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EFFECT OF METALLOTHIONEIN LEVELS IN *Gammarus pulex* EXPOSED TO COPPER AND COPPEROXIDE NANOPARTICLES

ABSTRACT

With the increase in the use of products containing nanoparticles (NP) (<100nm), the food chain including aquatic systems and organisms is at risk due to the effect of NPs released as a result of anthropogenic activities. In this study, metallothionine (MT) biomarker responses were determined by exposing Gammarus pulex, an indicator species living in freshwaters, to metal-based copper (Cu) and copper oxide (CuO) NPs. In the experiment, 4 groups (Control, 10ppm, 20ppm, 40ppm) were formed and studied with three replications. It was determined that the Cu-NP application samples caused an increase ($p<0.05$) in MT levels compared to the control at the 24th and 96th hours samples. It was determined that CuO applied samples caused increases in MT levels compared to the control group ($p<0.05$), but this increase in 96-hour samples was lower than the increase at the 24th hour samples. As a result of the study, it is predicted that Cu and CuO NPs cause an increase in MT levels in the organism, and this increase may cause oxidative stress.

Keywords: Metallothiyonein, Gammarus pulex, Biomarker, Copper Nanoparticle, Copper Oxide Nanoparticle

1. INTRODUCTION

Metallothioneins (MT) act as metallo-chaperones by transferring metal ions to other proteins. They control the free ion concentration of the essential trace elements such as zinc and copper. They act as detoxification agents of heavy metals. They are free radical scavengers [1]. Excessive accumulation of essential metals such as copper, molybdenum, zinc, and non-essential elements such as cadmium, mercury, and lead in organs, tissues, and cells causes toxication. In the prevention of this toxicity, MTs are involved in the translocation and transcription stages [2]. Therefore, Zn and Cu are neutralisated, and heavy metals such as Cd, Hg, and Pb are passivated [3]. The molecular weight of Metallothionein and glutathione proteins are low that reduce toxicity by binding toxic heavy metals and preventing them from binding to compounds with high molecular weight such as enzymes. The main functions of MTs are that providing homeostasis of essential metals, taking a role in detoxification of I and II group heavy metals, protecting cells and organs from the toxic effects of metals [4 and 5], and acting as a free radical scavenger due to its high thiol content [6].

Copper (Cu) is one of the important micronutrients for humans and animals and also an essential component of many oxidation-reducing enzyme systems such as cytochrome Cu oxidase, uricase, tyrosinase, superoxide dismutase, amine oxidase, lysyl oxidase and ceruloplasmin [7]. The functions of copper in the central nervous system are its necessity in iron metabolism for melanin pigment formation, and it is an important

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element for normal connective tissue metabolism [8, 9 and 10]. It also has mandatory duties in the biological, physiological, and immune responses of aquatic organisms. There are scientific studies on quantitative Cu requirements for aquatic organisms [11, 12, 13, 14 and 15]. In addition to all these essential functions in organisms, high Cu levels can be toxic to fish and crustaceans as it increases cellular oxidative stress due to the formation of reactive oxygen species (ROS) [16]. Copper and Copperoxide NPs have the feature of causing oxidative stress by crossing the plasma membrane and interacting with intracellular organelles [17, 18, 19 and 20]. It shows that Cu and CuO-NPs released directly or indirectly to the environment as waste which is a potential risk and this situation should be investigated comprehensively [21 and 22]. However, there are not enough studies on the ecotoxicity of Cu-CuO NPs in aquatic organisms [23, 24 and 25].

The use of nanotechnological (1-100 nm) materials and innovations in this field allow the production of a very wide variety of nanoparticle (NP) materials. Fact that this NP material is very small, and quantum dots (NP artificial nanostructures) exhibit magnificent optical, magnetic, and chemical properties [26]. Today, it is an industrial area where rapid progress is seen in nanomaterials science [27]. The increased production and use of metal NPs for different applications lead to adverse health effects [28]. In toxicological tests, the relations of living things with each other and with their environment should be taken into account, and it is important to choose the appropriate test organism together with the appropriate test type to obtain current and meaningful outputs [29]. Organisms used in bioassay or toxicity researches have an important place in studies. Most of industrial waste are dumped untreated into freshwater or marine systems directly or indirectly. Due to possible toxic effects, it is important to make possible toxicological evaluations on organisms found in both ecological systems.

2. RESEARCH SIGNIFICANCE

The aquatic ecosystem, which is the final destination of pollution in the ecosystem, is the most affected by the pollution. Organisms in the aquatic ecosystem are highly affected directly or indirectly by the pollution exposed to waste. Increasing industrial activities in recent years brings an increase of the release of wastes to the environment. This causes the inability of the environment to tolerate these wastes bring environmental pollution. Organisms in the ecosystem were threaten directly or indirectly from this pollution through the food chain. In particular, this study was designed to determine the effect of heavy metal pollution on organisms in aquatic ecosystems with a model organism and biomarker. Gammarides, an aquatic crustacean family, is among the commonly used species for toxicity tests due to its high sensitivity to pollutants, abundance, and easy availability [30]. For all these reasons, we chose the *G. pulex* organism as a model in this study. In the study, MT level changes were measured semi-quantitatively in Gammarus pulex exposed to metal-based Cu and CuO NPs. It was aimed to determine the changes in the MT levels of Cu and CuO NPs at the end of the 24th and 96th hours and to evaluate their usability as a biomarker.

Highlights:

- Selection of Gammarus pules in the aquatic ecosystem as a model.
- Determining the effect of heavy metal pollution on organisms in aquatic ecosystems with a model organism and biomarker.
- Determination of changes in MT level in Gammarus pulex and its use as a biomarker.

3. EXPERIMENTAL METHOD-PROCESS

3.1. Nanoparticles

The metal-based Cu-NP (60-80nm) chemical (Sigma-Aldrich) was purchased, and CuO-NP (40nm) was purchased from SkySpring (Houston, TX, USA) No purification and analytical reagent classification have been done to Cu-CuO NP chemicals. In the bioassay studies, the shape and size data declared by the manufacturer were taken as a reference.

3.2. Bioassay Organism

The *G. pulex* organisms were cultured under appropriate conditions. While selecting the organisms, healthy male individuals of the same size with no movement restrictions were preferred.

3.3. Preparation of Nanoparticle Suspensions

Cu and CuO NP stock suspensions were dissolved in ultrapure water at 10% (m/v) after vortexing for 5 minutes, sonication in an ultrasonic bath for an average of 15-20 minutes to ensure the highest NP distribution. *G. pulex* organisms were exposed to Cu-CuO NP concentrations by preparing appropriate solutions at the concentrations determined by the experiment design.

3.4. Toxicity Bioassay Setup

In the experimental design, 4 groups were formed, one of them was the Control group, in which Cu-CuO NPs were not applied and The experimental application concentrations of NP in the study were determined by considering the release rates to the environment. Application concentrations related to the effect of nanoparticles on *G. pulex* were established (Table 1). At the beginning of the experiment, 15 individuals were placed in each 1500 mL aquarium, and all experiments including the control and all other groups repeated 3 times, it is seen that the total number of individuals was approximately 180 at the beginning (Table 1).

Table 1. Bioexperimental design of organisms exposed to Cu and CuO nanoparticles

	Control	Group1	Group2	Group3
NP-Cu/CuO (mg/L)	0	10	20	40
G. pulex (piece)	15	15	15	15
Repetition	3	3	3	3

3.5. Metallothionine Analysis

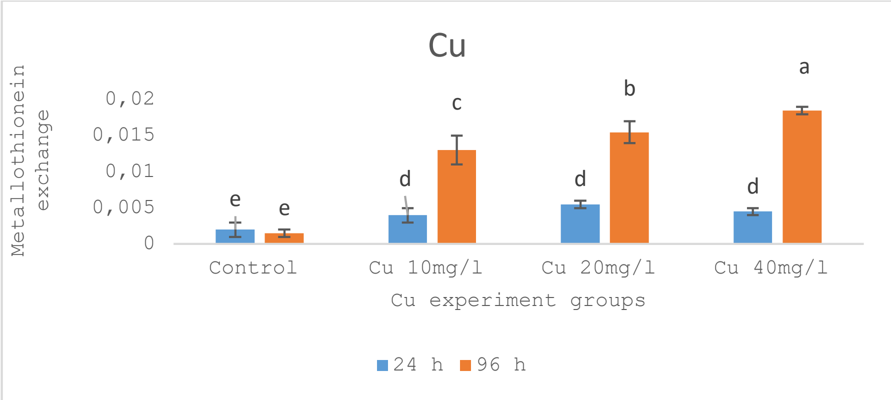
In the study, Cayman brand semi-quantitative biomarkers (Item No: 10008659) were used. This kit is based on the detection of the MT biomarker using a suitable monoclonal or polyclonal antibody in indirect antibody capture ELISA format in fish samples containing a set of Enzyme-Linked Immunosorbent Assay (ELISA) reagents for semi-quantitative detection of the MT biomarker [31].

3.6. Statistical Analysis

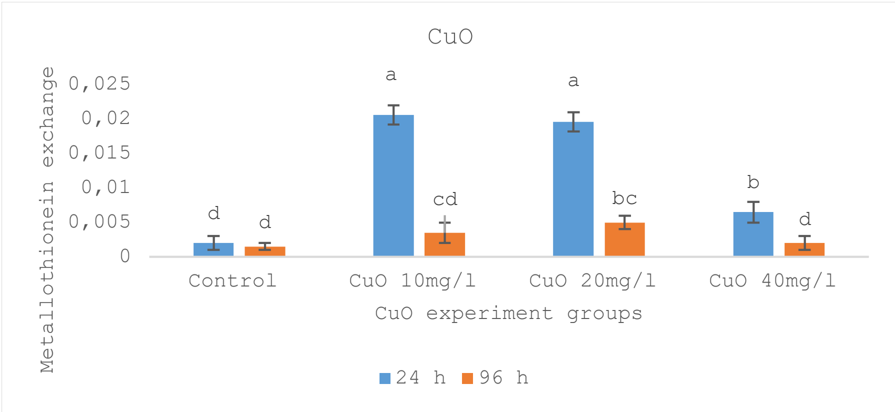
All experimental groups designed in the study were independently repeated three times. SPSS/24.0 packaged software was used, one-way analysis (ANOVA) and Tukey were chosed in statistical analysis and ($p<0.05$) test was preferred for significant differences between groups.

4. RESULTS AND DISCUSSION

The effects of exposure to Cu-NP and CuO-NP on MT levels expressed as a percentage of the control values are presented in Figure 1a and Figure 1b.



a (Cu-NP)



b (CuO-NP)

Figure 1. a) MT levels in G. pulex exposed to Cu-NP, b) MT levels in G. pulex exposed to CuO-NP

Statistically significant differences were detected in *G. pulex* exposed to Cu-Np at 24 and 96 hours compared to the control (Figure 1a). For Cu-NP, exposure time and increasing concentrations showed a linear increase in MT levels. A statistically significant increase in MT levels was detected in *G. pulex* exposed to CuO-NP compared to the control (Figure 1b). However, there is a decreas on MT levels depending on exposure time to CuO-NP. It was aimed to investigate the effects of Cu-NPs and CuO-NPs on the aquatic ecosystem in case of possible mixing with the aquatic ecosystem, their response to MT biomarkers in a model organism, *G. pulex*, and their potential for use in ecotoxicological studies. Non-lethal concentrations of both NPs have been determined by preliminary studies. Metallothioneins are ubiquitous, heat stable, low molecular weight proteins characterized by a very high cysteine content of 22-33% and a selective capacity to bind heavy metal ions such as mercury, cadmium, copper, and zinc [32]. Although the variation between different living and different tissues, the variation between different MT-metal components varies according to the metal type, duration, concentration, and exposure method. The affinity of MTs to bind different metals is $Zn^{+2}<Pb^{+2}<Cd^{+2}<Cu^{+2}<Ag^{+2}=Hg^{+2}=Bi^{+3}$, respectively [33 and 34]. Tissues that are active in the direct uptake, storage, and excretion of metal have a high MT synthesis capacity [35]. Although copper is an essential metal for living organisms in trace amounts, it can be toxic when it reaches a high concentration [36].

Copper is known to be both an MT inducer and an LP-producing pro-oxidant in many animal species, including aquatic organisms [37, 38, 39, 40, 41 and 42]. Both metal NPs showed similar induced rearrangements. The MT concentration gradually increased with the increase of the Cu-NP metal concentration. However, it was slightly different in individuals exposed to CuO-NP. Although the MT level of the CuO-NP concentration increased with time compared to the control group, it decreased with increasing concentration in the CuO-NP applied groups.

Zhao et al. (2010) [43], reported in their study that they could induce MT synthesis at application concentrations in *Ruditapes philippinarum* crustaceans exposed to Cu and Cd metals, but the induction was limited to organisms. Correia et al. (2002) [42], investigated the responses of MT and LP in *G. locusta* exposed to Cu and their potential to be used as biomarkers of Cu exposure and toxicity, in integrated ecotoxicological studies. They reported that for different exposure concentrations, Cu increased between 1.36 and 1.93 times at varying exposure times compared to the control. Although Cu is a less potent inducer of MT in some crustaceans than some other metals [44 and 45], high concentrations of MT have been reported following exposure to this metal in several crustacean groups, including crabs [46, 47 and 48], lobsters [49 and 50], and copepods [51]. Vellinger et al. (2013) [52], investigated the single and combined effects of Cd and arsenic on *G. pulex*. They reported that the increase in GSH and MT concentrations in *G. pulex* exposed to Cd caused the increased activity of the used model organism in antioxidant defense systems together with metal stress. However, a few studies have emphasized that there is no increase in internal MT concentration for aquatic organisms acutely exposed to metals [53 and 54]. For Cu and Cd exposure, the MT content increased linearly with exposure time. In general, high concentrations of essential or non-essential metals in aquatic organisms tend to increase MT concentration in their cells [55], reflecting their bioavailability at high metal exposure [56, 57, 58 and 35]. Similarly, in this study, MT biomarker levels of increasing Cu-NP and CuO-NP concentrations increased compared to the control.

5. CONCLUSION

With the determination of the metal-binding properties of metallothioneins, it was thought that they might have potential roles in heavy metal detoxification, and this idea was supported by many in vivo and in vitro studies [59]. After exposure to various metals, it was determined that MT synthesis increased in organs such as the kidney, liver, and intestine, and there was MT accumulation in these organs. Since MTs are direct metal holders, they prevent the metals from circulating freely in the cell and damaging the cell. Metallothioneins detoxify heavy metals not only by their metal-binding capacity, but also by protecting cells from oxidative damage. Metal detoxifications of MTs vary according to the type of cell to which they are exposed, the properties of the metal, and the concentration of the metal [60 and 61]. By using *G. pulex* as the biological matrix, the exposure of MT biomarkers to Cu-NPs and CuO-NPs, which are metallic NPs, can be beneficial for the use of MT biomarkers in monitoring programs. Accordingly, when the NPs was taken by the organisms in the food chain accumulate in the tissue, they may cause oxidative stress and may pose a danger to higher consumer organisms. A control mechanism should be established for the use and contamination of these particles and extensive studies such as genotoxicity should be carried out by using them in different organisms.

CONFLICT OF INTEREST

The author declared no conflict of interest.

FINANCIAL DISCLOSURE

The author declare that this study has received no financial support.

DECLARATION OF ETHICAL STANDARDS

The author of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

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