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Effects of Copper Oxide Nanoparticles on Immune and Metabolic Parameters of *Galleria mellonella* L

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Abstract

In this study, the effects of dietary CuO nanoparticles (NPs) on metabolic enzyme activity, biochemical parameters, and total (THC) and differential hemocyte counts (DHC) were determined in *Galleria mellonella* larvae. Using concentrations of 10, 100, 1000 mg/L and the LC10 and LC30 levels of CuO NPs, we determined that the NPs negatively impacted metabolic enzyme activity and biochemical parameters in larval hemolymph. Compared with the control, the greatest increase in THC was observed in larvae fed on diets with 100 mg L^{-1} of CuO NPs. Plasmatocytes and granulocytes were among the most numerous hemocytes in all treatments. These results suggest that dietary CuO NPs effects the metabolic metabolism and immune system of *G. mellonella* and provide indirect information regarding the toxic effects of CuO NPs in mammalian immune system given similarities between mammalian blood cells and insect hemocytes.

Keywords Nanoparticles · Transferase enzyme · Hemocyte · *Galleria mellonella*

Introduction

Nanotechnology is an example of any materials that have structures or components that are 100 nm or less in a least one dimension (Dowling 2004; Nowack and Bucheli 2007). Among these novel nanomaterials (NMs), nanoparticles (NPs) with 3 dimensions between 1 and 100 nm are central in the advance of nanotechnology (Ju-Nam and Lead 2008). Due to the extensive use in different industrial areas such as electronics, biomedical, pharmaceutical, cosmetic, energy, environmental, catalytic and material applications, metal oxide NPs have received considerable attention and massively produced over the last years (Tiede et al. 2009). The increased use of metal nanoparticles in many fields of science has created a need to study the environmental impact of nanoparticles.

Although hemocytes have similar roles in immunity in all species, the nomenclature of different hemocyte types differs more or less between species and taxa. The classification of hemocytes in species belonging to the order Lepidoptera consists of six different types; prohemocytes, granulocytes, plasmotocytes, adipohemocytes, spherulocytes and eeocytoids (Lavine and Strand 2002).

Due to the comparative analyses of the cellular components of the insect and mammalian immune systems, insects have become popular models to evaluate the immunological effects of microbial pathogens, antimicrobial agents, heavy metals, chemicals. The insect immune response demonstrates a number of structural and functional similarities to the immune system of mammals. Insect hemocytes recognize pathogens and phagocytose material in a similar manner as neutrophils in mammalian immunity (Browne et al. 2013).

Cell damage in insects increases due to environmental pollution factors such as pesticides, heavy metals and nanoparticles (Aslan et al. 2019; Tuncsoy et al. 2019).

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Metabolic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) are used to determine the level of cell damage (Sertçelik et al. 2018). Disrupted energy metabolism due to cell damage is regulated by metabolic enzymes such as creatine kinase (CK) and alkaline phosphatase (ALP). In addition, biochemical parameters such as total protein (TP), glucose (GLU), urea (UREA) of insects are adversely affected due to the toxic effects of chemicals (Etebari et al. 2007; Sugeçti and Büyükgüzel 2018).

The toxicity of CuO NPs was examined in several invertebrates model organisms such as *Daphnia magna* (Heinlaan et al. 2008; 2011), *Daphnia culex* (Griffitt et al. 2009), *Mytilus galloprovincialis* (Gomes et al. 2011) and also in our previous study with *G. mellonella* (Tuncsoy et al. 2019). The different mechanisms of free copper ion levels on the antioxidant system can be explained by the results obtained from both forms of copper. However, factors other than soluble Cu^{+2} ions released by the dissolution of nanoparticles contribute to the toxicity of CuO NPs (Fahmy and Cormier 2009; Ahamed et al. 2010; Buffet et al. 2011; Heinlaan et al. 2011). In fact, some studies have reported that CuO NPs have low solubility (< 1% dissolved Cu) in the application environment and a high tendency to form large aggregates as a result of increased exposure time (Gomes et al. 2011; Maria and Bebianno 2011). Due to their small size, large surface area and potential to cause oxidative stress, CuO NPs may be internalized by the digestive system and released into cells at higher damaging concentrations. This toxic mechanism of nanoparticles is described as trojan horse mechanism (Karlsson et al. 2008; Studer et al. 2010). In addition, particles can be trapped in the extracellular part where toxicity mechanisms such as direct contact between the cellular membrane and particles or other surface processes can cause damage due to the nanoparticle effect (Griffitt et al. 2009; Heinlaan et al. 2011).

In recent years, *Galleria mellonella* is frequently used as a model organism in experimental studies in many fields. Due to the difficulties of using mice in experimental studies, species such as *G. mellonella* have became models of choice, as their production is simple and not expensive and suitable for commercialization. In fact, *G. mellonella* larvae can be easily mass-reared at room temperature with a short generation time, which distinguishes this species from other invertebrates that may be artificially reared. *G. mellonella* also has an immune system similar to that of mammals, making it a suitable model organism for various studies (Gwokyalya and Altuntaş 2019).

The objective of the present study was to determine the toxic effects of CuO NPs on the metabolic enzyme activities of ALT, AST, CK, LDH, biochemical parameters of TP, GLU, UREA, direct bilirubin (BILD), total bilirubin (BILT) and uric acid (UA) in the hemolymph and immune system (total hemocyte number) of the model insect *G. mellonella*.

Materials and Methods

G. mellonella larvae were reared at 30 ± 1°C, 65 ± 5% RH on a diet composed of bran, honey, glycerol, honeycomb and distilled water (Bronksill 1961). Newly-hatched larvae were reared through the fourth instar on the artificial diet, when they were transferred into jars containing the diet with CuO NPs and fed until last instar (approximately 15 days, 150–200 mg; n = 20 for each replicate). Commercially available CuO nanopowder (from Sigma-Aldrich) was used in this study, which according to the supplier has a mean particle size of < 50 nm. More details on the characterization methods are described in Gomes et al. (2011). CuO NPs were incorporated into the artificial diet at the concentration of 10, 100 and 1000 mg/L. Cu concentrations were measured in nutrient media as 9.98 ± 0.02, 99.76 ± 0.24, 999.97 ± 0.03 mg/L. Larvae were reared on diets without CuO NPs as controls. Treatments were replicated 3 times with 20 last-instar larvae per replicate (Gomes et al. 2011).

The lethal effects of various concentrations of CuO NPs were determined. These data were used for probit analysis of the lethal activity of CuO NPs to fourth instar larvae of *G. mellonella*. Six groups of larvae were exposed to CuO NPs in the following concentrations: control, 0.1, 10, 100, 1000 and 10,000 µg/mL. After the treatment, number of dead larvae were calculated and LD50 values were determined using SPSS 21.00, Probit Analysis programme (Finney 1971).

Prior to assay, larvae were chilled on ice for 5 min and surface sterilized in 95% ethanol. Larval hemolymph was collected into cold Eppendorf tubes and stored at − 80 °C. A few crystals of phenylthiourea (PTU) were added to each sample to prevent melanization. Analyses were performed using the appropriate kit with Roche Hitachi Cobas c501 instrument (Roche, Germany). ALT (Roche, Germany, Kit no: 23641701), AST (Roche, Germany, Kit no: 22310601), LDH (Roche, Germany, Kit no: 23767001), CK (Roche, Germany, Kit no: 25703501) BILD (Roche, Germany, Kit no: 15104901), BILT (Roche, Germany, Kit no: 20341501), GLU Roche, Germany, Kit no: 56831600), TP (Roche, Germany, Kit no: 19727001), UA (Roche, Germany, Kit no: 24202101), UREA (Roche, Germany, Kit no: 24887901).

For determining THC, 36 µl of anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.017 M Na2EDTA and 0.041 M Citric acid, pH = 4.5) in 4 µl hemolymph obtained from the larva was transferred to eppendorf tubes. Then, 10 µl of the cell suspension was taken and loaded on the Neubauer hemocytometer. Hemocytes were counted under light microscope (Leica DM750) to determine the number of hemocytes in one milliliter of hemolymph. The counted

hemocytes were calculated using the Jones (1962) method. As for DHC, hemolymph was obtained from larvae and quickly spread on slides without allowing melanization, then allowed to dry at room temperature. The slides were kept in methanol: acetic acid (3:1) solution for 5 min for the fixation of the hemocytes. Freshly prepared Giemsa dye solution was used for staining the hemocytes. In order to prepare the staining solution, 3 ml of giemsa dye solution (MERCK Giemsa's Azure Eosine Methylene Blue Solution) was slowly mixed in slide staining containers with 57 ml PBS (pH 7.4). After the fixation, the dried slides were taken into the giemsa dye solution and stained for 15 min. After staining, the hemocyte types were determined under microscope according to the Jones (1967).

Data on the biochemical parameters and metabolic enzymes were analyzed by one-way analysis of variance (ANOVA). The least significant difference (LSD) test determined significant differences among treatment means when the $F > 0.05$ (SPSS 1997). Regression analysis was used to identify correlations between dietary treatments (SPSS 1997). The statistical analyses of the data were carried out by a series of Analysis of Variance and Student-Newman Keul's (SNK) test.

Table 1 Characterization of CuO nanoparticles using Transmission Electron Microscope (TEM). Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)Values are mean ± std

Particle characteristic	Method	Milli-Q W	Tap water
Size (nm)	TEM	< 50[a]	–
Particle size distribution (nm)	TEM	33.0 ± 8.3[b]	–
Hydrodynamic diameter (nm)	DLS	586.6 ± 89.4[b]	812.8 ± 98.8[b]
Zeta potential (mV)	ELS	− 17.1 ± 0.4[b]	− 18.5 ± 2.1[b]

[a]Information from Sigma-Aldrich

[b]100 mg L^{-1} CuO NPs

Results

Characterization of CuO NPs

The size and shape of CuO NPs were determined by TEM and DLS analysis (Table 1); the characterization was described in detail in Tuncsoy et al. (2019). The size of CuO NPs reported by the manufacturer is < 50 nm, which is in agreement with the size obtained by TEM (33.0 ± 8.3 nm). The hydrodynamic size obtained 586.6 ± 89.4 nm and zeta potential determined − 17.1 ± 0.4 mV. CuO NPs tends to aggregate producing suspensions with both small and large aggregates.

The data obtained from the toxicity tests was calculated using SPSS 21.00, Probit Analysis Method. The LC10 and LC30 values of CuO NPs for the fourth instar of *G. mellonella* were found to be 0.826 mg/L and 43 mg/L, respectively. 95% confidence limits were between 0.690–3.242 and 16.34–90.13 mg/L for LC10 and LC30 values of CuO NPs, respectively.

Effect of dietary CuO NPs on metabolic enzymes in hemolymph of *G. mellonella*

The greatest increases in transaminase enzyme activities (e.g., ALT and AST) in the hemolymph of *G. mellonella* larvae occurred with the LC30 concentration of CuO NPs, which were 2.54 and 2.34-fold higher in comparison to the control group (1000 mg/L). ALT activity was measured as 80.5 ± 2.01 U/L in the control group. ALT activity was measured as 67.5 ± 1.34 U/L at the highest CuO NPs concentration (1000 mg/L) added to artificial diet. AST activity in the control group was 290 ± 1.62 U/L; in diets containing 1000 mg/L of CuNPs, AST activity was decreased to 415.75 ± 2.20 U/L. These activities were statistically significant when compared with the control diet. The greatest increase in CK activity occurred following consumption of the diet containing 1000 mg/L CuO NPs, while the greatest increase in LDH activity was seen in the group exposed to the LC30 concentration of CuO NPs (e.g., 3.18 and 1.68-fold

Table 2 Effects of CuO NPs on some metabolic enzyme activities in hemolymph of last instar larvae of *G. mellonella*

CuO NPs Concentrations (mg/L)	Control	10	100	1000	LC10	LC30
ALT (U/L) (Mean* ± S.E)†	80.5 ± 2.01[a]	56.5 ± 1.82[b]	42.75 ± 1.19[c]	67.5 ± 1.34[d]	62.5 ± 2.32 b[d]	204.25 ± 2.61[e]
AST (U/L) (Mean* ± S.E)†	290 ± 1.62[a]	352.5 ± 2.14[b]	207.25 ± 2.01[c]	415.75 ± 2.20[d]	272.25 ± 1.51[e]	679.75 ± 2.88[f]
CK (U/L) (Mean* ± S.E)†	77.25 ± 1.78[a]	177.25 ± 2.11[b]	200.1 ± 1.74[c]	245.75 ± 1.67[d]	78.75 ± 1.98[a]	199 ± 2.37[c]
LDH (U/L) (Mean* ± S.E)†	162.5 ± 1.34[a]	204.25 ± 2.33[b]	151.25 ± 1.29[c]	172.75 ± 1.19[a]	164.25 ± 1.43[d]	273.5 ± 1.61[e]

*Mean of four replicates per treatment with 20 larvae per replicate

†Means within a line followed by the same lowercase letter are not significantly different ($P > 0.05$)

§Control Diet (CuO NPs free)

increases, respectively). CK activity was significantly increased from that of the control diet by diets containing CuO NPs concentrations of 1000 mg/L and LC30. CK activity was measured 77.25 ± 1.78 U/L in the control group, and CK activity increased to 245.75 ± 1.67 U/L, 199 ± 2.37 U/L for 1000 mg/L and LC30 concentration, respectively. LDH activity was significantly increased from 162.5 ± 1.34 to 273.5 ± 1.61 U/L at the LC30 concentration when compared to the control group. (Table 2).

Effect of dietary CuO NPs on biochemical parameters in hemolymph of *G. mellonella*

TP increased statistically in all treatments compared to the control. In comparison to the controls, glucose decreased in the 10 and 100 mg/L CuO NPs treatments; however, an increase was observed in the treatment of the LC30 concentration of CuO NPs. The amount of glucose was measured at the LC30 concentration to 118 ± 3.48 g/dL. These contents were statistically significant when compared with the control group. The highest concentration of CuO NPs caused a statistically significant BILD and BILT increase in comparison to the control. Amount of BILD and BILD were measured as 0.025 ± 0.001 mg/dL and 0.064 ± 0.002 mg/dL at the highest CuO NPs concentration, respectively. Amount of UREA was significantly increased from 0.43 ± 0.015 to 0.66 ± 0.015 mg/dL at the highest CuO NPs concentration when compared to the control group. Amount of UA also was statistically increased than the control ($p < 0.05$) (Table 3).

Effect of dietary CuO NPs on total and differential hemocyte number in hemolymph of *G. mellonella*

The number of hemocytes significantly increased to 76 and 102% of the number observed in the control following exposed to 10 and 100 mg/L, respectively. The greatest increase in total hemocyte count occurred in the group exposed to 100 mg/L CuO NPs. Decreases in total hemocyte counts decreased following consumption of the LC10 and LC30 values of CuO NPs when compared to the control group ($p < 0.05$) (Fig. 1). Consumption of CuO NPs also resulted in a change in differential hemocyte number. Significant increases in granulocyte number were observed in those larvae exposed to diet containing the LC10 concentration of CuO NPs. Numbers of plasmatocyte were significantly higher in all treaments in comparison to the control. The greatest increases were observed in spherulocyte and prohemocyte numbers following consumption of the diet containing the LC10 concentration of CuO NPs, while

Table 3 Effects of CuO NPs on some biochemical parameters in hemolymph of last instar larvae of *G. mellonella*

CuO NPs concentrations (mg/L)	Control	10	100	1000	LC10	LC30
GLU (g/dL) (Mean* ± S.E)†	63.25 ± 1.74^{a}	47.01 ± 1.16^{b}	27.5 ± 1.15^{c}	63.01 ± 1.12^{a}	52.5 ± 1.14^{b}	118 ± 3.48^{d}
TP (g/dL) (Mean* ± S.E)†	1.46 ± 0.03^{a}	3.03 ± 0.08^{b}	1.62 ± 0.02^{c}	2.53 ± 0.02^{d}	1.52 ± 0.01^{ac}	3.36 ± 0.02^{e}
UA (mg/dL) (Mean* ± S.E)†	0.049 ± 0.002^{a}	0.072 ± 0.002^{b}	0.083 ± 0.001^{c}	0.062 ± 0.001^{d}	0.094 ± 0.003^{e}	0.063 ± 0.002^{d}
UREA (mg/dL) (Mean* ± S.E)†	0.43 ± 0.015^{a}	0.75 ± 0.016^{b}	0.32 ± 0.017^{c}	0.66 ± 0.015^{d}	0.29 ± 0.013^{c}	1.06 ± 0.02^{e}
BILD (mg/dL) (Mean* ± S.E)†	0.018 ± 0.0011^{a}	0.033 ± 0.0015^{b}	0.014 ± 0.0014^{a}	0.025 ± 0.001^{c}	0.017 ± 0.001^{a}	0.08 ± 0.0014^{d}
BILT (mg/dL) (Mean* ± S.E)†	0.031 ± 0.0013^{a}	0.015 ± 0.001^{b}	0.012 ± 0.0011^{b}	0.064 ± 0.0015^{c}	0.022 ± 0.0016^{d}	0.024 ± 0.0013^{d}

*Mean of four replicates per treatment with 20 larvae per replicate

†Means within a line followed by the same lowercase letter are not significantly different (P > 0.05)

§Control Diet (CuO NPs free)

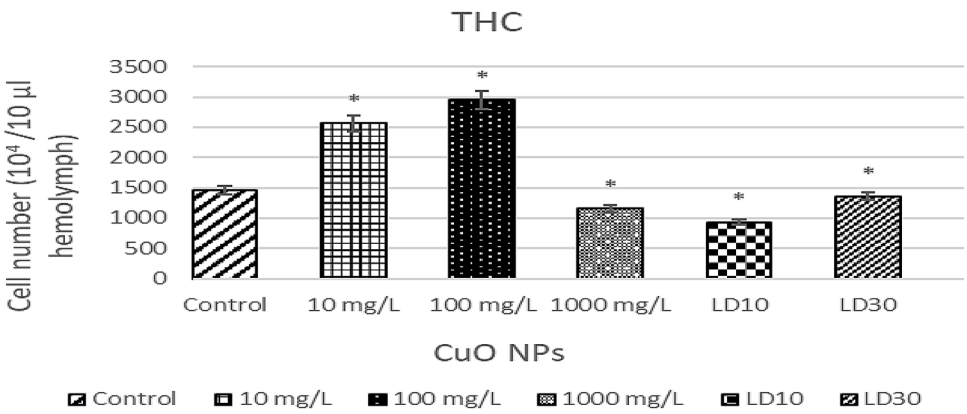


Fig. 1 Effects of CuO NPs on total hemocyte number in hemolymph of last instar larvae of *G. mellonella* (n = 20)

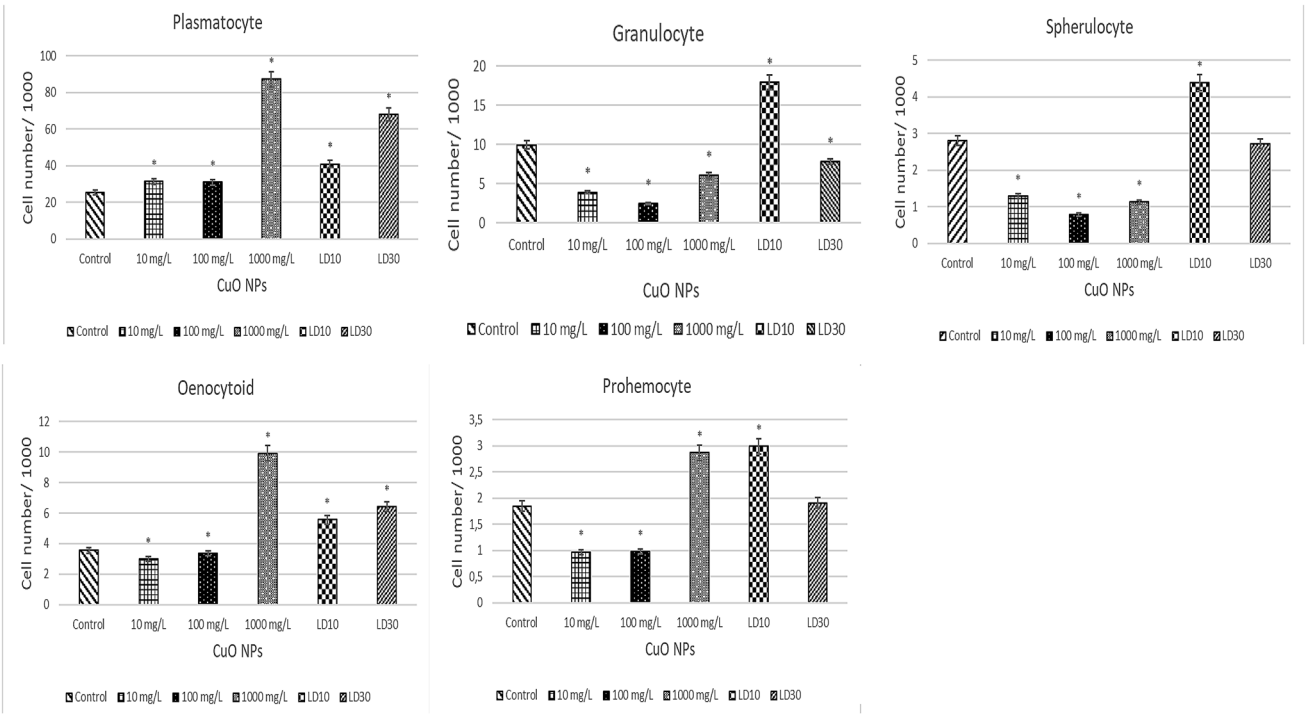


Fig. 2 Effects of CuO NPs on differential hemocyte number in hemolymph of last instar larvae of *G. mellonella* (n = 20)

oenocytiod numbers were greatest in the 1000 mg/L treatment (Fig. 2) ($p < 0.05$).

Discussion

In recent years, changes in AST and ALT enzyme activities have been used as markers in determining the oxidative effects of chemicals with high toxic effects in insects (Sugeçti and Büyükgüzel 2018; Sertçelik et al. 2018). Studies have indicated that ALT and AST enzyme activities increase in connection with cell damage (İçen et al. 2005; Sugeçti and Büyükgüzel 2018). In this study, it was determined that ALT enzyme activity significantly decreased in hemolymph of *G. mellonella* larvae fed with the highest concentration of CuO NPs (1000 mg/L) added to an artificial diet. At the LC30 concentration, ALT activity significantly increased when compared to the control group. AST enzyme activity increased significantly at the highest concentration of CuO NPs (1000 mg/L) and the LC30 concentration. Changes in AST and ALT enzyme activities may have resulted from impaired metabolic metabolism of *G. mellonella* larvae due to the oxidative effect of CuO NPs. AST and ALT enzymes transfer the amino group from amino acids to α-keto acids in order to provide transition between energy source molecules in the tissues of insects belonging to the order Lepidoptera, allowing insects to adapt to oxidative stress (Hakkak et al. 2018). Also, amino acids in insect hemolymph are involved in many metabolic pathways, such as maintaining osmotic pressure, regulating energy provided through proline metabolism, and neurotransmission (Mirhaghparast et al. 2015). In this study, the enzymes responsible for amino acid transfer increased the AST and ALT activities, showing the demands for amino acids to achieve physiological adaptation of *G. mellonella* larvae exposed to CuO NPs. Sugeçti and Büyükgüzel (2018) reported that oxfendazole (1.5%) added to artificial diet causes oxidative stress in *G. mellonella* larvae and increases hemolymph metabolic enzymes AST and ALT activities.

LDH is an important glycolytic enzyme that plays a role in carbohydrate metabolism in many tissues (Shen et al. 2016). LDH enzyme activity increases depending on the oxidative stress level and is used as a cell damage marker (Wand et al. 2013). In this study, it was determined that LDH activity increased at the highest concentration of CuO NPs added to artificial diet, but there was no statistical difference. The LDH activity significantly increased with the LC30 concentration. The reason for this increase may be cell damage due to the oxidative effect of CuO NPs. In another study with similar results, the oxidative effects of *Artemisia annua* L. (Asterasea) extracts on *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) were investigated. In this study, the extract was applied to the leaves at concentrations of 0.09, 0.22, and 0.44 µg/mg to feed the insect. Then, the hemolymph of the

insect was taken after 24, 48 and 72 h to measure metabolic enzymes. It was found that the enzyme activity of AST, ALT and LDH significantly increased in the hemolymph of the insect fed with the highest concentration of extract after 72 h (Zibaee et al. 2011). Mirhaghparast et al. (2015) reported that 4th instar larvae of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) exposed to the insect growth regulator hexafluoride (0, 50, 180 and 470 µg/ml) displayed LDH, AST, and ALT activities that were statistically higher after 24 h. Data from these studies show that biological and chemical agents cause similar results on the same insect.

CK is a metabolic enzyme that increases energy production to protect biosystems during oxidative damage (Skitek et al. 2014). In our study, it was determined that the CK enzyme activity significantly increased in the hemolymph of *G. mellonella* larvae fed with diet containing the highest concentration of CuO NPs (1000 mg/L) added to artificial diet. CK enzyme activity did not change statistically at the LC10 concentration when compared to the control group, but significantly increased at LC30 concentration. The reason for the increase in CK enzyme activity may be the physiological adaptation of the insect to the oxidative effect of CuO NPs. In another study with similar results to our study, the effect of Diaquabis (N, N-diethylnicotinamide-χN1) bis (4-formylbenzoato-χO) cobalt (II) metal complex on the metabolic enzymes of *G. mellonella* larvae was investigated. In this study, hemolymph was isolated 2, 4, 6 and 8 h after the injection of the metal complex. It has been stated that AST, ALT, CK and LDH enzyme activities are significantly increased in hemolymph isolated 8 h after injection (Sertçelik et al. 2018).

Biomolecules, such as protein, lipid and carbohydrate, are very important for the vital activities of insects. These biomolecules are damaged by the oxidative effects of chemical and biological agents (Sugeçti et al. 2016; Kastamonuluoğlu et al. 2020). In this study, the amount of UREA and TP in the hemolymph of *G. mellonella* larvae fed with diets containing the highest concentration of CuO NPs (1000 mg/L) increased significantly compared to the control, but there was no statistical difference in the amount of GLU. The amount of GLU, TP and UREA significantly increased at the LC30 concentration. Changes in the amount of these biomolecules are due to balanced physiological conditions in insects. The amount of TP may have increased in insects as a result of the insect's adaptation to oxidative damage. Çelik et al. (2019) reported that oxyclozanide at different concentrations added to artificial diet significantly increased the amount of TP of *G. mellonella* larvae. Some studies have shown that synthetic chemicals, herbal pesticides, and antibiotics are effective on the insect's biochemical parameters by changing the amount of lipids, carbohydrates, and proteins (Wang et al. 2005; Sezer and Ozalp 2011). The amount of UREA is used in the evaluation of nitrogen metabolism (Hirayama and Nakamura 2002), and increases in the amount of UREA may be due to the deterioration of nitrogen metabolism in the hemolymph of *G. mellonella* larvae of CuO NPs added to artificial diet. In another study, it was stated that nicotinamide reduced the amount of UREA and TP in *B. mori* larvae (Etebari et al. 2004). Etabari et al. (2007) reported that pyriproxyfen (1, 10, 75, 150 and 500 ppm) had no effect on the amount of UREA in *B. mori* larvae. These studies show that different chemicals have different effects on insect's biochemical parameters.

Insects eliminate the oxidative effects of insecticides and environmental pollutants with antioxidant defense system (Kumar et al. 2019). Bilirubin, UA, ALB and glutathione are important non-enzymatic antioxidants (Sharma et al. 2016). In this study, the amount of UA, BILD, and BILT significantly increased in the hemolymph of *G. mellonella* larvae that fed the diet containing the highest concentration of CuO NPs (1000 mg/L) added to artificial diet. The reason for this increase in non-enzymatic antioxidant amounts is the indicator of the antioxidant response developed by the insect against the oxidative effect of the nanoparticle. The amount of BILD and BILT at the LC30 concentration significantly decreased. Cell damage biomarkers AST, ALT and LDH activities significantly increased. Antioxidant defense may be suppressed with high cell damage at the LC30 concentration. Many studies have suggested that there is a negative relationship between oxidative damage and antioxidant defense (Erdem et al. 2016; Aslan et al. 2019; Foret et al. 2019).

Hemocytes, which play an important role in the immune system of insects, are impacted by starvation, parasitism, diseases, and exposure to insecticides, as well as changes in the growth of the insects. Thus, it is known that THC may change under the influence of stress factors. In this study, the number of hemocytes was significantly increased when exposed to 10 and 100 mg/L CuO NPs. The increase of THC may be due to the promotion of hematopoiesis or the release of hemocytes that adhered on surfaces (sessile hemocytes) within the hemocoel (Ghasemi et al. 2014; Kurt and Kayış 2015). Also, the increase of THC may be due to the activation of mitotic division of hemocytes which was activated in response to nanoparticles. Mir et al. (2020) showed that THC of *Bombyx mori* larvae increased immediately after zinc oxide nanoparticle (ZnO NPs) exposure and reached the highest value after 12 h (Mir et al. 2020). Otherwise, another study with *G. mellonella* determined that feeding with ZnO NPs did not show significant changes in all treatment groups (0.5, 1, 2.5, 5 µg/10 µL) (Keskin et al. 2019).

In the present study, it was also determined that the numbers of hemocytes in the group exposed to the highest concentration of CuO NPs (1000 mg/L) was decreased significantly in comparison to the control. Hemocyte density is influenced by the mitotic division of the circulating

hemocytes (Er et al. 2010, 2011). Also, the reduction of THC may result the inhibition of larval hematopoietic function and cell proliferation (Zhu et al. 2012) which may be attributed to the cytotoxicity of metal nanoparticles. Keskin et al. (2019) demonstrated that metal nanoparticles can induce a decrease of hemocyte counts due to the toxic effects of nanoparticles resulting in an increase the number dead hemocytes. Li et al. (2017) also determined that after the exposure to the three nanomaterials (cadmium telluride, silicon dioxide and carbon nanomaterials), hemocyte counts were significantly decreased. The decrease of THC may be due to the damage of hemocytes by the highest concentration of CuO NPs and destruction of the secretion of hemocytes by the hematopoietic organ. Previous studies in marine invertebrates and vertebrates have reported that after a low-level heavy metal exposure, immune responses may increase for a short time, but longer exposure or higher concentrations may inhibit the same responses (Galloway and Depledge 2001). These studies are consistent with our results. In this study, the reason for the decrease in THC count of *G. mellonella* larvae fed a diet with high concentration of CuO NP may be hemocyte apoptosis caused by high concentrations of CuO NPs. This hypothesis is supported by other studies showing increased hemocyte apoptosis in *Spodoptera litura* (Boisd.) (Lepidoptera: Noctuidae) larvae fed with high concentrations of nickel or zinc added to the diet (Xia et al. 2005; Sun et al. 2011). We observed that plasmatocyte numbers increased due to the CuO NPs exosure, whereas granulocyte numbers decreased. It is known that THC and DHC may be enhanced due to stress factors, such as insecticides and heavy metals. Zibaee et al. (2012) showed that THC and numbers of plasmatocytes and granulocytes decreased in *Eurygaster integriceps* adults after exposure to pyriproxyfen which includes in a chemical insecticide group of juvenile hormone analogs. Ghasemi et al. (2014) also reported that THC, plasmatocyte, prohemocyte and spherulocyte numbers decreased in *Ephestia kuehniella* larvae, while granulocyte and oenocytiod numbers increased when exposed to pyriproxyfen. We also determined that prohemocyte numbers decreased with the 10 and 100 mg/L CuO NPs treatments, while a significant increase in the number of plasmatocytes occurred. The immediate response to NP challenge was the differentiation of prohemocytes into phagocytes. This lowered the percentage of prohemocytes and increased that of professional phagocyte called plasmatocytes (Mir et al. 2020).

In conclusion, the results obtained in the present study showed that metabolic enzymes and biochemical parameters are strongly affected by the intoxication of insects with metal nanoparticles. Moreover, metal nanoparticles are also immunotoxins for insects and they affect the morphology and hemocyte index. We assume that metabolic enzymes, biochemical and immune parameters are key biomarkers in assessing tissue injury due to CuO NPs treatments in insects. Adverse effects that may occur in any organisms of ecosystems will be affected by the rest of the ecosystem by food chain or interaction among species. Due to the increased uses of nanoparticles, it should be investigated in all organisms physiologically and morphologically.

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Declarations

Conflict of interest The authors declare no known conflict of interest.

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