Effects of Cu, Zn and their mixtures on bioaccumulation and antioxidant enzyme activities in *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract

The effects of Cu, Zn and their mixture on bioaccumulation and antioxidant enzyme activities of midgut and fat body of *Galleria mellonella* larvae were investigated. Exposure to mixtures of both metals showed a synergistic effect and the accumulation levels were increased in both tissues. When the metals were exposed separately the concentration of Zn increased in both tissues, whereas the concentration of Cu increased in midgut and decreased in fat body. Also, it was determined that, oxidative stress occurred in the midgut and fat body when *G. mellonella* larvae were fed singly and in a mixture with different concentrations of Cu and Zn. In addition, significant changes were observed in antioxidant and detoxification enzyme activities, which are an indicator of oxidative stress. Larvae of *G. mellonella* showed immune responses similar to vertebrates, and could be used as bioindicator species due to being grown easily in the laboratory and reduced research costs Understanding the detoxification mechanism in insects is an important parameter for future ecotoxicological studies on the genotoxic, cytotoxic and physiological effects that different environmental pollutants such as heavy metals can cause.

Keywords Accumulation · Antioxidant enzyme · *Galleria mellonella* · Heavy metals

Introduction

The use of heavy metals and industrial wastes has increased due to the rapid development of technology and industry. Toxicity caused by exposure to heavy metals in organisms can cause adverse effects, such as death, depending on the type of material and its interaction with other metals (Tuncsoy and Erdem 2014). Some metals are essential elements for the continuation of life, reproduction and the maintenance of the immune system’s activities in many organisms. However, they show toxic effects after a certain concentration in living organisms. Once heavy metals enter living organisms, they can interact with proteins and inhibit them by changing their structure and enzymatic function, they can induce toxic effects by replacing essential elements, or some metals can interact with proteins and cause intracellular accumulation (Tuncsoy et al. 2016).

Every living organism regulates its homeostasis, and metals are key players in many transport processes, and enzymatic activities. Zinc (Zn) is a cofactor of thousands of metalloenzymes and proteins. It is also a crucial element, forming active sites in a wide range of enzymes, particularly metalloproteinases and peptidases, in carbonic anhydrase, superoxide dismutase, and naturally the zinc fingers of many DNA-binding proteins (Dow 2017). Copper (Cu) is a catalyst in the reaction of oxidation and reduction, and defense of the cell against oxygen radicals (Yazgan and Yazgan 2014).

Metals can cause oxidative stress by inducing redox reactions and the production of reactive oxygen species (ROS). ROS are produced during normal metabolic processes and are regularly eliminated by the antioxidant defense system. These species cause oxidation of proteins, damage to nucleic acids, lipid peroxidation and activation of the immune system (Hermes-Lima and Zenteno-Savín 2002; Gavrilovic et al. 2017). Antioxidant enzymes eliminate or transform free radicals and ROS, inhibit the reactions that cause the formation of free radicals, or repair...
damage originating from them. Elements such as Cu and Zn are also required for their enzymatic antioxidant activity. Superoxide dismutase (SOD) needs metals such as zinc, copper and manganese for maximum effectiveness, catalase (CAT) needs transition metals such as iron, and glutathione peroxidase (GPx) needs selenium cofactors, but they can also cause oxidative stress by inducing free radical formation when taken in excess.

The increase of ROS impairs the absorption of nutrients and may cause oxidative damage in the midgut cells of insects. Insects, like other eukaryotes, have various biochemical mechanisms which restrict damage of ROS (Bla-gojevic and Grubor-Lajsic 2000; Boardman 2012; Gavrilovic et al. 2017). SOD is the first detoxification enzyme and a powerful antioxidant produced in cells. GPx plays a major role in preventing lipid peroxidation, preserving cells from oxidative stress (Gill and Narendra 2010). Acetylcholinesterase (AChE), Cytochrome P450 (CytP450) and Glutathion-S-Transferase (GST) are charged with detoxification of xenobiotics in organisms. AChE is an important enzyme in nerve impulses for vertebrates and invertebrates (Frasco et al. 2006). The Cyt P450 enzyme plays a part in the growth and development of insects and their resistance and tolerance to pesticides. GST plays an important role in detoxifying toxic compounds and removing oxidative stress caused by them. Increases in these enzyme activities are used as biomarkers to determine the toxic effect of contaminants (Tuncsoy and Mese 2021).

_Galleria mellonella_ L. (Lepidoptera; Pyralidae), which were used as a model organism, show a similar metabolic reaction to mammals. Moreover, due to being reared easily in a short time, it reduces the cost of researches (Cook and McArthur 2013; Maguire et al. 2017; Lange et al. 2018). Researchers mostly prefer to use _G. mellonella_ as a model organism because it is ubiquitous, and can be reared inexpensively, in large quantities, under laboratory conditions without any special equipment (Wojda 2017). Moreover, it is very useful for biochemical research, as it is possible to easily obtain 20–40 μl of hemolymph from one larva (Wojda et al. 2020). The aim of the study is to determine the toxic effects of Zn and Cu, singly and in mixtures, on antioxidants, on detoxification enzyme activities, and on the accumulation of these metals in midgut and fat body of _G. mellonella_.

**Materials and methods**

**Stock culture and application of heavy metal concentrations**

_G. mellonella_ larvae were reared on semi-synthetic diet (bran, honey, glycerol, honeycomb and distilled water) according to Bronksil (1961) in laboratory conditions at 28 ± 2 °C, 70 ± 5% RH. The maintenance of the stock culture was ensured by the hatching of the eggs laid by the females as a result of the mating of _G. mellonella_ adult female and male insects left in the food jars. Experimental solutions were prepared using Zinc (ZnCl2; 30 mg/L) and copper (CuSO4; 10 mg/L) and applied singly and in mixture at 1:1 ratio to the combs via dipping method. Larvae were reared on combs with distilled water as a control. For each replicate, 500 mg combs were used. Control and experimental groups were treated for 72 h. Experiments were run in triplicate with 20 fifth instar larvae in each replicate.

**Bioaccumulation of heavy metals in midgut and fat body**

The dissected midgut and fat body tissues of six larvae were placed in a drying oven at 150 °C for 48 h. Dried tissues were weighted and burnt in nitric acid and perchloric acid mixture (2/1; v/v) at 105 °C for 3 h in fume cupboard. Afterwards, samples were transferred to polyethylene tubes. Total volume completed 5 ml with distilled water. Metal levels in tissues were determined using an ICP-MS Spectrophotometer (PERKIN ELMER) (Muramoto 1983).

**Isolation and homogenization of tissues**

Midgut and fat body samples of six larvae for each replicate were homogenized in a phosphate buffer (20 mM; pH 7.6) at 4 °C by ultra turrax and ultrasonic homogenizer. The homogenate was centrifuged at 500 g for 15 min (+4 °C). Supernatants were put into another tube and recentrifuged at 12000 g for 45 min (+4 °C). Then, the filtration procedure was performed to eliminate low molecular weight protein. Sephadex® G-25 gel columns were used for filtration (Gonzalez-Reya et al. 2014). Sephadex® G-25 gel columns were used for filtration (Gonzalez-Reya et al. 2014). The samples were stored at −80 °C until the determination of enzyme activities.

**Determination of enzyme activities**

SOD activity was based on the decrease in cytochrome c by the xanthine oxidase / hypoxanthine system at 550 nm (McCord and Fridovich 1969). CAT activity was based on the measurement of reduced absorbance released by the degradation of H2O2 at 240 nm (Greenwald 1985). GPx activity was measured in the presence of glutathione reductase, reduced glutathione and cumene hydroperoxide as substrate, following oxidation of NADPH at 340 nm (Lawrence and Burk 1976). GST activity was determined by measuring the total GST activity catalyzing the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with
reduced glutathione at 340 nm (Habig et al. 1974). Tris HCl (100 mM, pH: 8.0) solution at a ratio of 1: 5 and 10% Triton in a volume 10 times the amount of Tris HCl were added onto the midgut and fat body. Samples were centrifuged at 12,000 g for 30 min at 4 °C. AChE activity was detected by Ellman et al. (1961). The samples were measured kinetically in the spectrophotometer at 405 nm absorbance. Cyt P450 enzyme activity was determined using PNOD (4-nitroanisole) as substrate at 405 nm (Rose et al. 1995). Total protein was evaluated according to the Bradford (1976) method using Bovine serum albumin as a substrate.

Statistical analysis

The statistical differences between the control and experimental groups were detected by a series of analysis of variance (ANOVA) and Student-Newman’s (SNK) procedure by using the SPSS 21.00 program. Differences were considered significant when $p < 0.05$.

Results

Metal accumulation in fat body and midgut

While a decrease in the accumulation level was detected in the fat body of the Cu singly applied group, no statistical differences were observed in the group applied the mixture with Zn (Fig. 1; df: 2; $F$: 3696.5; $p < 0.05$). Cu accumulation was increased in the midgut of G. mellonella larvae when exposed to Cu singly and in a mixture with Zn according to the control group (Fig. 1; df: 2; $F$: 398.24; $p < 0.05$).

Accumulation occurred in the fat body of the Zn singly applied group, while a significant decrease was observed when Cu and Zn were applied in mixture (Fig. 2; df: 6; $F$: 30,476.09; $p < 0.05$). In the midgut, accumulation was also seen in the groups that were treated with Zn alone and the mixture (Fig. 2; df: 2; $F$: 3696.5; $p < 0.05$).

Enzyme activity

Superoxide dismutase (SOD) activities

SOD activity in the fat body of G. mellonella larvae decreased by 56.9%, 10.8% and 68.2%, respectively, in the groups treated with Cu, Zn and mixture (df: 3; $F$: 431,930.18; $p < 0.05$). Significant increases were determined in the Cu and Zn applied groups (286% and 22.7%, respectively) in the midgut (df: 3; $F$: 1576134). However, a significant decrease was observed (42.4%) in the mixture applied group in the same tissue (Fig. 3; $p < 0.05$).

Catalase (CAT) activities

CAT activity in the fat body of G. mellonella larvae increased by 68.5% in the groups treated with Cu, whereas significant decreases were observed in the Zn and mixture applied groups (9.8% and 38.8%, respectively) (df: 3; $F$: 119.145). In the midgut, a significant increase was determined in Cu (293%) while significant decreases were detected in the Zn and mixture applied groups (55.3% and 60%, respectively) (Fig. 4) (df: 3; $F$: 14,378.58; $p < 0.05$).

Glutathion peroxidase (GPx) activities

Significant decreases were observed in midgut and fat body of G. mellonella larvae in all treatment groups compared to the control. GPx activities in fat body of G. mellonella larvae decreased by 40.2%, 20.5% and 19.5%, respectively, in all applied groups (df: 3; $F$: 1478.83; $p < 0.05$). In midgut, significant decreases were also observed in all applied groups according to the control group (62.3%, 29.75% and 58%, respectively). (Fig. 5) (df: 3; $F$: 241.15; $p < 0.05$).
Glutathione-S-transferase (GST) activities

GST activities were increased significantly in the fat body of larvae applied Cu and Zn singly (115% and 155%, respectively). A significant decrease was observed in the mixture applied group compared to the control group (81.7%) (df: 3; F: 219.48; p < 0.05). In the midgut, GST activity was significantly increased in the Cu applied groups, whereas a significant decrease was observed in the mixture applied groups (29.3%) (df: 3; F: 108.44; p < 0.05). GST activity of the mixture group showed a significant decrease in both tissues compared to Cu and Zn application groups (p < 0.05) (Fig. 6).

Acetylcholinesterase (AChE) activities

In the fat body, a significant increase in the AChE activity was observed in the Zn application group (168%), while there was a significant decrease in the Cu (43%) and mixture groups (75%) compared to the control (df: 3; F: 420.02; p < 0.05). In the midgut, it was observed that AChE activities were increased with Cu (62.5%) and Zn (62.6%) applied singly (df: 3; F: 19.01; p < 0.05). AChE activity showed a significant decrease in the mixture treatment groups in both tissues compared to Cu and Zn groups (p < 0.05) (Fig. 7).

Cytochrome P450 (Cyt P450) activities

In the fat body, Cyt P450 activity was decreased in the mixture applied group compared to to the control (31.5%), while a significant increase was determined in the Zn applied group (86.14%) (df: 3; F: 38.99; p < 0.05). In the midgut, significant increases were observed in the Cu and Zn applied groups compared with the control group (435.2% and 480%, respectively) (df: 3; F: 62.34; p < 0.05) (Fig. 8).

Discussion

Heavy metals are known to have an effect on insect growth rates and mortality. Moreover, they have a direct effect on...
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Fig. 7 Effects of Cu, Zn and their mixtures on the AChE enzyme activity of G. mellonella in midgut and fat body. Data shown are means ± SE (Student–Newman–Keul’s test; asterisks indicate significant differences respect to control at p < 0.05)

Fig. 8 Effects of Cu, Zn and their mixtures on the CytP450 enzyme activity of G. mellonella in midgut and fat body. Data shown are means ± SE (Student–Newman–Keul’s test; asterisks indicate significant differences respect to control at p < 0.05)

some innate insect resistance mechanisms such as the immune and detoxification systems. Antioxidant and detoxification enzymes are good indicators of oxidative damage caused by metals. Some parameters are used as biomarkers in toxicity measurements such as bio-transformation enzymes (CytP450) (Tuncsoy and Mese 2020), oxidative stress (CAT, SOD, GPx) (Tuncsoy et al. 2018), hematological (transaminases) (Sugecti and Buyukguzel 2018), neuromuscular (AChE) (Hualing et al. 2020) and genotoxic (DNA damage) (Javed et al. 2017) parameters. Biomarkers provide information about the potential xenobiotic mechanisms. In this study, the effects of G. mellonella larvae fed comb with Cu (10 mg/L), Zn (30 mg/L) and their mixture on the antioxidant enzyme activities and accumulation levels in the midgut and fat body were investigated.

Similar studies have shown that the accumulation of heavy metals in tissues cause toxic effects when they reach critical values (Li et al. 2005; Tkachenko and Kurhaluk 2012). In insects, large amounts of accumulated metals are taken up by metal-binding proteins and stored in intracellular spheres to prevent them from interacting with biomolecules and reactions in midgut tissues. When midgut cells reach the metal storage capacity, cells break down and the accumulated metals are released into the lumen and excreted with feces. This process damages the epithelial cells and disrupts the midgut processes (Perić-Mataruga et al. 2018). Moreover, the gut is the first organ to be exposed to metals and other environmental xenobiotics taken up by insects and to defend against them. In addition, it also contains antioxidant effects, detoxification, reform of digestive enzyme activities, and expression of different enzyme isoforms (Wang et al. 2020).

The fat body of insects is analogous with liver in vertebrates, and it is known that xenobiotics mostly accumulate in this tissue (Yang et al. 2007). According to the results of this study, metal accumulation was higher in mixture than in single exposures to Cu in the fat body, while the Cu accumulation was lower in mixture compared to the Cu singly applied group in the midgut. In the fat body, the metal mixture had a synergistic effect on metal accumulation compared to the Cu singly applied group, while an antagonistic effect in the midgut was observed. Moreover, metal accumulation in the fat body decreased in the mixture group compared to the Zn singly applied group, while an increase occurred in the midgut. The mixture of metals showed an antagonistic effect in fat body compared to the Zn singly applied group, while a synergistic effect occurred in the midgut. Hence, it is thought that these differences between tissues may have occurred due to the different metabolic activities in the tissues. In addition, the decrease in metal accumulation may be due to the fact that Cu and Zn may have been competing as trace elements (Duran et al. 2015).

Long-term exposure to metals also causes metals to accumulate in organisms. The accumulation of metals increases the formation of high superoxide radicals (O2−) or derivatives such as hydrogen peroxide (H2O2) (Gopi 2019). SOD, CAT and GPx are the first defense antioxidants to neutralize any molecules that have the potential to become free radicals or any free radicals that can trigger the production of other radicals (Ighodaro and Akinloye 2018). SOD catalyzes the transformation of O2− to H2O2 and O2 (McCord and Fridovich 1969). H2O2 transforms into OH− (hydroxide) radical by Fenton and Haber Weiss reaction in the presence of metal ions (Gomes 2012). CAT transforms toxic hydrogen peroxide generated as a result of superoxide dismutase activity into water and oxygen (Duthie et al. 1989). GPx plays an important role in the process of preventing lipid peroxidation (Gill and Narendra 2010). In this study, SOD activity was increased in the Cu and Zn groups in the midgut, while it decreased in the fat body of G. mellonella larvae compared to the control. CAT activity increased in both tissues in Cu applied groups compared to
the control, while it decreased in the midgut in Zn applied group. It is thought that due to the high toxic effect of Cu, the SOD produces excessive H$_2$O$_2$ and the CAT activity may have increased to eliminate the excessively produced H$_2$O$_2$. In *Metaphire posthuma* (Annelida: Clitellata: Oligochaeta), it was reported that different concentrations of CuSO$_4$ and CuO NPs caused a decrease in SOD activity and the lowest decrease occurred at the highest concentration (Gautam 2018). Additionally, it was demonstrated that there was a significant increase in the CAT activity of *Spodoptera littoralis* (Lepidoptera: Noctuidae) fed with the extract of castor leaves treated with ZnO NPs and AgNPs (Ibrahim and Ali 2018). Another study with *G. mellonella* demonstrated that larvae fed with increasing concentrations of Cr and Pb caused an increase in CAT and SOD enzyme activity compared to control (Wu and Yi 2015). In this study, a significant decrease in GPx activity was observed in all experimental groups compared to control. Moreover, it was observed that GPx activity in the group treated with Cu was lower than CAT and SOD enzymes. In addition, GPx activity showed an increase in the Zn and mixture applications compared to SOD and CAT activity. Its known that activity of CAT is more effective in high concentrations of H$_2$O$_2$, while GPx activity is more effective in lower H$_2$O$_2$ concentrations (Duthie et al. 1989). Li et al. (2005) observed that in *Oxya chinensis* (Orthoptera Acridoidae), GPx activity decreased due to increase in Cd concentration. Zn is a cofactor of thousands of metalloenzyme and proteins (Weiss and Carver 2018) and also a crucial element, forming active sites in a wide range of enzymes and the zinc fingers of many DNA-binding proteins (Dow 2017). Additionally, although Cu is an integral part of many vital enzymes, it can cause oxidative damage (Nikolić et al. 2016) and accumulation in tissues at increasing concentrations. Moreover, it has been observed that antioxidant enzyme activities change according to the species, developmental stage and tissues.

In this study, Cyt P450 activity increased in the midgut when Cu and Zn were applied singly, while GST activity increased only in the Cu applied group. In the fat body, GST and CytP450 activities were increased in the Cu and Zn applied groups when compared to the control. P450 enzymes are mainly involved in the first stage of xenobiotic metabolism, while GST enzymes are mainly involved in the second stage and responsible for the modification and conjugation of polar compounds. GST and Cyt P450 enzymes are found in the tissues of many organisms, generally in the liver. GST plays a role in protecting cellular integrity, preventing oxidative stress responses and DNA damage by catalyzing endogenous and exogenous xenobiotics, and conjugation of glutathione with various electrophilic substrates (Casalino et al. 2004; Mao et al. 2018). In studies with different insect species, P450 enzyme activities were found to be at the highest levels, especially in the midgut and malpighi tubes (Tuncsoy and Ozalp 2021). In a study with *Helicoverpa armigera* (Lepidoptera: Noctuidae), as a result of treatment of pentamethylbenzene and naphthalene, it was observed that the amount of increase in Cyt P450 activity was higher in the midgut compared to the fat body (Qiu et al. 2003). In the present study, AChE enzyme activities in the midgut were increased in the Zn and Cu singly applied groups when compared with the control. In the fat body, AChE activity was also increased in the Zn applied group, while the enzyme activity was reduced in the Cu applied group. Also, it is thought that as a result of the toxicity of Cu and Zn, the increase in free radical formation and the decrease in AChE activity may result from the inactivation of this enzyme by free radicals. In addition, it was commented that increased free radicals might have reduced the activity of enzymes by interacting with metal ions, which are cofactors of these enzymes, or with active amino acids in the enzyme structure (Tuncsoy et al. 2018).

In this study, it was observed that there was a significant decrease in the enzyme activities in the midgut and fat body of the mixture applied group. Also, decreases in enzyme activities in both tissues were observed when the mixture applied group was compared with the singly applied metals groups. Excessive production of ROS with the effect of increased metal accumulation in tissues may have caused deficiency of defense systems or inhibition of enzymes. Moreover, it is thought that these differences may be due to applied time, different size and concentration of metals (Benavides 2016; Ibrahim and Ali 2018; Gautam 2018).

Innate immune parameters and antioxidant enzymes are used as indicators to determine the stress tolerance of invertebrates and environmental pollution. *G. mellonella* larvae and studies with other species show that heavy metals accumulate in tissues, and when accumulation reaches critical levels, may cause toxic effects and changes in antioxidant and detoxification enzymes, which are oxidative stress biomarkers. Changes in such enzymes are used as biomarkers in determining the toxic effects of xenobiotics.

**Conclusion**

In this study of *G. mellonella* larvae fed concentrations of Cu and Zn singly and in mixture, oxidative stress is observed in the midgut and fat body, with significant decreases and increases in antioxidant and detoxification enzyme activities.

Insects that can be exposed to heavy metals naturally or by humans, through air, water or food, can be used as bioindicators in determining environmental pollution levels. *G. mellonella* can be produced easily in the laboratory due to their short life cycles and rapid reproduction. Because of these
properties, they are widely used as an alternative organism to vertebrates in studies of human acute toxicity, bacterial and fungal pathogenicity, and antimicrobial pharmacokinetic effects. Understanding the reactions of G. mellonella, a model organism with immune system responses similar to vertebrates and bioindicator species, to metal detoxification systems, which are an important parameter in insect physiology, is thought to contribute to future toxicological, genotoxic, physiological and ecotoxicological studies.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

BT and YM analyzed the enzymatic activities and hemocyte numbers. All authors were the contributor in writing the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

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