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. The application of metals as a mixture showed a synergistic effect and the accumulation levels were increased in both tissues. Zn accumulation increased in midgut and fat body of *G. mellonella* larvae exposed to metal singly. On the other hand, Cu accumulation increased in midgut, while a decrease was observed in fat body exposed to Cu singly. Moreover, it was determined that oxidative stress was occurred in midgut and fat body of *G. mellonella* larvae with significant decreases and increases in antioxidant and detoxification enzyme activities when fed singly and in mixture with different concentrations of Cu and Zn. Understanding the reactions of *G. mellonella*, which is a model organism showing immune system responses similar to vertebrates and bioindicator species, to metals by detoxification systems, which is an important parameter in insect physiology, is thought to contribute to future toxicological, genotoxic, physiological and ecotoxicological studies. *G. mellonella* larvae, which were used as a model organism, shows a similar reaction to mammals.

Introduction

The use of heavy metals and industrial wastes has increased due to the rapid development of technology and industry. In metal toxicity, which has gained importance recently, metal concentrations cause toxic effects that can go up to death in living things, depending on the type of metal and its interaction with other metals. 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. Heavy metals, which are resistant to environmental conditions, accumulate in foods and biological systems. Metals taken into the organism can interact with proteins and inhibit them by changing their structural and enzymatic functions, may exert toxic effects by replacing essential elements, or some toxic metals may interact with proteins and cause intracellular accumulation (Tuncsoy et al. 2016) .

Every living organism regulate its homeostasis, and metals are key players in the transport processes, and many enzymatic activities. Zn is a cofactor of thousands of metalloenzyme 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). 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 as normal metabolic processes and are regularly eliminated by antioxidant defense system. These species cause oxidation of protein, damage to nucleic acid, lipid peroxidation and activation of immune system (Hermes-Lima and Zenteno-Savin 2002; Gavrilovic et al. 2017). Antioxidant enzyme eliminates free radicals and ROS or transform them to impair molecules, and

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. SOD needs metals such as zinc, copper and manganese for maximum effectiveness, CAT needs transition metals such as iron, and 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 have various biochemical mechanisms like other eukaryotes, restrict damage of ROS (Blagojevic and Grubor- Lajsic 2000; Boardman et al. 2012; Gavrilovic et al. 2017). Superoxide dismutase (SOD) is the first detoxification enzyme and high extensive powerful antioxidant produced in a cell. Glutathion peroxidase (GPx) plays a major role during prevention of lipid peroxidation and so the cell preserve from oxidative stress (Gill and Tuteja 2010). Acetylcholinesterase (AChE), Cytochrome P450 (CytP450) and Glutathion-S-Transferase (GST) are charged with detoxification of xenobiotics in organisms. AChE are important enzymes in nerve impuls for vertebrate and invertabrates (Frasco et al. 2005). The 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 xenebiotics or contaminants.

G. mellonella L. (Lepidoptera; Pyralidae), which were used as a model organism, shows a similar reaction to mammals. Thus they can rearrange easily and in a short time in vitro also reduces the cost of researches (Cook and McArthur 2013; Maguire et al. 2017; Lange et al. 2018).

The aim of the study is to determine the toxic effects of Zn and Cu singly and in mixture on antioxidant, detoxification enzymes activities and 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 in semi-synthetic diet (bran, honey, glycerol, honeycomb and distilled water) according to Bronksil (1961) in laboratory conditions at 28 ± 2 °C, $70 \pm 5\%$ RH. Copper 10 mg/L and zinc 30 mg/L and 1: 1 copper-zinc solutions were prepared for feeding the larvae. Copper and zinc solutions were applied to the combs by dipping method and the control group larvae were fed with combs with distilled water. 20 fifth instar larvae were used in the experiment. Control and experimental groups were treated for 72 h. Experiments were run in triplicate being 20 larvae in each replicate.

Isolation and homogenization of tissues

After dissection, midgut and fat body samples were homogenized at 4 °C by ultra turrax and ultrasonic homogenizer. The homogenate were 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). After 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). The samples were stored at -80 °C until the determination of enzyme activities.

Bioaccumulation of heavy metals in midgut and fat body

The dissected midgut and fat body tissues 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 (Muramoto 1983). 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).

Determination of enzyme activities

SOD activity is based on the measuring the decrease in cytochrome c by xanthine oxidase / hypoxanthine system at 550 nm (McCord and Fridovich 1969). CAT activity is based on the measurement of the 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 minutes at 4°C. AChE activity was detected by Ellman et al. (1961). The samples were measured kinetically in the spectrophotometer at 405 nm absorbance. CytP450 enzyme activity was determined using PNOD (4-nitroanisole) as substrate. Samples were measured 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 data between the control and test groups were calculated using the SNK test in the SPSS 21.0 program. P<0.05 was considered statistically significant.

Results

Metal accumulation

While a decrease in bioaccumulation level in fat body was detected in the group in which Cu was applied alone, no statistical differences were observed in the group applied in mixture with Zn. In midgut, it was

determined that bioaccumulation was occurred in the group treated with Cu alone and in a mixture with Zn when compared with the control group (Fig. 1 and Fig 2.; $p < 0.05$).

SOD activities

It was determined that the SOD activity in 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. Otherwise, significant increases were determined in Cu and Zn applied groups (286 % and 22.7 %, respectively) in midgut whereas a significant decrease was observed (42.4 %) in mixture applied group (Fig. 3.; $p < 0.05$).

CAT activities

CAT activity in 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). Otherwise, in midgut, a significant increase was determined in Cu (293%) while significant decreases were detected in Zn and mixture applied groups (55.3 % and 60 %, respectively) (Fig. 4.; $p < 0.05$).

GPx activities

Significant decreases were observed in midgut and fat body of *G. mellonella* larvae in all treatment groups compared according to the control. It was determined that GPx activities in fat body of *G. mellonella* larvae decreased by 40.2 %, 20.5 % and 19.5 %, respectively, in the all applied groups. 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; $p < 0.05$).

GST activities

While GST activity increased in Cu and Zn groups in fat body compared to control, it decreased in the mixture group and these differences were considered significant. It was determined that GST activities were increased significantly in fat body of larvae Cu and Zn applied singly (115 % and 155 %, respectively). Otherwise, a significant decrease was observed in mixture applied group according to the control group (81.7 %). In fat body, GST activity was increased in Cu applied groups whereas significant decreases were observed in Zn and mixture applied groups (3.6 % and 29.3 %, respectively).

GST activity of the mixture group showed a significant decrease in both tissues compared to Cu and Zn application groups (Fig 6; $p < 0.05$).

AChE activities

A significant increase 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. In midgut, it was observed that AChE activities were increased when Cu and Zn applied singly, whereas a significant decrease was observed in the mixture group.

determined in mixture applied group (9.4 %). AChE activity showed a significant decrease in the mixture treatment groups in both tissues compared to Cu and Zn groups.

Cyt P450 activities

Cyt P450 activity was decreased in mixture applied group according to the control (31.5 %), while a significant increase was determined in fat body of Zn applied group (86.2 %). Otherwise, in Cu and Zn applied groups, significant increases were observed compared with the control group (317 % and 153 %, respectively). Also enzyme activity showed a significant decrease in both tissues in the mixture group compared to Cu and Zn groups.

Discussion

Heavy metals are known to have an effects on insect growth rates and mortality. Moreover, they have a direct effect on 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 biotransformation enzymes (CytP450) and their products (protein, RNA, DNA), oxidative stress (CAT, SOD, GPx), hematological (transaminases), neuromuscular (AChE) and genotoxic (DNA damage) parameters. Biomarkers provide information about the potential xenobiotic mechanisms. In this study, the effects of *G. mellonella* larvae fed with different concentrations of Cu (10 mg /L), Zn (30 mg /L) and thier 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 (Peric-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 also defend against them, in addition, it also include many events such as an antioxidant effects, detoxfication, reformed of digestive enzyme activities, expression of different enzyme isoforms. (Wang et al. 2020).

Fat body of insects is analog with liver in vertebrates and it was known that xenobiotics mostly overaccumulate in this tissue. According to the results of this study, the implementation of metals as a mixture showed a synergistic effect and it has increased the accumulation in both tissues. When using metals singly, Zn levels increased in midgut and fat body. Moreover, Cu level increased in midgut whereas decreased in fat body. Long-term exposure to metals causes metals to accumulate in organisms. The accumulation of metals increases the formation of high superoxide radicals (O_2^-) or derivatives such as

hydrogen peroxide (H_2O_2) (Gopi et al. 2019). SOD, CAT and GPx are the first defense antioxidant to neutralize any molecule that has 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 O_2^- to H_2O_2 and O_2 (McCord and Fridovich 1969). H_2O_2 transforms into $\text{OH}^\cdot$ (hydroxide) radical by Fenton and Haber Weiss reaction in the presence of metal ions (Gomes 2012). Catalase transforms toxic hydrogen peroxide generated as a result of superoxide dismutase activity into water and oxygen (Duthie 1989). GPx plays an important role in the process of preventing lipid peroxidation (Gill and Tuteja 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 copper, the SOD produces excessive H_2O_2 and the CAT activity may have increased to eliminate the excessively produced H_2O_2 . In *M. posthuma*, it was reported that different concentrations of CuSO_4 and Cu NP caused a decrease in SOD activity and the highest decrease was occurred at the highest concentration (Gautam et al. 2018). Additionally, it was demonstrated that there was a significant increase in the CAT activity of *S. littoralis* 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 that an increase in Zn and mixture applications compared to SOD and CAT activity. It is known that activity of CAT is more effective in high concentrations of H_2O_2 , while GPx activity is more effective in lower H_2O_2 concentrations (Duthie et al. 1989). Li et al. (2005) observed that in *Oxya chinensis*, GPx activity decreased due to increase in Cd concentration. Zinc 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 copper is an integral part of many vital enzymes, it can cause oxidative damage (Nikolic et al. 2016) and accumulation in tissues at increasing concentrations. In addition, it has been observed that antioxidant enzyme activities change according to the species, developmental stage and tissues.

In this study, CytP450 activity increased in the midgut when Cu and Zn applied singly, while GST activity increased only in the Cu applied group. In fat body, GST activity was increased in Cu and Zn applied groups, while CytP450 only increased in the Zn applied group 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, conjugation of glutathione with various electrophilic substrates (Casalino et al. 2004; Mao et al. 2019). In studies with different insect species,

P450 enzyme activities were found to be at the highest levels, especially in the midgut and malpigi tubes (Tuncsoy 2017). In a study with *Helicoverpa armigera*, as a result of treatment of pentomethylbenzene and naphthalene, it was observed that the amount of increase in CytP450 activity was higher in the midgut compared to the fat body (Qiu et al. 2003). In this study, AChE enzyme activity in midgut has increased in both treatment groups compared with control, while in fat body increased in Zn treatment and reduced in Cu. In a study, it is thought that as a result of the toxicity of Pyriproxyfen and *B.*

Thuringiensis, 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, the authors 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 2018). In this study, it was observed that there was a significant decrease in the enzyme activities analyzed both in midgut and fat body of the larvae fed with the mixture applied diet. Also, when mixture group is compared with the other two metals, a decrease in enzyme activities in both tissues. Excessive production of ROS with the effect of increased metal accumulation in tissues may have caused deficiency of defense systems or inhibition of enzymes. Also, it is thought that these differences may be due to applied time, different size and concentration of metals (Benavides et al. 2016; İbrahim and Ali 2018; Gautam et al. 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 showed that heavy metals accumulate in tissues and when reached the accumulation concentration critical levels, may cause toxic effects and also changes in antioxidant and detoxification enzymes, which are oxidative stress biomarker. Changes in such enzymes are used as biomarkers in determining the toxic effects of xenobiotics.

Conclusion

In the study, it was determined that *G. mellonella* larvae, when fed singly and in mixture with different concentrations of copper and zinc, cause oxidative stress in the midgut and fat body, with significant decreases and increases in antioxidant and detoxification enzyme activities, which are an indicator of oxidative stress.

Insects that can be exposed to heavy metals naturally or human-induced by air, water and oral way and can be used as bioindicators in determining environmental pollution levels. Besides, they can be produced easily in the laboratory due to their short life cycles and rapid reproduction. Due to these properties, it is 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*, which is a model organism showing immune system responses similar to vertebrates and bioindicator species, to metals by detoxification systems, which is an important parameter in insect physiology, is thought to contribute to future toxicological, genotoxic, physiological and ecotoxicological studies.

Declarations

Funding

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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

Authors' contribution

BS 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.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

Figure 1

Accumulation of Cu in midgut and fat body of *G. mellonella* exposed to metals singly and mixture. Data shown are means $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P<0.05$).

Figure 2

Accumulation of Zn in midgut and fat body of *G. mellonella* exposed to metals singly and mixture. Data shown are means $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Figure 3

Effects of Cu, Zn and their mixtures on the SOD enzyme activity of *G. mellonella* in midgut and fat body. Data shown are means $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Figure 4

Effects of Cu, Zn and their mixtures on the CAT enzyme activity of *G. mellonella* in midgut and fat body. Data shown are means $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Figure 5

Effects of Cu, Zn and their mixtures on the GPx enzyme activity of *G. mellonella* in midgut and fat body. Data shown are means $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Figure 6

Effects of Cu, Zn and their mixtures on the GST enzyme activity of *G. mellonella* in midgut and fat body. Data shown are means $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Figure 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 $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Figure 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 $\pm$ SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).