Synergistic Effects of Zn, Cu, and Ni and *Bacillus Thuringiensis* On the Hemocyte Count and the Antioxidant Activities of *Hyphantria Cunea* Drury (Lepidoptera: Arctiidae) Larvae

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Abstract

Insects are model organisms for immunological studies. The cellular and the antioxidant enzyme responses of insects are major bioindicators against environmental stresses (metal exposure, infection, etc.). In our study, the differences in the hemocyte counts and the antioxidant enzyme activities of *Hyphantria cunea* larvae exposed to the different amounts of zinc, copper, and nickel and *Bacillus thuringiensis* infection were determined. With metal exposure, the superoxide dismutase, catalase, and glutathione peroxidase activities increased, but the hemocyte counts decreased. Additionally, both the hemocyte counts and the enzyme activities increased with *Bacillus thuringiensis* infection. As a result of this study, we found that the superoxide dismutase, catalase, and glutathione peroxidase and the hemocyte counts varied in response to both metal exposure and bacterial infection.

Introduction

Metals formed as a result of the natural processes and the anthropogenic activities are among the most important causes of water, soil, and plant pollution. While low amounts are essential for life, they show toxic effects at high concentrations (Cabassi 2007). Therefore, the balance of metals in the environment is very crucial. Metals affect the growth rate and the survival of herbivores (Ali et al. 2019), as well as immune function (Borowska and Pyza 2011; Pagliara and Stabili 2012). However, they can cause oxidative stress by increasing the amount of reactive oxygen species (ROS) (Koivula and Eeva 2010) (Fig. 1). To prevent ROS damage, the living organisms have complex defence mechanisms that contain antioxidants (Howe and Schillmiller 2002). Antioxidant enzymes are crucial in removing ROS from biological systems. The main antioxidant enzymes in insects are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (Mittapalli et al. 2007). SOD converts the superoxide radicals into molecular oxygen and H_2O_2 , while both CAT and GSH-Px convert H_2O_2 to oxygen and water (Ma et al. 2017). Also, the hemocytes are essential headliners of the insect immune system occurring at the cellular level, so they are important indicators in determining the cellular immune response.

Hyphantria cunea Drury (Lepidoptera: Arctiidae) is an extreme polyphagous insect (Firidin et al. 2008). Its high fecundity, short generation time, and high starvation resistance facilitate its spread and potential to damage crops (Xu et al. 2019). The insect is a significant pest in many parts of the world (Ji et al. 2003) and also causes a loss of many crops in Turkey. In our study, we have chosen the *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*), which is the most widely used microbial control agent (Saruhan et al. 2014). In this study, we selected zinc (Zn), copper (Cu), and nickel (Ni) from the most common and studied metals (van Ooik and Rantala 2010; Cheruiyot et al. 2013) in nature. We aimed to investigate how *B. thuringiensis* infection affected both the hemocyte counts and the antioxidant enzyme activities of *H. cunea* larvae, which consumed diets containing metals at different amounts.

Materials And Methods

Obtaining larvae and preparing artificial diets

From Çarşamba District of Samsun Province of Turkey, *H. cunea* larvae were collected in 2020. They brought to the laboratory were kept at $25\pm 2^{\circ}\text{C}$ and 70% humidity (16 h light/8 h dark) and were let to feed a control diet (CD), developed by Yamamoto (1969) until they reached the pupal stage. The larvae of the 2nd generation obtained from the 1st generation were used for the experiment. Zn and Cu used in the study were purchased from Sigma-Aldrich (Darmstadt, Germany) and Ni was purchased from Merck (Darmstadt, Germany). By the purpose of the research, various diets were prepared by adding 0.788 g L^{-1} , 1.576 g L^{-1} , and 2.364 g L^{-1} zinc, copper, and nickel. We used the metal amounts we determined in our previous study (Topkara and Yanar 2019). With the addition of zinc, copper, and nickel to the control diet, a total of 20 different diets were obtained (Table 1).

Bacterial culture conditions

Btk was used in larval infection. The strain was obtained from culture collection of microbiology laboratory at Karadeniz Technical University. The *Btk* was grown overnight at 30°C in nutrient broth (AppliChem, Darmstadt, Germany). The optical density of the growing culture was measured at a wavelength of 600 nm and set to $\text{OD}_{600} = 1.89$ (Danismazoglu et al. 2012). For infected groups, 1 mL of the bacterial suspension at this density was sprayed onto artificial diets.

Experimental setups

For both the control and the infected groups, 100 larvae were used to determine the enzyme activities whereas 50 larvae were used to determine the hemocyte counts. The larvae in the control groups were fed control diet for five days, and then the hemolymph of the larvae were taken by cutting third legs of the larvae. After five days, 1 mL of *Btk* suspension was sprayed into the diet of the larvae to be infected and continued to be fed for two more days. Then, the hemolymph of the larvae was taken, enzyme analyses were performed, and the hemocytes counted.

Giemsa staining

The insect hemolymphs to be used in the hemocyte counting were placed in the Eppendorf tubes, and 10 μL of the hemolymph was spread on the each slide. After drying the hemolymphs spread on the slide, staining steps were started with Giemsa. After staining, the fully protected preparations were obtained, and the hemocytes were counted with a microscope.

Enzyme analysis

The hemolymph samples taken from the larvae were homogenized with an ultrasonic processor (VCX 130 Sonics, Newtown, CT, USA). The homogenates, 20 mL each, were centrifuged for 20 minutes at 15000 rpm a refrigerated centrifuge (model 3500, Kubota, Tokyo, Japan). Protein determination in the study was made according to the method of Lowry et al. (1951). For this process, the intensity of the color caused by amino acids in the side chain of the reduced copper and proteins by reducing the Folin-Phenol reagent was measured spectrophotometrically at 595 nm. Superoxide dismutase activity was

determined by the method of Flohé and Ötting (1984) and the spectrophotometric method of McCord and Fridovich (1969). To determine the SOD activity, the reduction of cytochrome c by the xanthine/xanthine oxidase system was spectrophotometrically measured at 550 nm. While catalase activity was determined by the Lück (1963) method, glutathione peroxidase activity determination was carried out by the method of Lawrence and Burk (1976). CAT activity was determined spectrophotometrically with the decrease in 240 nm absorbance due to H_2O_2 degradation. The GSH-Px activity was measured spectrophotometrically at 340 nm under the cofactor of glutathione reductase and NADPH in the reaction medium. A UV/Vis spectrophotometer (model T70, Pharma Test Apparatebau, Hainburg, Germany) was used to determine enzyme activities.

Statistical analyses

Two independent sample t-tests were used to determine the relationship between the hemocyte counts and the enzyme activities depending on the diet content. SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for these tests.

Results

Hemocyte counts

Among the control groups, while the lowest hemocyte count was found to be in the larvae fed on the T diet (1269 ± 3.3 , $t = -6.6$, $p < 0.001$), the highest hemocyte count was obtained in the larvae fed on the control (A) diet (2519 ± 17.1 , $t = -6.7$, $p < 0.001$). The hemocyte counts of all groups infected with bacteria increased compared to the control ones. Among the infected groups, the lowest hemocyte count was in the larvae fed on the U diet (1336 ± 9.5 , $t = -6.6$, $p < 0.05$), and the highest was in the B diet group (2778 ± 34.5 , $t = -6.7$, $p < 0.001$) (Fig. 2).

Superoxide dismutase activities

Among the control groups, the highest SOD activity was in the group containing $1.576 \text{ g L}^{-1} \text{ Zn}$ (222 ± 1.3 , $t = 7.6$, $p < 0.001$), while the lowest activity was in the A diet group (125 ± 2.7 , $t = 7.9$, $p < 0.001$). In infected groups, it was determined that the highest SOD activity was in the F diet group (236 ± 1.4 , $t = 7.6$, $p < 0.001$) whereas the lowest activity was in the larvae fed on the U diet (141 ± 1.3 , $t = 3.4$, $p < 0.05$) (Fig. 3).

Catalase activities

Among the control groups, while the lowest CAT activity was found to be in the larvae fed on the A diet (222 ± 2.7 , $t = 3.2$, $p < 0.05$), the highest one was obtained in the larvae fed on the E diet (288 ± 1.5 , $t = 7.3$, $p < 0.001$). Among the infected groups, the lowest CAT activity was in the U diet group (230 ± 1.0 , $t = -5$, $p < 0.001$) and the highest one was in the F diet group (304 ± 1.6 , $t = 7.3$, $p < 0.001$) (Fig. 4).

Glutathione peroxidase activities

Among the control groups, the highest GSH-Px activity was in the G diet group (109 ± 1.8 , $t = 3.5$, $p < 0.05$), the lowest activity was in the control diet group (68 ± 0.8 , $t = 8.2$, $p < 0.001$). In the infected groups, the highest GSH-Px activity was 124 ± 1.5 , $t = 8.3$, $p < 0.001$ at the F diet group, while the lowest one was 77 ± 0.7 , $t = 8.2$, $p < 0.001$ at the B diet group (Fig. 5).

Discussion

Differences in the hemocyte counts of insects can be used to measure the immuno-suppressive or -stimulating effects (Fallon et al. 2011; Browne et al. 2013). Environmental contaminants (such as metals and insecticides) can induce structural abnormalities in the hemocytes and/or change their counts. Results obtained from studies with different species have shown that, as a result of contaminants, the hemocyte counts change (Renwranz 1990; Anderson et al. 1992; Coles et al. 1994). In our study, among the control groups, the highest hemocyte count was found in the larvae fed on the control diet. Studies showed that the hemocyte counts decreased with nickel and copper added to the diet (Sun et al. 2010; Kara et al. 2020). In our study, the result that the hemocyte count of the larvae decreased (except M) with increasing amounts of zinc, copper, and nickel in the diet among the control groups was consistent with the results of these studies. It was shown in various studies that the hemocytes could be affected by pathogens (Anderson et al. 1992; Oubella et al. 1993). In our study, we found that with the application of *Btk*, the hemocyte counts of all groups increased compared to the controls; this result coincided with the study by Dubovskiy et al. (2008) found that *B. thuringiensis* increased cellular immune response in *Galleria mellonella*. This increase may be due to the fact that the hemocytes fight bacteria in different ways (phagocytosis, nodulation) in response to the infection.

Since superoxide dismutase is an enzyme involved in the reduction of superoxide radicals (Ali et al. 2017), the increase in the activity of this antioxidant enzyme is an indicator of oxidative stress. In our study, SOD activities increased with the presence of metals added to the diet. This situation proved that metals caused oxidative stress and consequently increases in SOD activities occurred. In a study with *Spodoptera littoralis* larvae (Abd El-Wahab and Anwar 2014), it was found that zinc and copper nanoparticles significantly increased SOD activity. In our study, it was determined that the groups with the highest SOD activities were the groups containing zinc and copper, and this result was consistent with the mentioned above. The reason for this increase is that the presence of zinc and copper is essential for SOD activity because these metals are the catalytic and structural components of the SOD enzyme. It was found that enzyme activities increased at 1.576 g L^{-1} of all three metals compared to 0.788 g L^{-1} in both the control and the infected groups, but the activities decreased in groups with the maximum metal amount. Studies have found that the SOD activities of *Drosophila simulans* fly infected with *Wolbachia* and *G. mellonella* larvae infected with *B. thuringiensis* were higher than controls (Brennan et al. 2012; Sezer-Tunçsoy and Ozalp 2016). The result that we found in our study that the SOD activities of all groups infected with *Btk* were higher compared to their controls coincides with these results.

Hydrogen peroxide (H_2O_2) can transform into a highly reactive hydroxyl radical in the presence of reduced metal atoms. In this case, CAT efficiently converts H_2O_2 to water and oxygen (Tasaki et al. 2017). In our

study, it was found that the highest CAT activity among the control groups was found in the larvae fed with a diet containing 1.576 g L⁻¹ zinc (E diet). Similar to superoxide dismutase activities, it was found that CAT activities peaked at 1.576 g L⁻¹ of all three metals in both the control and the infected groups, but the activities decreased in the groups with the maximum metal amount. Compared to the control group, the increase in CAT activities in parallel with the SOD activities with the presence of metal is the evidence that the H₂O₂, which is formed as a result of SOD, is reduced by CAT, that is, these two enzymes work in a complementary manner. It was found that the hemolymph CAT activities of *G. mellonella* larvae infected with *B. thuringiensis* were higher compared to the control (Sezer-Tunçsoy and Ozalp 2016). This result was consistent with what we found that all groups infected with bacteria had high CAT activities compared to the controls (except U).

Metals can alter various aspects of immune function (Brousseau et al. 2000). Glutathione can prevent damage to important cellular components caused by ROS such as metals (Pompella et al. 2003), so it is crucial for cells. Sezer-Tunçsoy et al. (2019) found that copper oxide nanoparticles increased GSH-Px activities of *G. mellonella* larvae compared to the control. In our study, it was determined that the enzyme activities increased with the addition of copper, zinc, and nickel to the diet compared to the control groups. We found that the GSH-Px activity level was lower than SOD, suggesting that CAT may have a priority role in scavenging H₂O₂ than GSH-Px (Meng et al. 2009). In our study, it was also found that the infection increased the GSH-Px activities compared to the control groups. The result that *G. mellonella* larvae infected with *B. thuringiensis* had higher GSH-Px activity compared to control larvae (Sezer-Tuncsoy and Ozalp 2016) was consistent with what we found in our study.

Conclusions

Insects have been successfully used as bioindicators of environmental pollution in industrial and even urban areas. The immune system of insects protects themselves against invasive microorganisms, pathogens, and toxins (Kingsolver et al. 2013). Antioxidant enzymes like SOD, CAT, and GSH-Px play a crucial role in oxidative stress defences of cells by eliminating ROS. In our study, the cellular and the enzymatic responses of *H. cunea* were determined after exposure to zinc, copper, and nickel and the bacterial infection. It was found that the enzyme activities increased, but the hemocyte counts decreased with metal exposure. Besides, both the hemocyte counts and the enzyme activities increased with the bacterial infection. As a result, it was concluded that the hemocyte counts and the antioxidant enzymes of *H. cunea* were affected by metal exposure and bacterial infection. In this context, our study will shed light on immunological studies with other species.

Declarations

Funding: Not applicable.

Conflicts of interest/Competing interests: Not applicable. **Availability of data and material:** The data generated and/or analyzed during the current study are available from the corresponding author.

Code availability: Not applicable.

Authors' contributions: OY contributed to the study design; EFT, FGS, and SM perform the data analyzes. All authors helped write the manuscript.

Ethics approval: Not applicable.

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Tables

Table 1 is not available with this version.

Figures

Figure 1

Heavy metal toxicity for cells

Figure 2

Comparison of hemocyte counts of *Hyphantria cunea* larvae in the control and the infected groups. Data are expressed as mean $\pm$ S.E. Two independent samples t-test, $p < 0.001$

Figure 3

Comparison of superoxide dismutase activities of *Hyphantria cunea* larvae in the control and the infected groups. Data are expressed as mean $\pm$ S.E. Two independent samples t-test, $p < 0.001$

Figure 4

Comparison of catalase activities of *Hyphantria cunea* larvae in the control and the infected groups. Data are expressed as mean $\pm$ S.E. Two independent samples t-test, $p < 0.001$

Figure 5

Comparison of glutathione peroxidase activities of *Hyphantria cunea* larvae in the control and the infected groups. Data are expressed as mean $\pm$ S.E. Two independent samples t-test, $p < 0.001$