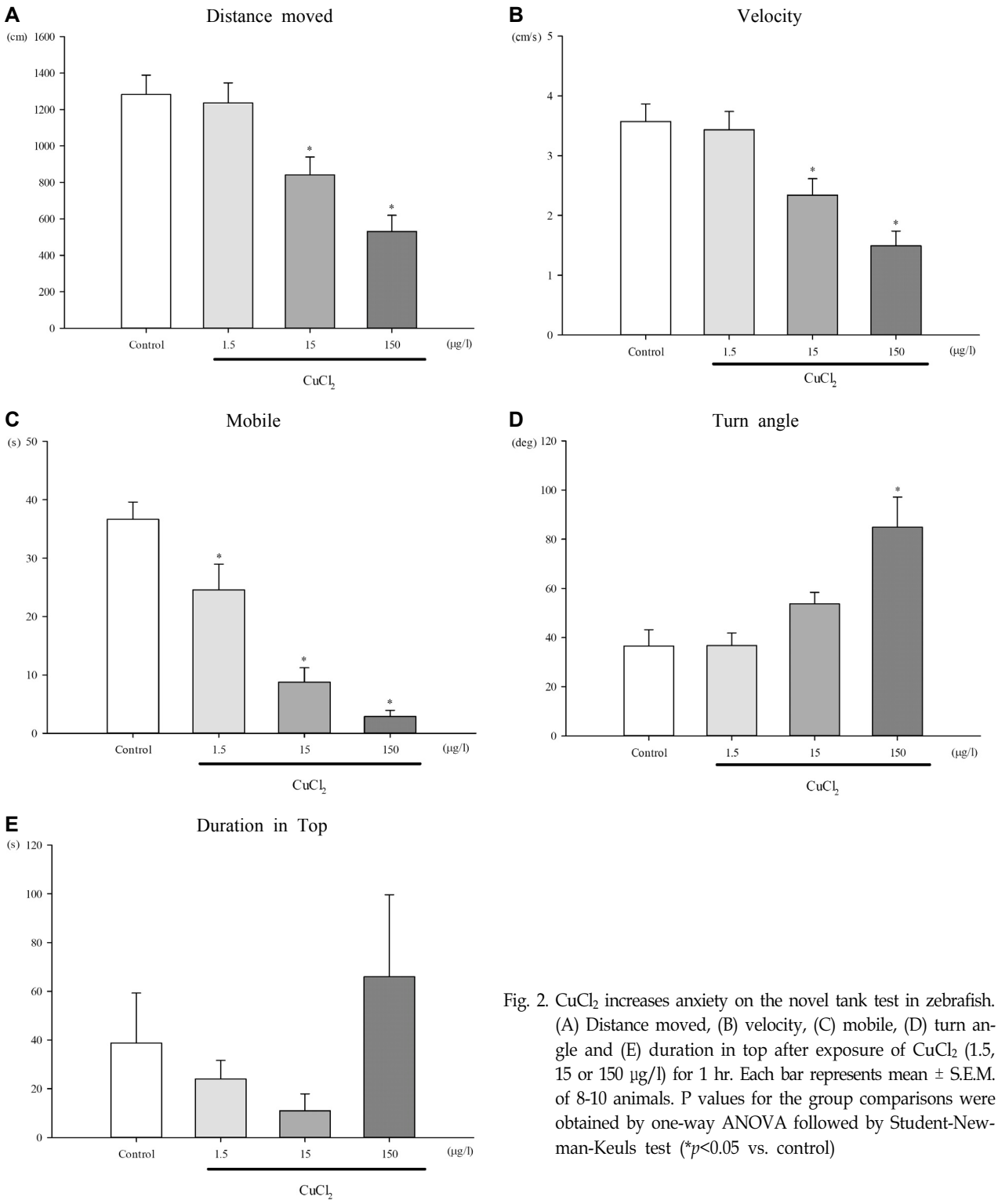

Fig. 2. $CuCl_2$ increases anxiety on the novel tank test in zebrafish. (A) Distance moved, (B) velocity, (C) mobile, (D) turn angle and (E) duration in top after exposure of $CuCl_2$ (1.5, 15 or 150 μg/l) for 1 hr. Each bar represents mean ± S.E.M. of 8-10 animals. P values for the group comparisons were obtained by one-way ANOVA followed by Student-Newman-Keuls test (*p<0.05 vs. control)

creased compared with control group (p<0.05; Fig. 3A).

As a result of measuring the velocity for 6 min in the OFT, the velocity values were respectively 4.7±0.2, 2.5±0.5, 2.9±0.3 or 1.5±0.2 cm/s at the concentrations of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 1.5~150 μg/l, the velocity values significantly decreased compared with control group (p<0.05; Fig. 3B).

As a result of measuring the mobile duration for 6 min in the OFT, the mobile duration values were respectively

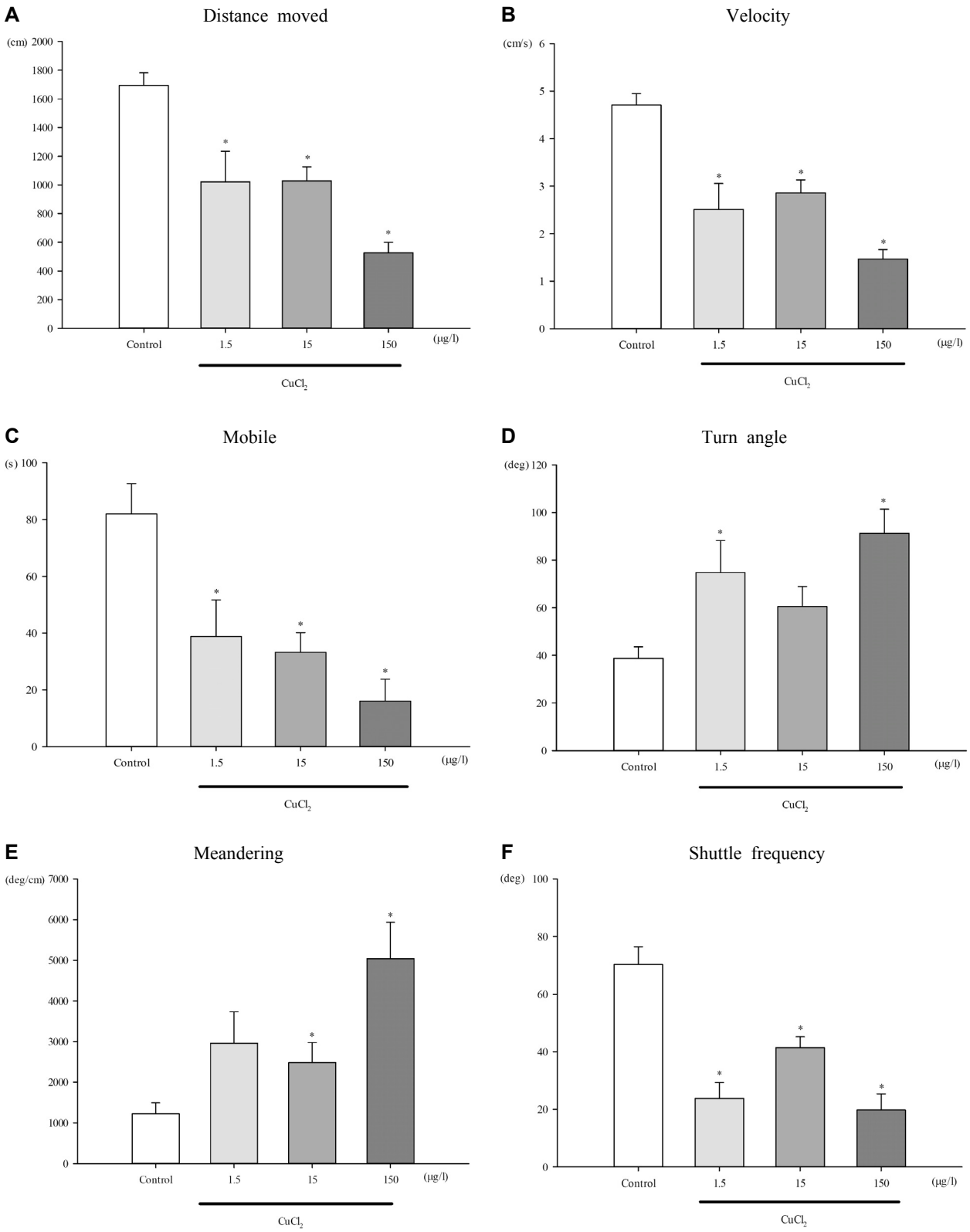

Fig. 3. $CuCl_2$ increases anxiety on the open field test in zebrafish. (A) Distance moved, (B) velocity, (C) mobile, (D) turn angle, (E) Meandering and (F) shuttle frequency after exposure of $CuCl_2$ (1.5, 15 or 150 μg/l) for 1 hr. Each bar represents mean ± S.E.M. of 8-10 animals. P values for the group comparisons were obtained by one-way ANOVA followed by Student-Newman-Keuls test (*p<0.05 vs. control).

82.0±10.7 second, 38.8±12.9 second, 33.2±6.9 second or 16.1±7.7 second at the concentration of the control group, 1.5 μg/l, 15 μg/l or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 1.5-150 μg/l, the mobile duration values significantly decreased compared with control group (p<0.05; Fig. 3C).

As a result of measuring the turn angle for 6 min in the OFT, the turn angle values were respectively 38.8±4.9, 74.8±13.4, 60.5±8.5 or 91.3±10.1 deg at the concentration of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at concentrations of 1.5 and 150 μg/l, the turn angle values significantly increased compared with control group, but not significantly increased at the concentration of 15 μg/l of $CuCl_2$ compared with control group (p<0.05; Fig. 3D).

As a result of measuring the meandering movement for 6 min in the OFT, the meandering movement values were respectively 1,227.1±268.5, 2,959.8±773.0, 2,481.7±494.4 or 5,039.1±895.2 deg/cm at the concentration of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at concentrations of 1.5 and 150 μg/l, the meandering movement values significantly increased compared with control group, but not significantly increased at the concentration of 15 μg/l of $CuCl_2$ compared with control group (p<0.05; Fig. 3E).

As a result of measuring the shuttle frequency which is the number of time zebrafish move from wall to the central arena, the shuttle frequency values were respectively 70.3±6.1, 23.8±5.6, 41.4±3.8 or 19. 8±5.68 deg at the concentration of the control group, 1.5, 15 or 150 μg/l of $CuCl_2$. In the $CuCl_2$-treated groups at the concentrations of 1.5 ~ 150 μg/l, the shuttle frequency values significantly decreased compared with control group (p<0.05; Fig. 3F).

Effect of $CuCl_2$ on Whole-body cortisol

The whole-body cortisol levels collected from control and three treatment groups are showed in the Fig. 4. Whole-body cortisol level of control was 30.63±4.14 ng/g. Whole-body cortisol level of three treatment groups were respectively 53.3±2.4 ng/g, 55.0±6.0 ng/g or 41.2±8.0 ng/g at the concentration of the 1.5 μg/l, 15 μg/l or 150 μg/l. In the $CuCl_2$-treated groups at the concentrations of 1.5 and 15 μg/l, the whole-body cortisol level values significantly increased compared with control group, but not significantly increased at the concentration of 150 μg/l of $CuCl_2$ compared with control group (p<0.05).

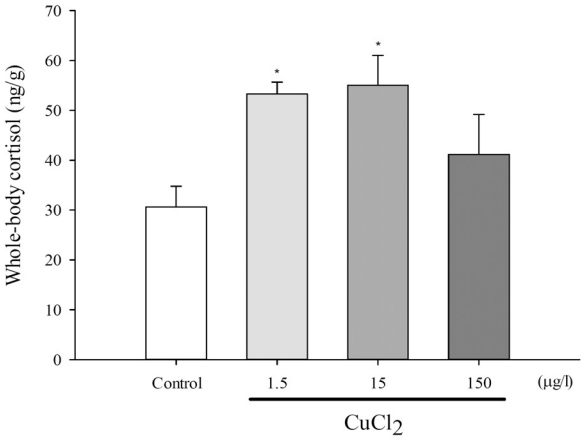

Fig. 4. $CuCl_2$ increases whole-body cortisol level. Zebrafish were exposed by $CuCl_2$ (1.5, 15 or 150 μg/l) for 1 hr. Each bar represents the mean ± S.E.M. of the whole-body cortisol level of 3-4 animals. P values for the group comparisons were obtained by one-way ANOVA followed by Student-Newman-Keuls test (*p<0.05 vs. control).

Discussion

Copper, which is an essential micronutrient, exists in the form of Cu^+ or Cu^{2+} *in vivo* as a structural cofactor of a variety of catalytic and structural enzymes which have a part of energy metabolism, mitochondrial respiration, and antioxidant defense [34]. Although copper is also an essential micronutrient on the aquatic organisms, it should be approached with further cautions because aquatic organisms are 10-100 times more susceptible to the toxic effects of copper than are mammals [36]. We performed measurement of median lethal concentration, whole-body cortisol level and behavioral tests to investigate toxicity of copper in zebrafish. As a result of the above-mentioned experiment, The LC_{50} values of $CuCl_2$ were 30.3, 25.3 or 14.8 μg/l in the 24 hr, 48 hr or 96 hr, respectively (Fig. 1), and the related mobility parameters including distance moved, mobile duration and velocity are significantly decreased compared with control group (p<0.05; Fig. 2 and Fig. 3). Interestingly, whole-body cortisol levels were significantly increased by exposures at concentrations of 1.5 μg/l and 15 μg/l of $CuCl_2$ compared with control group, but not in 150 μg/l of $CuCl_2$ (p<0.05; Fig. 4).

In this study, $LC_{50\text{-}48h}$ value of $CuCl_2$ was 25.3 μg/l though $LC_{50\text{-}48h}$ value of $CuCl_2$ was 130 μg/l in the study of Griffitt *et al.* [20]. Many studies demonstrated that hardness of water affected the mortality by toxicities of heavy metals in zebrafish [2]. Pascoe *et al.* demonstrated that cadmium is less toxic

in hard water than in soft water in toxicity tests with rainbow trout [30]. Moreover, Pourkhabbaz *et al.* reported that the toxicity of Co and Ni increased with decreasing water hardness in *Capoeta fusca* [32]. Actually, in low hardness of water, toxicities of heavy metals were revealed more strongly. Therefore, we assume that lower LC_{50} value was observed because of using distilled water (hardness: 3.4×10^{-4} mmol/l) as solvent. In addition, LC_{50} values of $CuCl_2$ decreased time-dependently. It might be considered that the toxic effect is severe by extension of exposure time to $CuCl_2$.

Exposures to environmental threats induce some abnormal behaviors that reflect the condition of animals and their response for escape [24]. When they are exposed to unaccustomed or a potentially perilous environments, anxious reaction is not only natural response but also essential response to survive [9, 13]. Behavioral tests are used to measurement of various condition such as smell relation behavior [26], anxiogenic [44], addiction [14], social behavior [15, 40], sleep [14], memory [45] and toxic responses [11, 41] in zebrafish.

NTT is one of zebrafish behavioral test that is an experiment to observe whether zebrafish is diving to make steady state or behaving vertically for exploration, or observing information about mobility. NTT is very useful to observe behavior parameters, such as distance traveled, turn angle, swimming speed and duration at the top [7, 38]. Pharmacological agents could change behavior parameters, for example buspirone [6], fluoxetine [19] and diazepam [42] which are anxiolytic drugs are increasing duration in top. On the other hand, anxiogenic drugs or induction of stress tend to decreasing duration at top, mobility and increasing erratic movements [43]. As a result of the NTT for 6 min, the distance moved, mobile and velocity were significantly decreased, on the other hand the turn angle values were significantly increased. Interestingly, the duration at the top were not significantly changed by exposure to $CuCl_2$. Also, exposure of $CuCl_2$ had a strong influence on mobility. We observed that conflict results in two parameters related to this anxiety of NTT. Above mentioned, anxiogenic drugs or induction of stress results in coherent changes of parameters related to this anxiety, whereas exposure of $CuCl_2$ do not.

OFT is an experimental method to observe the movement of experimental animals in the open space and OFT is using to confirm the basic motility and anxiety on various experimental animals [21, 35, 39]. In the OFT, stress-induced zebrafish causes abnormal swim with less normal straight-line swim shaking to the left and right. Above mentioned, the increase in meandering movements or turn angle is used as a measurement of abnormal psychological state such as anxiety or stress responses [8, 33]. As a result of the OFT for 6 min, distance moved, mobile, velocity and shuttle frequency were significantly reduced and the turn angle and meandering were significantly increased. In OFT, it was observed that the hypo-locomotion of zebrafish was induced by the exposure of $CuCl_2$. Besides, meandering movement and turn angle, parameters related to anxiety increased in OFT as the NTT. Interestingly, the shuttle frequency in OFT, which is an index related to exploration and anxiety, showed a significantly decrease in contrast with NTT.

Many studies mentioned an important relativeness between stress hormone and behaviors [12, 28, 31]. Increased cortisol has been used as a primary indicator of stress response in fish as human being [5, 37]. In this study, whole-body cortisol levels were significantly increased by exposure at concentrations of 1.5 and 15 μg/l of $CuCl_2$ compared with control group, but not in 150 μg/l of $CuCl_2$. Unfortunately, in this study, whole-body cortisol level was not significantly changed at the highest concentration of $CuCl_2$. We speculated that exposure of $CuCl_2$ induced endocrine disturbances. Similar shaped concentration–response curves have been reported after exposure of heavy metal. In previous study, the effects of lead nitrate on steroid profiles of *Heteropneustes fossilis* were examined *in vivo* and *vitro*. Cortisol elicited a concentration-dependent response of Pb^{2+} ion at low concentrations (1 or 0.1 μg/ml) were stimulatory, while the high concentrations were inhibitory. Based on these results, the authors suggested that Pb^{2+} ion could cause endocrine disruption [10]. In addition, adrenotoxic response have been observed in the reports on $CuSO_4$, previously. Gagnon *et al.* demonstrated that adrenocortical cells treated with $CuSO_4$ were inhibited the cortisol secretion in rainbow trout. According to authors, Cu^{2+} ion has a potential adrenotoxicity and impairs the secretory capacity of the adrenocortical cells [17]. Although we did not examine hypothalamus-pituitary-interrenal axis or adrenotoxicity in zebrafish, we assumed that $CuCl_2$ at high concentration could influence on release of cortisol in the interrenal gland. Many previous studies have shown obvious associations between stress hormones and behaviors. Because ambiguous correlation between stress hormone and behavior was observed under exposure to $CuCl_2$, further research is required to clarify whether $CuCl_2$ influences on endocrine disruption in detail.

In conclusion, the present study provides the evidences for the toxicity of copper. According to measurement of LC_{50} value, $CuCl_2$ could have more toxic effect on organism concentration- and time-dependently. As a result of behavioral tests showed that $CuCl_2$ was inducing hypo-locomotion through distance moved, velocity and mobile duration, and was inducing anxiety through change in meandering movement, turn angle and shuttle frequency. However, duration at the top, which is associated with anxiety, did not significant change suggest that additional study is needed as regards toxic stress. Taken together, this study suggests that excessive exposure to $CuCl_2$ induces endocrine disruption and abnormal behavioral changes.

Acknowledgement

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : $CuCl_2$ 노출에 의해 유도되는 제브라피시의 행동과 내분비계의 변화

성지원[1] · 이정원[2] · 이승헌[1,2,3]*

([1]제주대학교 해양과학대학 해양생명과학과, [2]제주대학교 해양과학대학 수산생명의학과, [3]제주대학교 해양과학연구소)

본 연구의 목표는 $CuCl_2$가 지브라피시에 미치는 영향을 조사하는 것이다. $CuCl_2$에 대한 지브라피시의 반수치사농도를 조사하기 위하여 각기 다른 시간에 따른 농도를 설정하여 노출시켰다. 본 연구에서는 스트레스 반응의 증가를 보기 위하여 whole-body cortisol level 측정과 행동약리학적 변화를 측정하기 위해서 open field test와 novel tank test를 실시하였다. Whole-body cortisol의 sample을 수집하거나 행동실험을 실시하기 전에 $CuCl_2$ 또는 vehicle을 1시간 동안 처리하였다. $CuCl_2$의 반수치사농도는 24시간, 48시간, 96시간에서 각각 30.3, 25.3, 14.8 μg/l으로 측정되었다. $CuCl_2$에 노출된 지브라피시의 novel tank test의 결과는 총 이동거리, 움직이는 시간 그리고 속도는 유의성이 있게 감소한 반면, turn angle은 최고농도에서 유의성이 있게 증가하였다(p<0.05). 다른 행동실험인 open field test의 결과에서도 총 이동거리, 움직이는 시간 속도가 $CuCl_2$의 노출에 의해서 감소하였으며, turn angle과 meandering이 유의하게 증가하였다(p<0.05). $CuCl_2$에 노출된 지브라피시의 whole-body cortisol은 1.5 or 15 μg/l의 농도에서는 유의하게 증가하였으나, 150 μg/l의 농도에서는 유의성 있는 변화를 발견하지 못하였다(p<0.05). 본 연구의 결과는 $CuCl_2$의 노출이 지브라피시에서 치사율 증가, 행동 및 내분비의 변화 등을 포함하는 독성 반응을 야기하는 것을 나타낸다.