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RESEARCH REPORT

# Effect of essential and non-essential elements on cellular immune system of cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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## Abstract

Three concentrations of 12.5, 25 and 50 mg/kg of cadmium and 25, 50 and 100 mg/kg of copper were added to artificial diet and fed to larvae for 7 days. Total hemocytes count (THC) at 12.5 mg/kg concentration of cadmium significantly declined, but at 50 mg/ kg of cadmium and all treatments of copper, the THC was significantly enhanced. As for differential hemocytes count (DHC) prohemocytes were reduced at 12.5 mg/kg of cadmium treatment and all concentrations of copper, while granulocytes in all treatments of cadmium were increased. The phenoloxidase activity and nodulation were increased versus entomopathogenic fungi, *Beauveria bassiana* (Balsamo) Vuillemin and latex beads. The adverse influence of stresses on immune responses of pest can be an effective solution for insect pest control.

**Key Words**: *Beauveria bassiana*; Cadmium; Copper; *Helicoverpa armigera*; Nodulation; THC; DHC

## Introduction

The rapid development of industrialization and urbanization has generated heavy metals contamination globally (Zhou *et al*. 2012). Some heavy metals such as cadmium (Cd), nickel (Ni), arsenic (As), chromium (Cr) and lead (Pb) have been increasing to dangerous levels for human, plant and animals (Sharma and Agrawal, 2005) but essential micronutrients; copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn) are required by organisms in low concentrations (Epstein, 1972; Marschner, 2011). Although organisms are able to regulate small amounts of essential metals generally, in excess these metals may become toxic (Kabata-Pendias, 2010; Chaffai and Koyama, 2011).

Among heavy metals, cadmium risks and toxicity are well documented. Cadmium accumulation is higher in leaves than other parts of plants (Marschner, 1983). Accumulation of cadmium in insects that feed on plants have been demonstrated (Lindqvist, 1992). Cadmium is involved in oxidative stress at the cellular level leading to the production of reactive oxygen (Mirčić *et al*., 2010) which causes many structural and functional disturbances such as decreased stability of the lysosomal membrane. They also cause increased lipid peroxidation (Korsloot *et al*. 2004), and decreased activity of anti-oxidative and detoxification enzymes (Lijun *et al*. 2005, Augustyniak *et al*., 2009). At the organismal level, cadmium affects feeding indices, food consumption and digestibility (Fountain and Hopkin, 2001, Van Ooik *et al*., 2007), secretion of neurohormones (Ilijin *et al*., 2009) and synthesis of hormone receptors (Cervera *et al*., 2006; Planelló *et al*., 2010).

Copper is a biostatic metal that in organisms has an important role in cytochrome oxidase C (Bertini *et al*., 1994) and the structure of proteins such as prophenoloxidase, hemocyanin and super oxide dismutase (SODs); nevertheless high concentration of copper may cause denaturation and dysfunction in proteins (Huang *et al*., 2012).

Insect immunity is divided into two major defense systems against infectious agents, cellular immune and humoral immune. In cellular immune, different types of hemocytes are active but in humoral immunity, anti-microbial peptides or phenoloxidase (PO) have been produced (Cerenius and Söderhäll, 2004; Stanley and Miller, 2006). There are indications that the humoral and cellular responses are well coordinated. There is an overlap between them, since several humoral factors affect the hemocytes function and these, in turn, are an important source of many humoral molecules such as phenoloxidase (Lavine and Strand, 2002; Jiravanichpaisal *et al*., 2006). Cellular immunity are phagocytosis, nodulation and encapsulation (Lavine and Strand, 2002). Hemocytes have been

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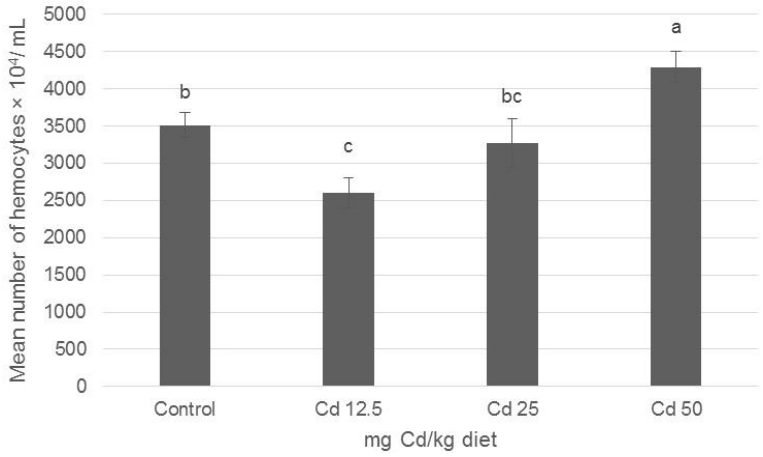


**Fig. 1** The effect of different concentrations of cadmium on THC of *H. armigera*. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05)

well studied in lepidopterous larvae such as *Pseudoplusia includens*, *Manduca sexta* and *Bombyx mori*, because they possess an adequate volume of hemolymph and a huge number of hemocytes which makes them a convenient system in which to study these cells. The hemocyte types described in Lepidoptera include prohemocytes, plasmatocytes, granulocytes, oenocytoids and spherule cells (Strand, 2008).

POs are vital enzymes that cause melanization. POs are presented as inactive precursor (PPO) in insect hemolymph and are activated in response to wounding or infection as a part of the innate immune response (Cerenius *et al.*, 2008).

Granulocytes and plasmatocytes are adhesive in nature. After the entrance of invaders to the hemocel, micro-aggregations of granulocytes and plasmatocytes are initiated on microorganisms. This process is started by changing of circulating hemocytes from non-adhesive to adhesive cells that are able to bind to microorganisms (Lavine and Strand, 2002). These micro-aggregations will eventually lead to the formation of nodules. In the later stages of nodule formation, melanization takes place within the nodules (Khosravi *et al.*, 2014).

It has been well documented that insect genotype (Rantala and Roff, 2006), sex and different stage (Rantala *et al.*, 2007; Jalali and Salehi, 2008), quality and quantity of food and feeding indices (Rantala *et al.*, 2003; Yang *et al.*, 2007; Baghban *et al.*, 2014), crowding (Wilson *et al.*, 2003) and physical activity (Ahtiainen *et al.*, 2006) affect the immune functions of insect. However, the role of immune system versus pollution has received less attention. If pollution affects the immune response (*e.g.,* nodulation) in insects, the enhanced or declined immune response may act as a different behavior resistance against entomopathogenic fungi, bacteria

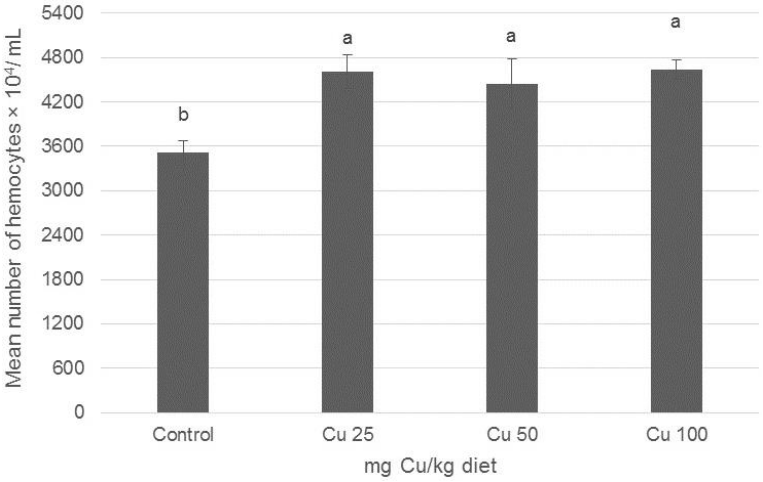


**Fig. 2** The effect of different concentrations of copper on THC of *H. armigera*. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05)

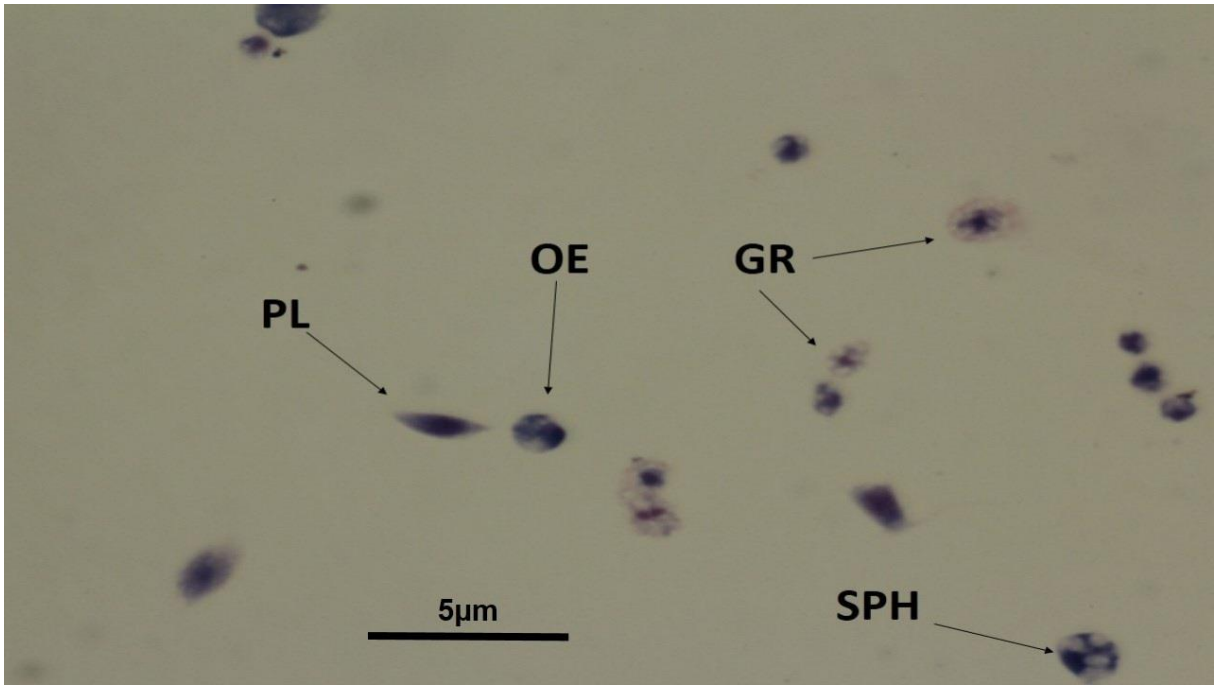


**Fig. 3** Hemocytes from American cotton bollworm *H. armigera* larvae stained by Giemsa for light microscopy observation. Plasmatocyte (PL), Granulocytes (GR), Oenocytoids (OE) and Spherolucyte (SPH)

or parasites. This could affect the parasitism rate in herbivorous insects in metal polluted areas. Hence, increasing or decreasing the number of hemocytes and type of them are indicator of response versus heavy metals. In this study, the effect of cadmium and copper were evaluated on total hemocytes count (THC); differential hemocytes count (DHC); PO activity and finally immune response versus entomopathogenic fungi, *Beauveria bassiana,* a common biological control agent and latex beads, the latex particles that cause cellular immune response on *Helicoverpa armigera* as an insect model due to its high fertility rate, non-diapausing larvae and ease of rearing on artificial diet.

**Materials and Methods**

*Experimental insect and treatments*

The larvae of *Helicoverpa armigera* were collected from tomato farms in Astaneh-ye Ashrafiyeh city (37°15′35″N 49°56′40″E) in the north of Iran. The larvae were reared on artificial diet (powdered cowpea, wheat germ powder, yeast, sorbic acid, ascorbic acid, sunflower oil, formaldehyde and water) (Shorey and Hale, 1965) in transparent plastic containers (10×5×5 cm) at 26 ± 2 °C, 65 ± 10% relative humidity and a photoperiod of 16L:8D and after rearing them for three generations in the laboratory then the third instar larvae were used for experiments. The chloride salt of cadmium and copper (Sigma-Aldrich Co., USA) were used and dissolved in distilled water to make stock solutions of 2000 ppm. The stock solutions then were diluted to make a series of different concentrations of heavy metals. The selected concentrations of heavy metals were considered as the amounts of these metals in different plants. Hence 12.5, 25, and 50 mg/kg diet of cadmium and 25, 50 and 100 mg/kg of copper diet were considered (Deng *et al*., 2004; Liu *et al*., 2007; Parizanganeh *et al*., 2010; Nazir *et al*., 2011). Newly hatched larvae reared in a plastic container on artificial diet until first and second instar. The third instar larvae were reared separately due to high cannibalism. Heavy metal treatment continued for 7 days until third instar larvae were 24 h old in all assays.

*Total hemocytes count (THC) and differential hemocytes count (DHC)*

For THC and DHC, the larvae were heat-fixed at 60 °C for 5 min following the method of Rosenberger and Jones (1960). This will prevent the adhesion of hemocytes to tissues and cause the release them to hemolymph. For THC, 10 µl hemolymph was mixed immediately in an Eppendorf with 290 µl of physiological saline (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA, 0.041 M Citric acid, pH 4.5) (Amaral *et al.*, 2010). Then, the total hemocytes numbers were counted on an improved Neubauer hemocytometer.

For DHC 5 µl of hemolymph were collected directly on a clean slide and a smear by another slide was made and dried at room temperature. The air-dried smears were stained by 1 to 10 diluted stock Giemsa (Merck, Germany) and after 14 min were washed by distilled water. In order to distinguish cytoplasm from nucleus of hemocytes,

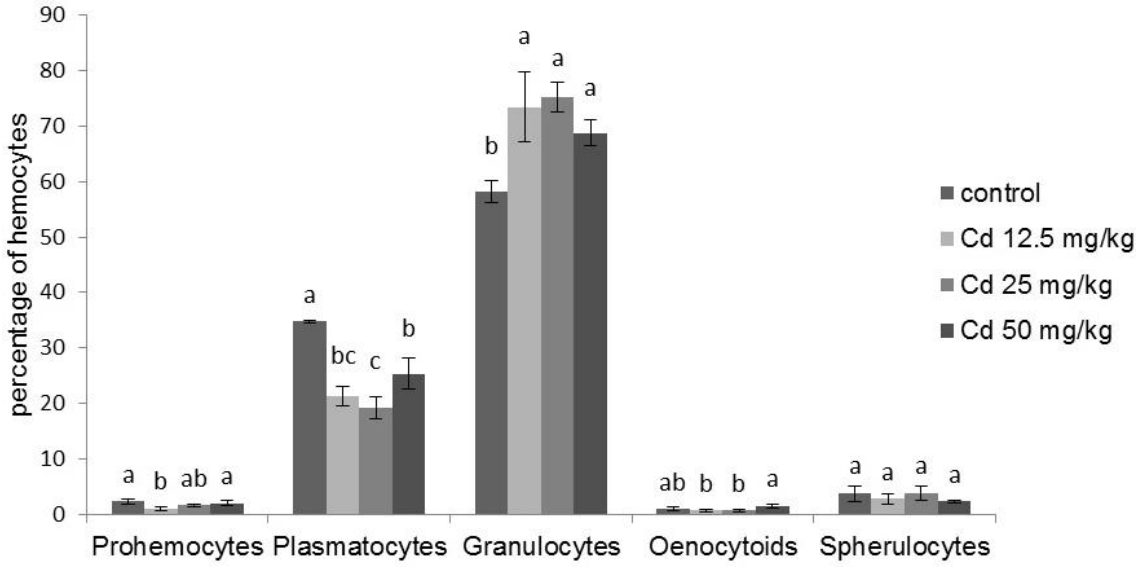


**Fig. 4** The effect of different concentrations of cadmium on DHC of *H. armigera*. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05)

smears were dipped in saturated lithium carbonate ($LiCO_3$) for 5 sec and then dried at room temperature. Finally, smears were fixed in Canada balsam (Merck, Germany) and DHC was performed by classifying 200 cells per smear (Gujar and Kalia, 2005; Jalali and Salehi, 2008, Wu *et al.*, 2016) based on the identification key provided by Gupta (Gupta, 1979).

*PO activity assay*

For measuring PO specific activity, 10 µl of hemolymph was diluted with 90 µl of ice-cold sterile phosphate buffered saline and then vortexed. Samples were frozen at -20 °C for 48h. We used L-DOPA (Sigma-Aldrich Co., USA) as substrate. Samples were centrifuged at 5,000 g at 4 °C for 5 min. Then 50 µl of hemolymph-buffer supernatant was mixed with 150 µl of L-DOPA (10 mM). PO activity was measured at 490 nm during the linear phase of the reaction. Specific activity was calculated by dividing absorbance with protein content in hemolymph using a microplate reader (Awareness Technology Inc., Florida, USA) (Catalán *et al*., 2012, Khosravi *et al*., 2014).

*Protein determination*

The method of Bradford (Bradford, 1976) was used for determining total protein, using bovine serum albumin (Bio-Rad, Munchen, Germany) as the standard.

Beauveria bassiana *culture*

*B. bassiana* isolate IRAN403C was grown in sterile petri dishes containing PDA (potato dextrose agar) at 25 ± 1 °C. After 14 days, spores were harvested from PDA plates with sterile scalpel and distilled water containing 0.01% of Tween-80 and finally, $1\times10^4$ spores/ ml concentration was adjusted by hemocytometer.

*Immune response assay*

After 7 days of treatment, larvae were immobilized on ice for 5 min and surface sterilized with 70% ethanol. After injection with $1\times10^4$ spores/ml and 1 to 10 distillated latex beads with distilled water by a 10 µl Hamilton syringe, the larvae were transferred to rearing jars and were provided with fresh diet. The control larvae were injected with distilled water containing 0.01% of Tween-80 (1 µl) alone. Effect of fungal spore on nodulation 24 h post injection were determined. Hemolymph was collected from each larva, then samples in 3 replicates were poured into a hemocytometer, and the number of nodules was counted (Franssens *et al.*, 2006, Seyedtalebi, 2017).

*Statistical analysis*

All data are presented as means ± SE and data were subjected to analysis of variance (ANOVA) using SAS 9.1 software. Differences among treatments were compared using Tukey's multiple range tests. Differences among means were considered significant at p≤ 0.05. All experiments have three replicates and each replicate with ten larvae.

## Results

*Effect of cadmium and copper on THC*

The results showed that while the number of hemocytes at 12.5 mg/kg concentration of cadmium was significantly decreased but at 25 and 50 mg/kg concentration of cadmium it was significantly increased compared to control (F= 18.56; df= 3; p≤ 0.05) (Fig. 1). On the contrary the THC after copper treatment showed an increasing trend and in all concentrations it was significantly higher than control (F= 16.46; df= 3; p≤ 0.05) (Fig. 2).

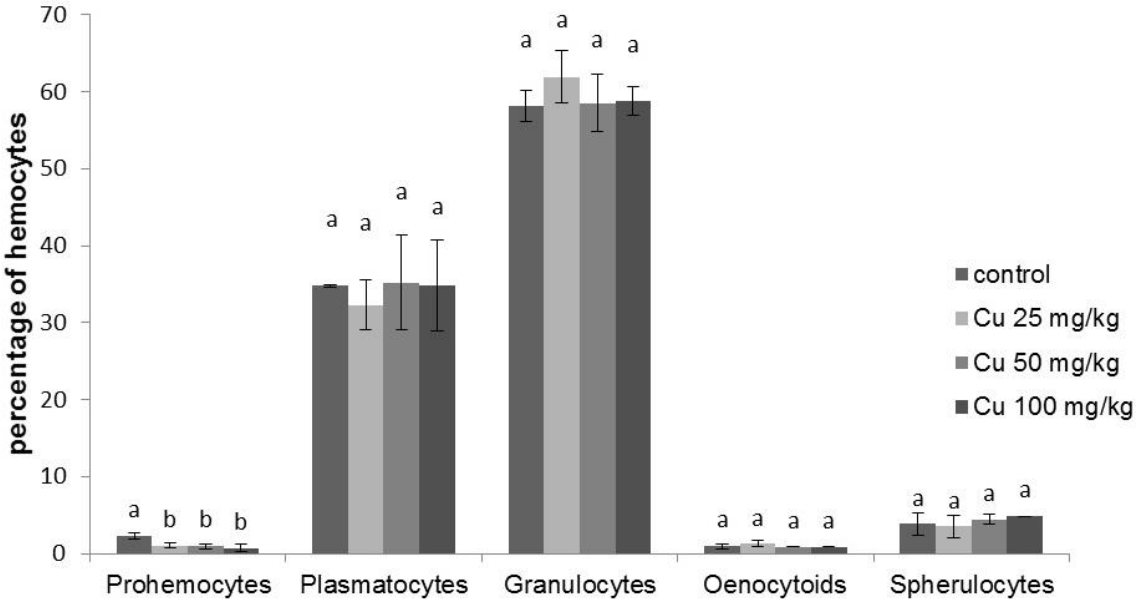


**Fig. 5** The effect of different concentrations of copper on DHC of *H. armigera*. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05)

*Effect of cadmium and copper on DHC*

Five forms of hemocyte were observed (Fig. 3). The DHC result demonstrated significant decline in prohemocytes at 12.5 mg/kg concentration of cadmium compared with the control (F= 7.07; df= 3; p≤ 0.05). While the number of plasmatocytes were significantly decreased (F= 26.7; df= 3; p≤ 0.05) and that of granulocytes were increased in all concentrations of cadmium compared with the controls (F= 12.5; df= 3; p≤ 0.05). On the other hand no changes in the number of oenocytoids and spherule cells were observed between treatments and their controls (Fig. 4). The prohemocyte DHC results in copper treatments declined significantly compared with the control (F= 11.94; df= 3; p≤ 0.05) but in all other hemocyte types no significant differences were seen between treatments and the control (Fig. 5).

*Effect of cadmium and copper on PO activity*

On the measurements of PO activity, a key enzyme in immunology and immune responses of all insects showed that there was significant enhancement in its activity at 50 mg/kg concentration of cadmium compared with the control but no significant difference was observed between 12.5, 25 mg/kg concentrations of cadmium and the control (F= 24.37; df= 3; p≤ 0.05) (Fig. 6). In case of copper treatments the activity of PO did not show significant changes and remained same in all treatments compared with control (F= 1.6; df= 3; p≤ 0.05) (Fig. 7).

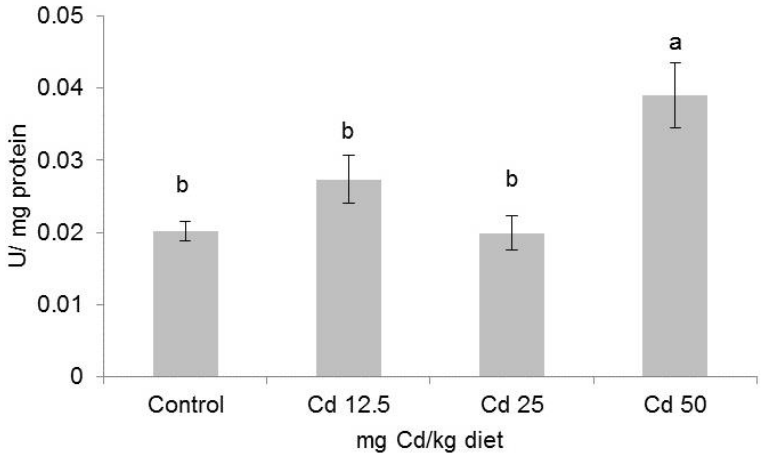


**Fig. 6** The effect of different concentrations of cadmium on PO enzymes activity of *H. armigera*. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05)

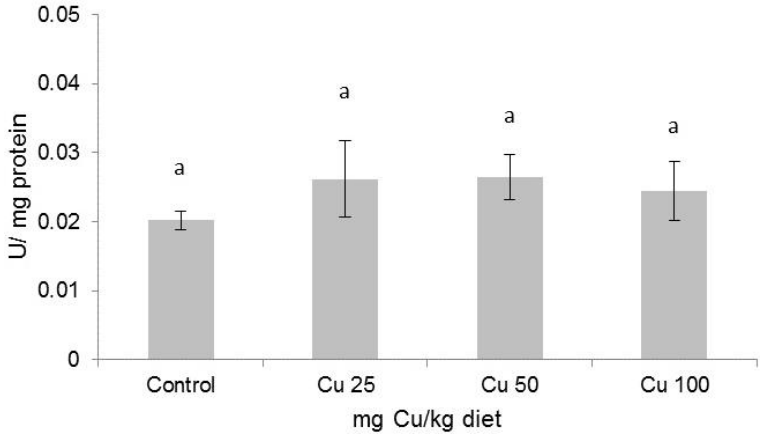


**Fig. 7** The effect of different concentration of copper on PO enzymes activity of *H. armigera*. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05)

*Effect of cadmium and copper on nodule formation*

The formation of nodules as an indicator of cellular response was also studied (Fig. 8). There were significant differences in the numbers of nodules after injection of *B. bassiana* and latex beads (F= 28.66; df= 3; p≤ 0.05 and F= 27.95; df= 3; p≤ 0.05, respectively) at maximal concentration of cadmium compared to control (Fig. 9). Copper treatment versus *B. bassiana* injection in all concentrations of copper showed no significant differences compared to control (F= 4.3; df= 3; p≤ 0.05). However, after injection of latex bead in all concentrations of copper, the nodule formation was sharply increased compared to control. Treatments with 25 and 100 mg/kg concentration of copper showed significant differences compared with the control (F= 7.77; df= 3; p≤ 0.5) (Fig. 10).

**Discussion**

Increase or decrease in the total number of blood cells are considered a common response to stressors present in the environment (Perez and Fontanetti, 2011). Pipe and Coles (1995) demonstrated that decrease in the total number of hemocytes under stress condition could be a consequence of cellular lysis, reduced replacement or migration of the cells from the circulation to the tissues. Our result showed that at 12.5 mg/kg concentration of cadmium the THC was significantly decreased, possibly because hemocytes in low concentrations of cadmium move from circulation to the tissues. It seems at low concentration of cadmium, midgut epithelium was damaged thus causing the migration of hemocytes to this tissue

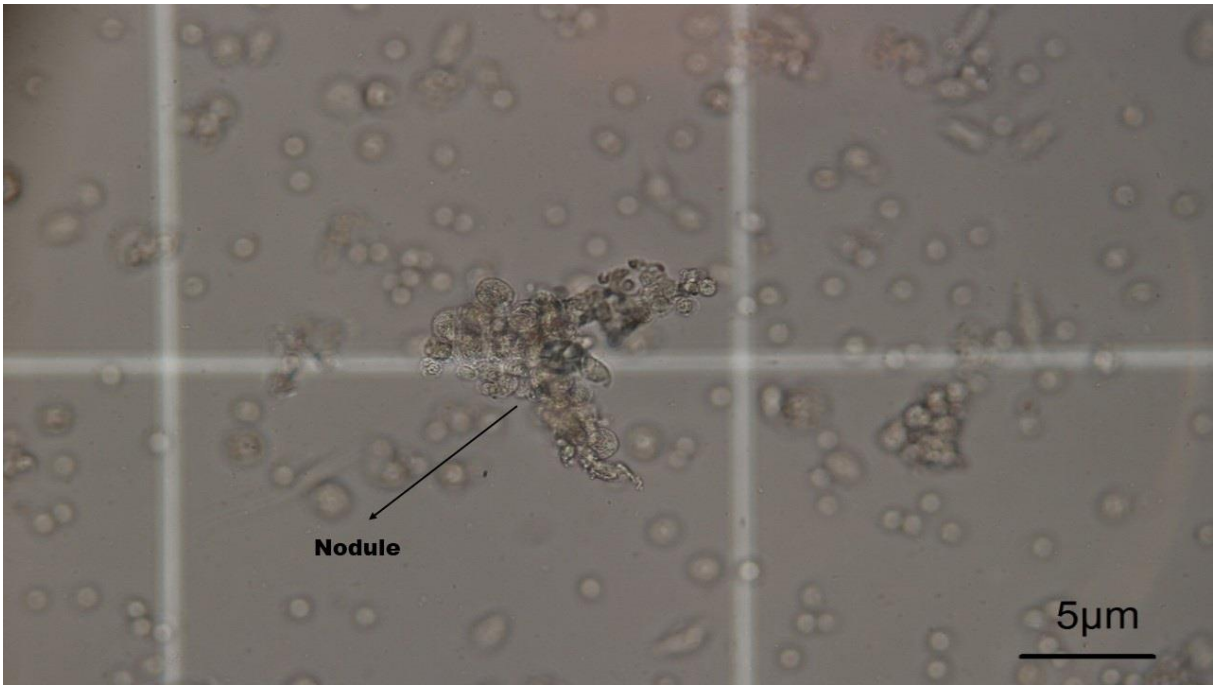


**Fig. 8** Nodule formation against *B. bassiana* spore in American cotton bollworm *H. armigera* larvae

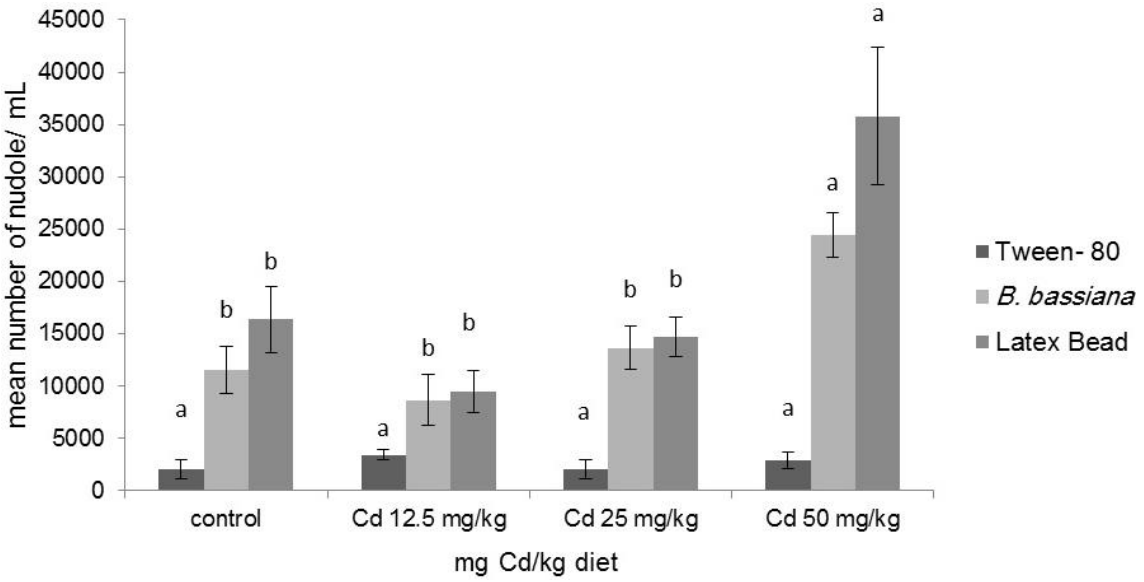


**Fig. 9** The effect of different concentrations of cadmium on immune response (Nodulation) of *H. armigera* versus entomopathogenic fungi, *B. bassiana* and latex bead. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05). Columns with the same color are compared together

henceforth leading to decline in the number of circulating hemocytes (Perez and Fontanetti, 2011). Some studies have shown an increase in the number of hemocytes in midgut tissue of exposed animals that indicated a tissue injury with inflammatory process (de Godoy and Fontanetti, 2010; Nogarol and Fontanetti, 2010; Perez and Fontanetti, 2011). The THC increase under heavy metal stress can be explained by their potential to produce metallothioneins under exposure to heavy metals (Roesijadi *et al*. 1997). Metallothioneins are cysteine rich proteins that bind to heavy metals and scavenger them (Kägi and Kojima, 1987; DeMoor and Koropatnick, 2000). In addition, some other studies have shown that hemocytes could transport heavy metals intracellularly (in lysosomal vesicles or within the cytoplasm) (Robinson and Ryan, 1988). Our previous report (Baghban *et al*., 2014) showed that the relative growth rate and the amount of lipids was reduced due to cadmium but reverse was true in case of as for copper treatment. Cadmium produced free radicals and it is possible at high concentrations of cadmium, hemocytes were activated due to high oxidative stress. Hence, migrated to injured epithelial cells and produced metallothioneins. It seems in all concentration of copper *H. armigera* have not a big challenge to heavy metal stress and increasing in THC was a response to enhancing growth (body size) (Ghasemi *et al*., 2013) and/or copper transport to fat body. Our previous report has shown significant increase of lipid storage and relative growth rate (RGR) in copper treatments (Baghban *et al*., 2014).

In the DHC assay, our results showed that at 12.5 mg/kg concentration of cadmium, the mean counts of prohemocytes were declined as the hemocytes developed into other hemocytes types. However, when the concentration of cadmium reached to toxic levels (50 mg/kg concentration of cadmium), hematopoietic organs produced stem cells (prohemocytes) for transformation to other hemocytes especially granulocytes. Probably granulocytes through their granules transported cadmium intracellularly and/or migrated to the injured tissue. In a similar study Victor (Victor, 1993) reported in *Paratelphusa hydrodromous* Herbst that cadmium induced change in the ratio of hemocytes thus increasing granulocytes and prohemocytes number. Our results also showed that in all concentrations of copper the percentage of granulocytes show no significant differences but prohemocytes were significantly reduced compared to the control. Based on THC and DHC results, we understand that the stress of copper may not have reached to the point causing significant changes in percentage of involved hemocytes and granulocytes or other hemocytes that normally react to increasing amounts of copper in hemolymph and carrier copper and/or produce metallothioneins.

Present results showed that PO enzyme activity significantly increased at 50 mg/kg concentration of cadmium but at 12.5 and 25 mg/kg of cadmium and all concentrations of copper no differences were observed. Our data support previous findings that have shown increasing PO activity in *Epirrita autumnata* Borkhausen larvae after exposure to heavy metals (van Ooik *et al*., 2007; van Ooik and Rantala, 2010). Dubovskiy *et al*. (2011) mentioned that probably enhanced PO activity may be the result of oxidative damage of the midgut epithelial cells and discharge of some immune mediators into the hemocel. This hypothesis is supported by the reports that the rate of hemocytes apoptosis in *Spodoptera litura* Fabricius larvae increases with an

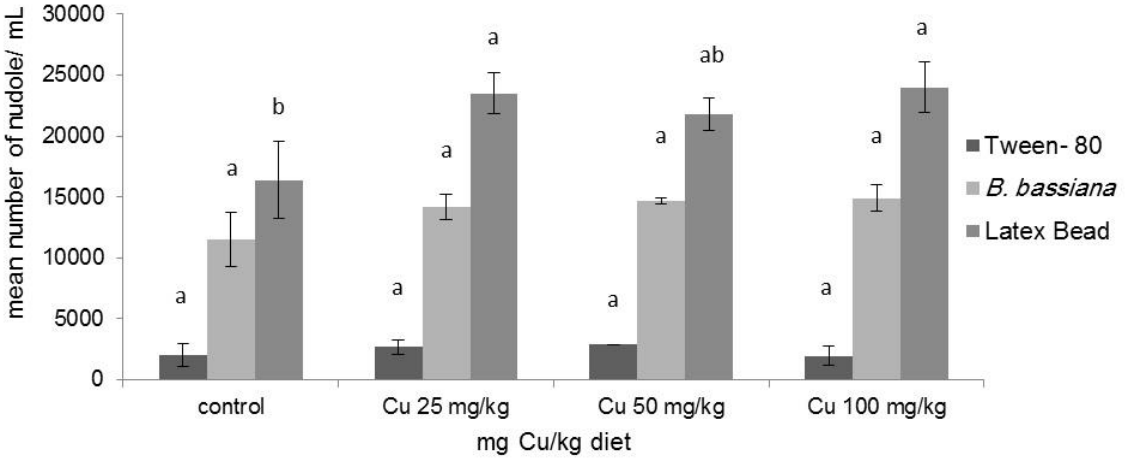


**Fig. 10** The effect of different concentrations of copper on immune response (Nodulation) of *H. armigera* versus entomopathogenic fungi, *B. bassiana* and latex bead. Treatment columns sharing the same letter are not significantly different under the Tukey tests (p≤ 0.05). Columns with the same color are compared together

increasing nickel concentration in the diet (Xia *et al*., 2005). A radical-related and antioxidant-dependent process such as apoptosis or oxidative stress can induce the level of melanization (Dubovskiy *et al*., 2011). On the other hand, our results showed treatment with cadmium could change THC and DHC. The fact that humoral and cellular immunity are closely dependent, has been demonstrated in several studies (Lavine and Strand, 2002; Jiravanichpaisal *et al*., 2006). However, some processes such as apoptosis that are radical-related and antioxidant-dependent ultimately cause changes in hemocyte counts. These changes have direct effect on immune response, as in Dubovskiy *et al*. (2011). There exists discrepancy between reports by various researchers on the subject which could be related to the nature of heavy metals, their concentrations incorporated, exposure time and the species chosen for the study (Lorenzon *et al*., 2001).

There is a positive correlation between immune response and the number of circulating hemocytes (Rantala *et al*., 2000). This reason probably is the strongest reason for the present result that indicates the high dose of cadmium cause enhanced nodulation reaction in the larvae of the cotton bollworm. Several studies have also reported that pollution by heavy metals can increase immune response (encapsulation) of insects (van Ooik *et al*., 2007; van Ooik and Rantala, 2010; Dubovskiy *et al*., 2011). Here we considered both PO activity and nodulation showing higher activity at maximal dosage of cadmium. Some studies have shown low-level and short-term metal exposure could increase immune responses, whereas higher concentrations or a longer exposure may inhibit the very same response (van Ooik *et al*., 2008). However, in the present study it was found that at high levels of cadmium and 25 and 100 mg/kg concentrations of copper, immune response were enhanced. Mirhaghparast *et al*. (2013) mentioned that the maximum number of nodules occurred 12 h after *B. bassiana* injection and 24 h after latex bead injection. It seems that due to enhanced oxidative stress in the higher concentration of cadmium immune functions may delay the responses to *B. bassiana* compared to treatments with copper.

The duration of treatment was 7 days and the response of *H. armigera* to heavy metal pollution showed itself in the changes in number of hemocytes. This response could affect the infection of entomopathogenic fungi or latex bead and enhanced nodulation rate. However, if the duration of exposure or if the concentration of heavy metals extends, the immune response might be differently affected.

## Conclusion

This study clearly showed that the immune responses of *H. armigera* are affected by cadmium and copper. Immune response of insect is affected by various factors which could alter their performance. The present results and other studies showed immune responses (like nodulation, encapsulation, and PO activity) are affected by environmental stress (in this case heavy metals pollution) and this effect was imposed variously. The nature of the contamination, the duration of exposure to pollution, contamination concentrations and animal species variously affect immune responses. Whether these responses favor organism or disfavor it has to be pointed and incorporated in our pest manipulation strategies.

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