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Determination of Oxidative Stress Responses Caused by Zinc Oxide Nanoparticle on *Gammarus Pulex*

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ABSTRACT

Zinc Oxide Nanoparticles (ZnO-NP) are inevitably released into the environment and penetrate into the aquatic environment during production, transportation, use and disposal processes. In this study, which aims to investigate the effect of ZnO mixed into the aquatic environment, *Gammarus pulex*, a good indicator species, was chosen as a model organism. To carry out the study, *G. pulex* individuals were exposed to 0 (control), 10, 20 and 40 ppm concentrations for 24 and 96 hours and elimination periods. Samples were taken at 24 and 96 hours and elimination periods and kept at-86 °C until oxidative stress and antioxidant biomarker parameter analyzes were performed. Model organisms were taken from the experimental environment after 96 hours and kept in the water provided from the living areas for 24 hours, elimination groups were created and changes in oxidative stress and antioxidant biomarker parameters were determined. Among the biomarker parameters, SOD, Catalase (CAT) activities and Glutathione (GSH) and Thiobarbituric acid (TBARS) levels were measured. Measurements were carried out with Cayman brand ELISA kits. Considering the study data, it was determined that ZnO-NP caused fluctuations in SOD activities, but there was no change in CAT activity, compared to the control. While there were decreases in GSH levels, it was observed that there were increases in TBARS levels.

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Keywords

Gammarus pulex; Zinc oxide; Oxidative stress; Biomarkers; Reactive oxygen species

Introduction

Zinc Oxide Nanoparticles (ZnO-NP) are white powders consisting of metal oxide nanoparticles. They possess the characteristic of being non-combustible and lacking any discernible scent. Titanium dioxide is widely used in various products, including sunscreens, cosmetics, paint, paper, plastics, and building materials, due to its exceptional stability, resistance to corrosion, and photocatalytic properties. However, the presence of nano-ZnO may pose a possible risk to the environment [1].

Zinc oxide (ZnO) is a potent antibacterial agent that exerts its effects through many methods involving diverse chemical species. According to the literature, there are three distinct mechanisms by which ZnO acts: Firstly, it generates Reactive Oxygen Species (ROS) as a result of its semiconductor properties; secondly, it disrupts ZnO in microbial membranes when it comes into direct contact with cell walls; and thirdly, ZnO releases Zn^{2+} ions in aqueous environments, which possess inherent antimicrobial properties. The presence of Zn^{2+} cations leads to the disturbance of protein structures and an elevation in the amounts of ROS within cells. This is caused by the interference with mitochondrial electron transport, as demonstrated by Xia and George [2,3].

Furthermore, the surface of ZnO nanoparticles has the ability to produce ROS as a result of redox reactions.Zinc cations (Zn^{2+}) have been demonstrated to have a detrimental impact on aquatic organisms, particularly fish, by interfering with the process of egg hatching [3,4].

According to Xiong, living organisms exposed to environmental contaminants can experience the presence of ROS [5].In addition, the generation of ROS results in oxidative harm to large molecules such as proteins, DNA, and lipids, ultimately resulting in damage to many cellular organelles [6].

Furthermore, DNA damage primarily arises from the hydroxyl radical and superoxide anion radical. This type of damage is particularly worrisome due to its potential to induce genetic consequences and disorders. In typical circumstances, the detrimental consequences of oxidative stress in living organisms are counteracted by antioxidant enzymes such as SOD and Catalase (CAT).

TBARS, a marker for the amount of lipid peroxidation, has been identified as one of the molecular pathways responsible for the toxicity caused by nanoparticles [7]. Its importance as a biomarker for oxidative stress has been acknowledged in several studies [8].

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GSH plays a crucial role in defending against oxidative damage caused by reactive oxygen species. It functions as a reducing agent and scavenges free radicals. GSH is also recognized as a cofactor substrate and is involved in the activity of GSH-related enzymes [9].

Zinc (Zn) is a vital trace metal for aquatic species when present in low concentrations. However, large amounts of Zn can be harmful and toxic to aquatic life, as stated by Eisler [10].

Aquatic creatures exhibit swift responses to environmental contaminants through the measurement of molecular and cellular biomarkers. These biomarkers serve as indicators to evaluate the health condition of organisms and can act as early indicators of potential harm to higher-level biological systems, before irreversible damage takes place [11].

Grammarus species exhibit a higher degree of sensitivity to water contamination compared to fish. The utilization of this taxonomic group in toxicological investigations is on the rise because to their heightened susceptibility to diverse contaminants, rapid production capacity, and ability to be amassed in substantial quantities [12-14].

G. pulex is an ideal organism for assessing the impact of environmental pollutants on freshwater species. This is because it has significant ecological importance and plays a crucial part in the food chain. An organism that is highly important and sensitive in terms of ecology and ecotoxicology, and serves as a food source for various creatures like frogs, fish, and birds, is considered suitable for conducting eco-ecotoxicological investigations on water at elevated concentrations [13,15].

The objective of this study is to investigate the impact of ZnO_3 nanoparticles on *G. pulex* by analyzing the activities of SOD and CAT enzymes, levels of GSH and TBARS, as well as the clearance rates, in order to assess the oxidative stress responses.

Materials and Methods

Nanoparticles

The NP materials used in the study were obtained from the ZnO_3 commercial company (SkySpring). The chemical, which is in the analytical reagent class, was used without any purification. The manufacturer's claimed shape and size data for NP were utilized in bioassay investigations, with accordance to the manufacturer's reported shape and size data.

Organism provision and adaptation

G. pulex individuals used in the study were collected from the side branches of Munzur Stream in Tunceli province with the help of a bottom scoop, and brought to the Munzur University Faculty of Fisheries research laboratory by supplementing air. *G. pulex* individuals were placed in 40 x 20 x 20 cm aquariums and adapted to laboratory conditions for 4 weeks. Environments suitable for natural habitats were prepared for the adaptation of *G. pulex* to laboratory conditions.

For this purpose, sediments taken from the natural environment of *G. pulex* were washed with pure water and placed in stock aquariums. Water brought from the natural environment of *G. pulex* was added to the aquariums. Stock aquariums were supplemented with oxygen using an air engine. A photoperiod of 12 hours of darkness and 12 hours of light was used for ambient lighting. The ambient temperature of the aquariums was fixed at 18°C with thermostatic air conditioning. After the adaptation environment was prepared, *G. pulex* collected from Munzur Stream were placed in stock aquariums.

G. pulex was allowed to adapt to laboratory conditions. 70% of the water in stock aquariums was renewed weekly. To feed *G. pulex*, shrub willow tree leaves were collected and left to rot.

Sublethal concentration selection and trial design

The concentration values to be applied were determined by reviewing the literature, taking into account their release into nature and their effects on aquatic organisms [16].

In all experimental stages of the research, 0.5 liters of non-chlorinated water taken from the natural environment of the creatures was used in 1-liter glass aquariums. 10 *G. pulex* were placed in these aquariums for each concentration.

Group 1: (Control (C)) water taken from the organisms' natural environment.

Group 2: 10 ppm ZnO_3 concentration was applied to (ZnO_3).

Group 3: A concentration of 20 ppm ZnO_3 was applied to (ZnO_3).

Group 4: (ZnO_3), 40 ppm ZnO_3 concentration was applied.

Biochemical analyzes

Tissue samples collected at 24 and 96 hours, as well as during the elimination period, were utilized. The samples were weighed and subsequently combined with PBS buffer (Phosphate-Buffered Saline Solution) at a weight-to-volume ratio of 1/5. The mixture was homogenized using an ice homogenizer to evaluate its antioxidant capabilities.

The samples were subjected to centrifugation at a speed of 17,000 revolutions per minute for a duration of 15 minutes. The liquid part obtained, referred to as the supernatant, was subsequently stored in a deep freezer at a temperature of-86°C until further tests were performed. The enzymatic functions of SOD and CAT, along with the quantities of TBARS and reduced GSH, were assessed using ELISA kits acquired from Cayman Chemical Company.

Statistical analysis

SPSS 24.0 package program one-way ANOVA (Duncan 0.05) was used to evaluate biochemical analyses.

RESULTS

SOD activity

The figure presented as Figure 1 displays the temporal variations in SOD activities in *G. pulex* when exposed to varying concentrations of ZnO_3. After 24 hours, there were significant enhancements in SOD activity compared to the control group, as evidenced by a p-value below 0.05. Likewise, there were notable reductions in SOD activity after 96 hours in comparison to the control group, with a p-value below 0.05. Significant modifications ($p<0.05$) were seen between the elimination and application groups (C1, C2, and C3) based on statistical analysis.

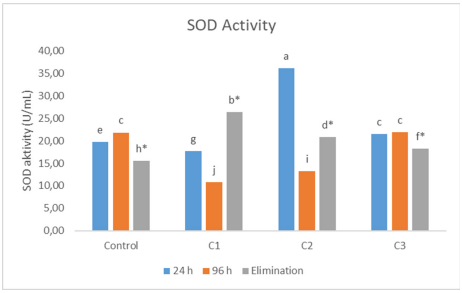

Figure 1. SOD (U/mL tissue) activities of *G. pulex* exposed to ZnO_3, different letters above the bar are statistically significant ($p<0.05$). The*sign indicates the statistical difference in the same group at different times ($p<0.05$).

CAT activity

The activities of CAT in *G. pulex* exposed to various concentrations of ZnO_3 at different time intervals are presented in Figure 2. The decrease observed in the C1 group at the end of 96 hours is statistically significant ($p<0.05$) when compared to the control group. However, it was concluded that there was no statistically significant alteration in CAT activity across any other groups ($p>0.05$). Significant reductions ($p<0.05$) in elimination quantities were seen in all groups compared to the control.

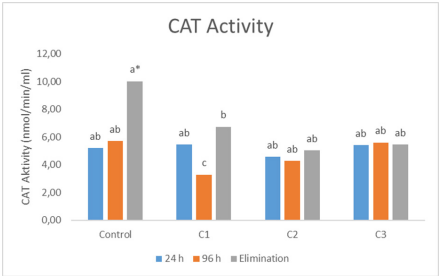

Figure 2. CAT (nmol/min/ml tissue) activities of *G. pulex* exposed to ZnO_3, different letters above the bar are statistically significant ($p<0.05$). The*sign indicates the statistical difference in the same group at different times ($p<0.05$).

GSH level

The levels of GSH in *G. pulex*, which were subjected to various doses of ZnO_3, are presented in Figure 3, with respect to time. Compared to the control group, the decreases in GSH levels and elimination amounts in all groups were found to be statistically significant ($p<0.05$).

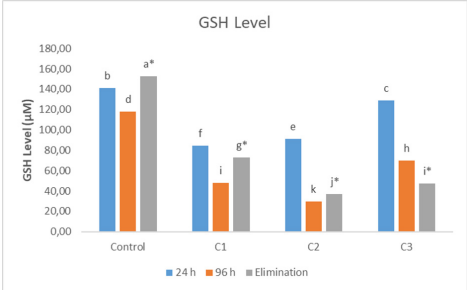

Figure 3. GSH (µM tissue) levels of *G. pulex* exposed to ZnO_3, different letters above the bar are statistically significant ($p<0.05$). The*sign indicates the statistical difference in the same group at different times ($p<0.05$).

TBARS level

The levels of TBARS in *G. pulex* subjected to various concentrations of ZnO_3 at different time intervals are presented in Figure 4. Statistically significant ($p>0.05$) increases in TBARS levels and elimination quantities were seen in all groups compared to the control group.

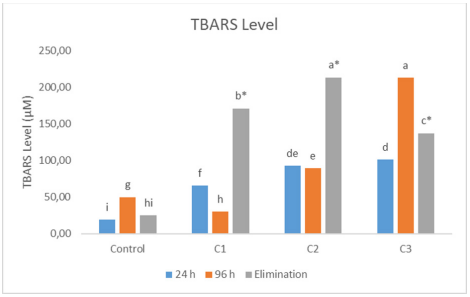

Figure 4. TBARS (µM tissue) levels of *G. pulex* exposed to ZnO_3, different letters above the bar are statistically significant ($p<0.05$). The*sign indicates the statistical difference in the same group at different times ($p<0.05$).

Discussion

SOD is an enzyme that acts as an antioxidant by converting the superoxide radical (O^{2-}) into hydrogen peroxide (H_2O_2) [17]. Catalase activity facilitates the enzymatic conversion of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) by reduction. The CAT enzyme is frequently associated with SOD activity [18]. Therefore, both enzymes work together to generate the initial defense mechanism against oxidative stress [19]. Fluctuations in the activity of SOD and CAT enzymes were detected in this study, which were dependent on factors such as tissue type, exposure time, and the size and concentration of NPs [1]. Results of SOD and CAT changes caused by nano-ZnO in *Cyprinus carpio* support our study Kaya, stated that fluctuations were observed in the SOD and CAT activity results of ZnO NP in *Oreochromis niloticus* [20]. Shahzad et al. observed changes in the SOD and CAT activities of ZnO in *Oreochromis mossambicus* [21]. Asghar et al. observed increases in ZnO NP-induced SOD activity of selenium in *Catla catla* [22]. Sanpradit et al. stated that ZnO decreased SOD activities in *Daphnia magna* [23]. Zhao stated that there were changes in SOD and CAT activities after ZnO NP exposure in zebrafish embryos [24]. Mahjoubian stated that mixtures of Ag NPs and ZnO NPs caused changes in SOD and CAT activities in *Danio rerio* [25]. Suman et al. observed that there were increases in SOD activities in Chlorella vulgaris due to ZnO NPs [26]. Abdelazim et al. observed that ZnO caused decreases in SOD and CAT activities in Nile tilapia [27]. Hong et al. they stated that ZnO exposure could increase SOD activity in Carassius carassius [28]. Sanpradit et al. stated that ZnO reduced SOD activities in *D. magna* with the effect of temperature [29]. Abdel-Daim et al. stated that there were decreases in SOD and CAT activities in Nile tilapia with the effect of ZnO [30]. Benavides observed that there were fluctuations in SOD and CAT activities as a result of the effects of ZnO and Al_2O_3 NPs [31]. Mohammady et al. stated that changesoccurred in SOD CAT activities in *O. niloticus* with the effect of ZnO [32]. Ma and Wang stated that there were changes in SOD and CAT activities as a result of $ZnSO_4$

and nZnO exposures in *Siganus fuscescens* [33]. Banaee et al. stated that there were changes in SOD and CAT activities in *Gambusia holbrooki* after exposure to microplastics and ZnO [34].

GSH and GSH-related enzymes serve as a crucial secondary defense mechanism against oxidative damage by effectively eliminating peroxide and free radicals [35]. GSH is a small molecule with a low molecular weight that acts as a non-enzymatic antioxidant. It effectively removes reactive oxygen radicals by utilizing the -SH group [20]. Under mild oxidative stress situations, the production of GSH leads to an increase in its levels. However, under severe oxidative stress conditions, the levels of GSH fall due to the suppression of ROS [36]. In a study that supports the decreases in GSH levels observed in our study, Hao et al. detected nano-ZnO GSH decreases in *C. carpio* [1]. Ali et al. reported that there were decreases in GSH levels in *Lymnaea luteola* due to the effect of ZnO [37]. Asghar they investigated the ZnO NP-induced GSH effect of selenium in *C. catla* and stated that GSH levels decreased [28]. Suman et al. stated that there were decreases in GSH levels in *C.vulgaris* as a result of ZnO NP exposure [26]. Abdelazim e and Abdel-Daim , stated that ZnO reduced GSH levels in Nile tilapia [27,30]. Abdel-Halim et al., they observed that ZnO caused decreases in GSH levels in *Monacha cartusiana* [38]. Cimen et al. stated that Cu and CuO caused decreases in GSH levels in *Artemia salina* [16].

Excessive amounts of oxygen radicals, beyond the protective capacity of the cellular defense system, readily interact with unsaturated fatty acids in the membrane structure, resulting in lipid peroxidation [39]. TBARS is a significant criterion utilized to assess the extent of oxidative stress induced by metabolic by products of lipid peroxidation in the body [4,16]. In the study, TBARS level was measured to determine the oxidative stress level and it was determined that ZnO_3 caused oxidative stress as the TBARS level increased [40-45]. Kaya et al. data on the increase in TBARS levels caused by ZnO NP in *O.niloticus* are parallel to our study [39]. There are other studies that support our study [46-52]. Ali et al., they stated that TBARS levels increased with the effect of ZnO in *L.luteola* [37]. Sanpradit et al., stated that ZnO caused increases in TBARS levels in *D. magna* [23]. Zhao et al., stated that there were increases in TBARS levels in zebrafish embryos after ZnO NP exposure [24]. Mahjoubian et al., observed that mixtures of Ag NPs and ZnO NPs caused increases in TBARS levels in *D. rerio* [25]. Hong et al., reported that ZnO exposure increased MDA levels in *C. carassius* [28]. Sanpradit et al., stated that ZnO causes increases in TBARS levels in *D.magna* with the effect of temperature [23]. Abdel-Daim et al.,stated that there were increases in TBARS levels in Nile tilapia due to ZnONP [30]. Banaee et al., stated that there were increases in TBARS levels in *Gambusia holbrooki* after exposure to microplastics and ZnO [34]. Cimen et al., observed increases in TBARS levels of Cu and CuO in *A.salina* [16].

Conclusion

ZnO_3, which is one of the various engineering and industrial nano materials, is used in many areas and causes negative effects on many living organisms as a result of mixing with the environment and aquatic environment. All kinds of pollutants mixed into the aquatic environment penetrate into the cells of aquatic organisms, causing damage to the cell defences in the organism's cell and causing oxidative stress, which can even cause death of the organism in long-term exposure. Our study results and literature data show that ZnO and its derivatives cause oxidative stress in many living species, even at different concentrations and under different conditions.

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