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RESEARCH ARTICLE

Influence of zinc oxide nanoparticles (ZnO NPs) on the hemocyte count and hemocyte-mediated immune responses of the Greater Wax Moth *Galleria mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT

In this study, we examined the effects of different doses (100, 500, 1000, 3000, and 5000 ppm) of zinc oxide nanoparticles (ZnO NPs) on the total hemocyte count and hemocyte-mediated immune responses of the Greater Wax Moth *Galleria mellonella* (Lepidoptera: Pyralidae). The results showed that NPs caused a decrease in hemocyte count at 1000, 3000, and 5000 ppm doses. To investigate the effects of ZnO NPs on the encapsulation and melanization response of *G. mellonella*, the pre-dyed Sephadex chromatography beads were injected into the hemolymph of each last-instar larva. Larvae were dissected in the 4th and 24th hours after the injection. The level of the encapsulation response and melanization status around the beads were determined under microscopy. The analyses of the beads injected into the insects as encapsulation targets revealed that the number of weakly encapsulated beads increased significantly at 100, 1000, 3000, and 5000 ppm doses when compared to the control group after a short (4-h) post-injection. The number of melanized beads increased significantly at 100, 1000, and 3000 ppm doses in comparison to the control group after the short (4-h) post-injection. Finally, the number of melanized beads decreased significantly at 1000 and 5000 ppm doses when compared to the control group after the long-term (24-h) post-injection.

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KEYWORDS
Galleria mellonella; encapsulation; melanization; hemocyte count; zinc oxide nanoparticle

Introduction

Nanoparticles (NPs) are defined as small particles with a size between 1 and 100 nm that may differ from the bulk substance (Hasan 2015). Thanks to their nanoscale structure, NPs can more readily enter different biological structures than non-NPs, which could have a harmful effect. They can quickly enter the human body, pass through numerous biological barriers, and possibly even reach the most sensitive organs due to their small size (Pourmand and Abdollahi 2012, Bahadar *et al.* 2016). Also, the potential for NPs to be hazardous to bacteria, algae, invertebrates, and vertebrates has previously been demonstrated before (Exbrayat *et al.* 2015).

The chemical composition and physical properties of NPs can exhibit cytotoxicity. The positive charge of NPs can disrupt membrane lipid bilayers (Exbrayat *et al.* 2015). In comparison to bigger particles, smaller NPs with a large surface area are more hazardous (Gebel 2012). Zinc oxide nanoparticles (ZnO NPs) are used in fertilizers, paints, sunscreens, sensor applications, and toothpaste for industrial purposes (Özalp *et al.* 2020). ZnO can release free zinc ions into the environment and biological systems, which is one of the reasons why ZnO NPs are harmful to organisms (Tang *et al.* 2018). The unconscious release of these NPs into nature as pollutants with their widespread usage makes it crucial to investigate their negative effects on the ecosystem and living things (Özalp *et al.* 2020). On a macro-scale, the increasing presence of ZnO NPs on an environmental level may also have negative consequences. Some NPs, including ZnO NPs, have been examined for their cytotoxic effects on the insect model organism, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Eskin *et al.* 2019, Tunçsoy 2020, Eskin *et al.* 2021a, Eskin 2022, Eskin and Bozdoğan 2022).

G. mellonella is an important model organism in the investigation of pollutants in the ecosystem, and also an important species for heavy metal and NP studies. *G. mellonella* has a similar natural immune system to mammals and its exposure to NPs indicates that it can be an appropriate model organism for understanding the immune system response at the organism level (Tunçsoy 2021).

The total number of hemocytes is a parameter that is an indicator of health and stress (Günal *et al.* 2018). Total hemocyte count (THC) is used as a parameter for the detection of toxic substances weakening the immune system power (Günal *et al.* 2018). Significant changes in THC may result from the immunologic response, inhibition of larval hematopoietic function, and decreased mitotic activity (Coskun *et al.* 2020).

The natural immune system in insects is divided into two categories: humoral immunity and cellular immunity (Lavine and Strand 2002). Humoral immunity includes the

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antimicrobial peptides, the production of phenoloxidase, the cascades regulating the coagulation and melanization of hemolymph, and oxygen and nitrogen which are reactive intermediates (Lavine and Strand 2002, Wojda *et al.* 2009, Sheykhnejad *et al.* 2014). Cellular immune responses include phagocytosis, encapsulation, and nodule formation that are generated by hemocytes (Lavine and Strand 2002, Sheykhnejad *et al.* 2014). Innate immune responses in larvae and vertebrates are quite similar, including phagocytic processes, cell surface receptors, and cell signaling pathways (Cutuli *et al.* 2019, Tsai *et al.* 2020, Xu *et al.* 2021).

Prohemocytes, plasmatocytes, granulocytes, adipohemocytes, oenocytes, and spherulocytes are the defined hemocyte types in *G. mellonella* (Ashhurst and Richards 1964, Kurt and Kayış 2015). Among these cell types, granulocytes and plasmatocytes are important ones in recognizing pathogens and parasites. Plasmatocytes are more dominant in phagocytosis behavior. Granulocytes, on the other hand, have little phagocytic activity (Lackie 1988, İzzetoğlu and Karaçalı, 2003). Oenocytes contain the cytoplasmic form of phenoloxidase and do not adhere to foreign bodies. The feature of non-adhesiveness is also seen in spherulocytes and prohemocytes. Spherulocytes are responsible for the mucopolysaccharide synthesis and transport of the cuticle. Prohemocytes are cells that are capable of mitotic division and can differentiate into specialized hemocyte types (Ribeiro and Brehelin 2006, Mizerska-Dudka and Andrejko 2014).

Encapsulation is the response of hemocytes to large targets such as parasites, protozoa, and nematodes. Encapsulation begins with the binding of host granulocytes to a foreign target surface. The granulocyte lyses or gets degranulated. It releases the contents in its granules onto the surface of the foreign object. It is considered that this process attracts the plasmatocytes toward the foreign object and again allows the plasmatocytes to adhere to the surface of the foreign object. The termination of capsule formation occurs when granulocytes adhere to the periphery of the capsule as a single layer. The capsule turns black due to melanization, and the process almost always results in the death of the organism due to exposure to reactive cytotoxic products or suffocation (Uçkan *et al.* 2010, Rosales 2011).

Melanization is the process of the formation of melanin pigment. The phenoloxidase enzyme which contains copper in its body plays an important role in this process (Rosales 2011). Melanization is dependent on tyrosine metabolism. Tyrosine is converted to DOPA (dihydroxyphenylalanine) by tyrosinase enzyme activity and activated by phenoloxidase. DOPA can be converted to either dopamine by being decarboxylated with DOPA carboxylase, or to dopaquinone by being oxidized with phenoloxidase (Tsakas and Marmaras 2010). As a result, melanin is produced on the surfaces of microbes or wounds, and at the same time, it is cross-linked with proteins on these surfaces (Schnitger *et al.* 2007).

Important cell-mediated immune defense responses in insects include encapsulation and the melanization response (Kaya *et al.* 2021). Changes in THC and hemocyte behavior (encapsulation–melanization) can be used as important markers to understand the effects of ZnO NPs on the immune systems of organisms. There is currently no research on the impact of 70 nm nanorod ZnO NPs on the encapsulation and melanization response of *G. mellonella* larvae exposed to this NP through diet. Here, we aimed at determining whether ZnO NPs affect the THC and the rate of encapsulation and melanization response of *G. mellonella* larvae. The main aim of this study was to explore the toxic effects ZnO NPs at the organismal and cellular levels by using the *G. mellonella* model system.

Materials and methods

Chemicals

ZnO NP (Alfa Aesar NanoShield®, 70 nm, ZN-3008C, 50% in water, pH: 6.9 cationic-dispersant colloidal dispersion, the specific surface area of feed powder: 14.0 m^2/g, the mean diameter of feed powder particles: 76 nm) was used as a chemical in the experiments in the study.

Characterization and preparation of ZnO NPs

Using a high-resolution transmission electron microscope (HRTEM, JEOL 2100, Tokyo, Japan) and scanning electron microscope (JEOL/JSM-6510LV-INCA/EDS, Tokyo, Japan), the sizes of ZnO NPs (Alfa Aesar, Zinc oxide, NanoShield®, 70 nm, ZN-3008C, 50% in H_2O, colloidal dispersion with cationic dispersant, Karlsruhe, Germany) were examined. Zeta sizer was used to measure the particles' Zeta potential (Malvern Instruments Inc., Malvern, UK). For the dispersion, stabilization, and production of the stock solution, varying dosages of ZnO NPs (100, 500, 1000, 3000, and 5000 ppm) were added to double-distilled water and homogenized with an ultrasonic homogenizer (amp: 60%, 15 min, Bandelin Sonopuls, HD 3200, Berlin, Germany).

Biological assays

G. mellonella pup and adults were grown in 27 ± 1 °C temperature, 60 ± 5% relative humidity, and constant darkness in the laboratory of the Biology Department, Marmara University, Istanbul, Turkey. Female and male moths were placed in a 1 L glass jar with 10 g of natural blackened honeycomb. The adults were allowed to lay eggs on the natural blackened honeycomb.

In all experimental processes, 1 mL of ZnO NPs was drawn from the solutions at different ppm doses (100, 500, 1000, 3000, and 5000 ppm) prepared in double-distilled water with an automatic pipette and added to each point of 1 g powdered blackened honeycomb (blended). Only 1 mL of double-distilled water was added to the honeycomb in the control group. The honeycombs were kept in laboratory conditions for one day to let the liquid solution inside dry. Then, the larvae of the second stage that hatched from these eggs were taken with a brush and taken into the petri dishes.

The last instar larvae (0.18 ± 0.02 g) of appropriate size were selected and used for the experiments. In the studies of the effects of the NPs on the hemocyte count and encapsulation–melanization response of the insect, these steps were repeated exactly. Also, multiple-dose experiments up to high doses were conducted to determine the LD_{50} (lethal dose) of

ZnO NP. 100, 500, 1000, 3000, and 5000 ppm ZnO NPs doses were determined as experimental doses and applied in the study since 50–90% larval death was not observed at the NP doses below 50 000 ppm and the NP formulation used in the study did not allow to work with doses above 50 000 ppm.

Effects of ZnO NPs on hemocyte counts of *G. mellonella*

The steps used in the studies conducted by Tojo *et al.* (2000) and Teramoto and Tanaka (2004) were applied in this study to determine the effects of NPs on THCs of *G. mellonella*. Hemolymph was collected from the last-instar larvae which reached a weight of 0.18 ± 0.2 g, and their hemocyte counts were determined. Five larvae from each experimental group and the control group were used for the experiment. Experiments were carried out at different times in three replicates in total. To determine the total number of hemocytes in different experimental groups and the control group, hemolymph was extracted from the first hind leg of the larva with a 4 μL hemolymph microcapillary tube (Sigma, St. Louis, MO). The obtained hemolymph was transferred to Eppendorf tubes containing 36 μL of anticoagulant solution (0.098 M NaOH, 0.186 M NaCl, 0.017 M Na_2EDTA and 0.041 M citric acid, pH 4.5) (Tojo *et al.* 2000, Teramoto and Tanaka 2004, Er *et al.* 2011, Yılmaz 2013). The cell suspension diluted at a ratio of 1:10 was mixed by drawing and releasing with a micropipette several times, and 10 μL of the cell suspension was drawn with the micropipette and loaded into a 0.100 mm-depth Neubauer hemocytometer (Improved Neubauer Hemocytometer; Superior, Durkheim, Germany). Hemocytes were counted under an Olympus BX51-phase-contrast microscope (Tokyo, Japan). The following formula was used for counting: number of cells/mL = number of cells counted in the big square × dilution coefficient (10)×10^4 (Prescott and Breed 1910).

Effects of ZnO NPs on the encapsulation and melanization responses of *G. mellonella*

Encapsulation and melanization experiments were performed to determine the effects of ZnO NPs on the insect's immune system (Figures 1 and 2). The experiments were based on the study conducted by Uçkan *et al.* (2010) so that the encapsulation responses could be formed by hemocytes. To this end, positively charged Sephadex DEAE A-25 (40–120 μm in diameter, Sigma Chemical Co., St. Louis, MO) chromatography beads were used. Before the beads were injected into the last-instar *G. mellonella* larvae, they were weighed up to 0.025 g and transferred to the centrifuge tube. 0.1% Coomassie blue (Brilliant Blue G Sigma) prepared with phosphate-buffered saline (PBS) was added to the beads that were previously transferred to the centrifuge tube with a micropipette for easy recognition *in vivo*.

Sephadex A-25 beads were kept in the dye solution for one hour during the staining process. After the one-hour dying process, the supernatant was removed and three freshly prepared PBS was added to the beads to completely wash them (Uçkan *et al.* 2010). Then, PBS was added to the completely washed beads until filling the tube up to 2.5 mL. The prepared beads were injected into the bodies of last-instar *G. mellonella* larvae by drawing 10 μL of PBS containing 15–20 Sephadex A-25 beads using a 50 μL Hamilton microinjector with a 22-gauge injector tip. The samples were dissected under a TTT-ECHNI-C stereo microscope at a ×40 magnification in the 4th and 24th hours of the injection. After the dissection of the larvae which was performed with five larvae at each of the three replicates at different times, the beads found were collected with a dissection needle and placed on slides containing one drop of PBS. In this way, as a result of both NP treatments, 150 beads were examined in both the experimental groups and the control group in both 4-h and 24-h experiments. After that, the beads were covered

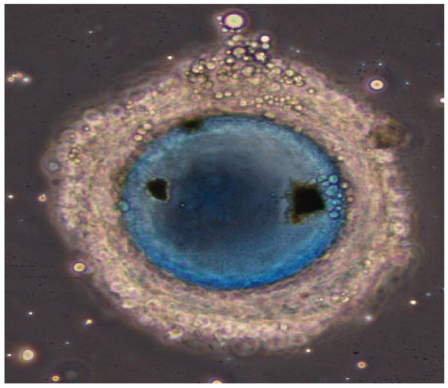

Figure 2. Melanization in the last-instar larva of *G. mellonella* (24th hour).

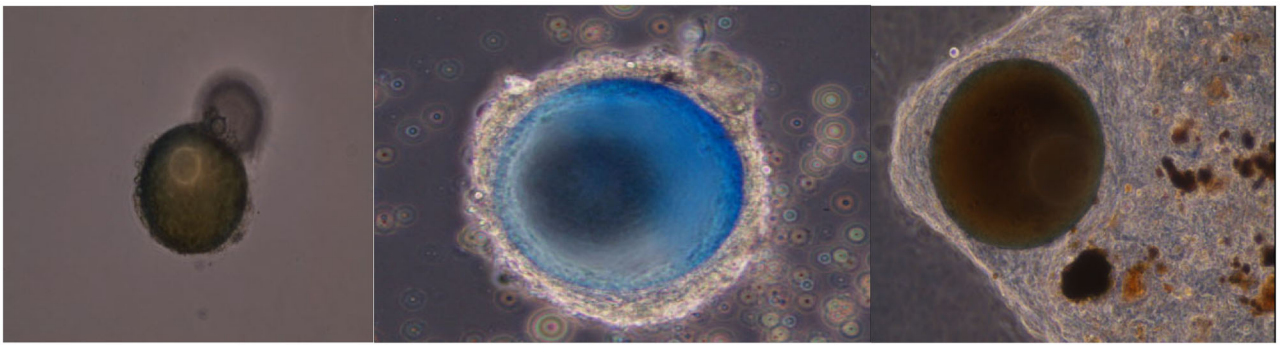

Figure 1. Encapsulation in the last-instar larva of *G. mellonella* (24th hour): (A) no encapsulation; (B) weak encapsulation; (C) strong encapsulation.

with a coverslip and examined under the Olympus brand CX31 model phase-contrast microscope in terms of their encapsulation response and melanization status. According to the results of the examination,

- The bead was not encapsulated or when there were a few hemocytes on it, it was not encapsulated (Uçkan *et al.* 2010) (Figure 1).
- When there were 4–9 layers of hemocytes on the bead, it was weakly encapsulated (Uçkan *et al.* 2010) (Figure 1).
- When there were 10 or more hemocyte layers on the bead, it was considered to be strongly encapsulated (Uçkan *et al.* 2010) (Figure 1).

Statistics

The SPSS (version 20.0, SPSS Science, Chicago, IL) program (SPSS Inc. 2010) was used to compare the means of the data received from the experimental and control groups. First, it was determined whether the data were distributed normally. Second, an ANOVA, a type of parametric test, was used to compare the analysis of variance of the normally distributed group means. Third, if the variance was uniform, the differences between the means were examined using the Tukey HSD test; otherwise, the Tamhane T2 *post hoc* test was used. In all statistical analyses, $p = 0.05$ was regarded as significant. The control group was designated as '0' in the tables of all statistical findings from this study.

All values given in percentages in the analyses of variance determining the effect of ZnO NP treatments obtained from the experimental and control groups on the hemocyte counts and the encapsulation and melanization responses in *G. mellonella* were subjected to statistical analysis after normalizing them by taking their arcsine square roots. All results were presented in percentages. SPSS statistical program (SPSS, version 20.0, SPSS Science, Chicago, IL) was used for data analysis. The significance level was taken as 0.05 in all statistical tests.

Results

Characterization of ZnO NPs

According to the obtained images shown in figure, it was understood that the ZnO NP consisted of nanorods and particles with a polymorphic structure (see Figure 3(A–D)).

As a result of the measurements in the study, the zeta potential value was measured as +23.0 mV (Figure 4). This

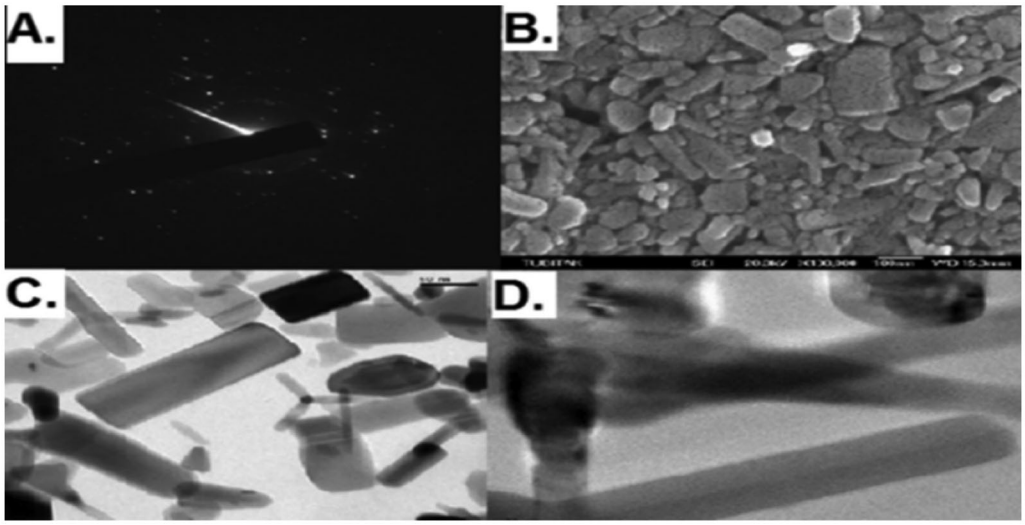

Figure 3. Electron diffraction pattern image of ZnO nanorods (A), SEM image of ZnO NP at a ×100 000 magnification (the bar indicates a length of 100 nm) (B), TEM image of ZnO NP (the bar indicates a length of 100 nm) (C), and TEM image of ZnO NP (the bar indicates a length of 20 nm) (D).

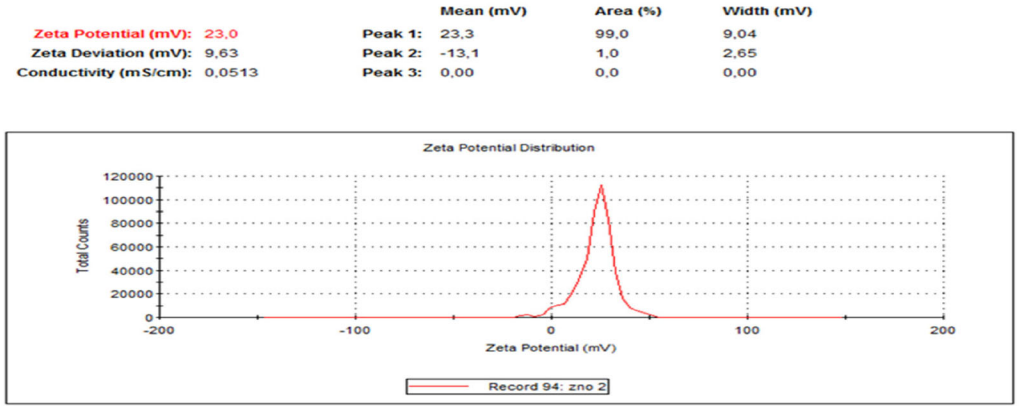

Figure 4. Zeta potential value of ZnO NP.

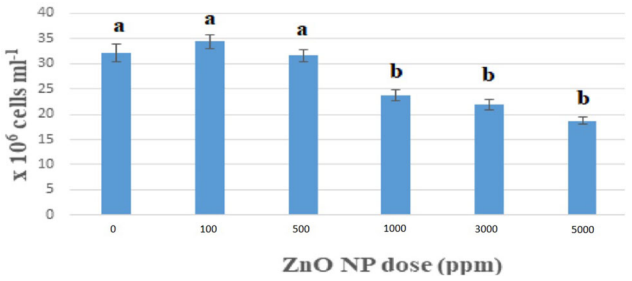

Figure 5. Effects of different doses of ZnO NPs on the total hemocyte counts of the last-instar *G. mellonella* larva.

Table 1. Effects of different doses of ZnO NPs on the total hemocyte counts of the last-instar *G. mellonella* larva.

Dose (ppm)	Min.–Max.	Hemocyte counts/mL (mean ± SE)
0	18.30–42.30	32.21 ± 1.78^{a}
100	26.00–42.00	34.39 ± 1.36^{a}
500	25.50–41.10	31.61 ± 1.18^{a}
1000	15.30–29.90	23.72 ± 1.12^{b}
3000	14.40–23.40	21.90 ± 1.03^{b}
5000	14.40–42.40	18.72 ± 0.72^{b}

Different letters (a-b) indicate statistically significant differences (Tamhane's T2 test, $p < 0.05$).

value (+23.0 mV) that we obtained was determined as stable because if the zeta potential is above ±60 mV, the particles are at a very good level and is stable. It is stable between ±20–30 mV if the synthesized NPs are within this range of values if not, they are unstable and tend to settle by agglomeration (Kavaz 2011).

Effects of ZnO NPs on the hemocyte count of *G. mellonella*

The findings regarding the effects of different doses of ZnO NPs on the THCs of the last-instar *G. mellonella* larva are shown in Figure 5 and Table 1.

In the groups exposed to 1000 ppm, 3000 ppm, and 5000 ppm doses in ZnO NP treatment, the mean hemocyte counts decreased significantly when compared to the control group ($F = 26\ 968$, df $= 5$, $p = 0.00$). The findings showed that the treatment of ZnO NP caused a significant decrease in the number of hemocytes of *G. mellonella* larvae (Table 1).

Effects of ZnO NPs on the encapsulation and melanization responses of *G. mellonella*

Figures 1 and 2 include the example photos illustrating encapsulation degrees and melanization responses of *G. mellonella* larva.

The results of the effects of ZnO NPs on the encapsulation and melanization responses of *G. mellonella* were obtained by examining a total of 150 beads (10 beads per larva) in three replicates for both the control and experimental groups in the experiments are given in Figures 6–9 and Tables 2–5.

According to the results of the one-way analysis of variance, in the 4th-hour encapsulation response; although the experimental groups exposed to different ZnO NP doses displayed various changes compared to the control group, these changes were found to be statistically significant except in the weak encapsulation response, and no statistically significant results were obtained in the 24th hour for encapsulation response (Figures 6 and 7; Tables 2 and 3).

When the results of the fourth-hour encapsulation response were examined; the number of unencapsulated beads increased in all doses (>38%) (*F*: 1.510; *p*: 0.195) except for the 5000 ppm ZnO NP dose (35.33%) compared to the control group. The percentage of poorly encapsulated beads was higher in the 100, 1000, 3000, and 5000 ppm dose groups than that in the control group, and this increase was found to be statistically significant (>20.66%) (*F*: 5.007; *p*: 0). Strong encapsulation percentages, on the other hand, decreased in general in all doses (<40%) (*F*: 2.551, *p*: 0.063) compared to the control group (Table 2).

When the 24th-hour encapsulation results are evaluated, the percentage of unencapsulated beads increased over the control group at all doses (>14%) with the highest percentage seen at 3000 ppm dose (34.67%). However, these increases were found to be statistically insignificant (*F*: 1.566, *p*: 0.179). The percentage of weakly encapsulated beads did not increase or decrease in general in all doses by being close to the control group. The greatest decrease was observed at 500 ppm (a decrease by 7%) (*F*: 0.992; *p*: 0.428). At 100 ppm dose, there was an increase by 4% compared to the control group, and the percentage of weakly encapsulated beads was recorded as 33.33%. With regard to the situation in strongly encapsulated beads, the highest decrease was seen at 3000-ppm ZnO NP dose (a decrease by 21% compared to the control group) (*F*: 1.528; *p*: 0.190) (Table 3).

Out of the results of the experiments in which the effects of different doses of ZnO NP on the melanization response of *G. mellonella* hemocytes were investigated, the results obtained in the 4th hour are given in Figure 8 and Table 4, and the ones obtained in the 24th hour are given in Figure 9 and Table 5.

The applied ZnO NP doses resulted in a statistically significant difference between the 4th and 24th-hour results in terms of the number of melanized and non-melanized beads in comparison with the control group (Figure 8 and Table 4). The number of melanized beads increased significantly at 100, 1000, and 3000 ppm doses after the short (4-h) post-injection (*F*: 3.482; *p*: 0.007) (Figure 8 and Table 4). At the 24th hour, in the 1000 and 5000 ppm ZnO NP dose groups, the percentages of non-melanized beads increased compared to the control group (*F*: 3.536, *p*: 0.006). The percentage of melanized beads in the same dose groups decreased in comparison with the control group (*F*: 3.423, *p*: 0.007). These increases and decreases are statistically significant (Figure 9 and Table 5).

Discussion

Nanotechnology has recently become more prevalent in a variety of scientific sectors, including health and agricultural studies (Ibrahim and Ali 2018). Numerous formulations based on NPs, including nano-sized insecticides, herbicides, fungicides, fertilizers, and sensors, have been studied for

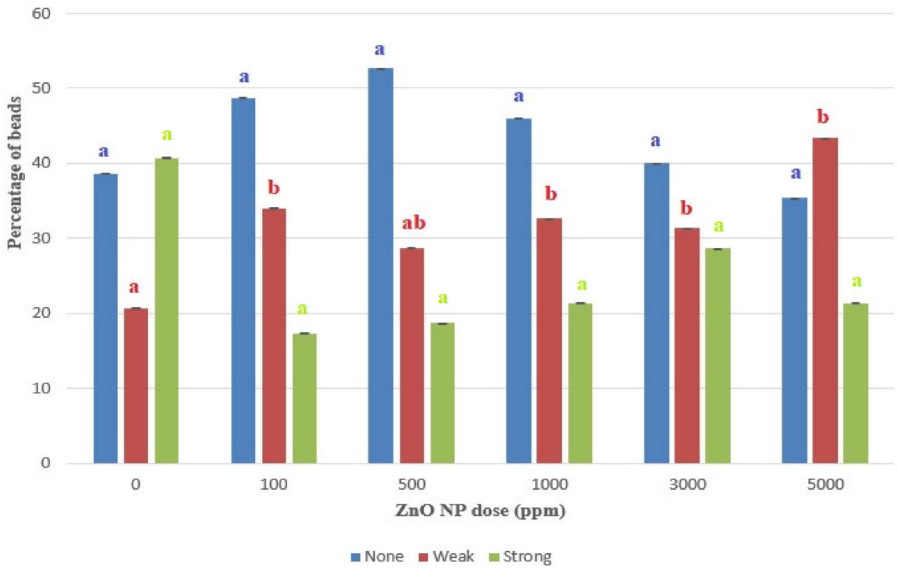

Figure 6. 4th-hour results of the effects of ZnO NP on the encapsulation responses of *G. mellonella*.

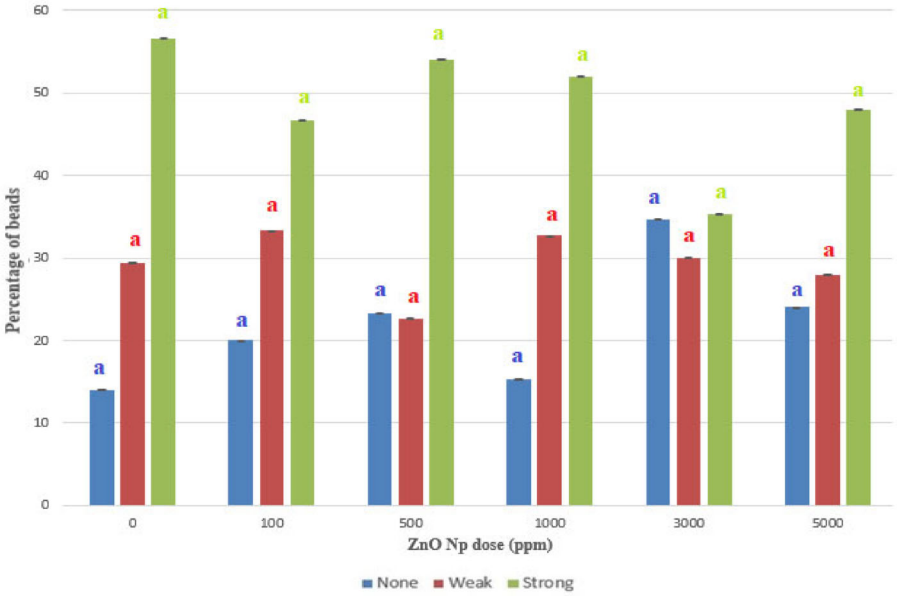

Figure 7. 24th-hour results of the effects of ZnO NP on the encapsulation responses of *G. mellonella*.

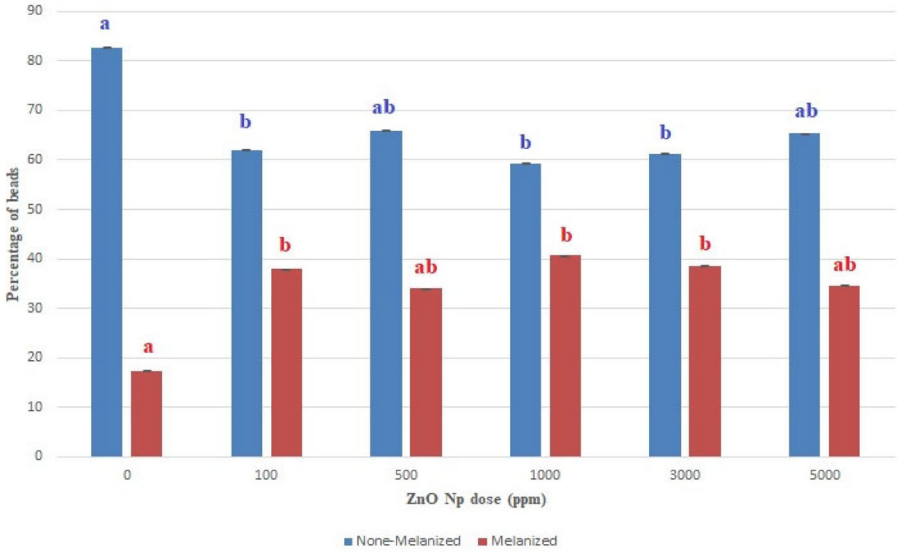

Figure 8. The effects of ZnO NPs on the melanization responses of *G. mellonella* in 4th-hour.

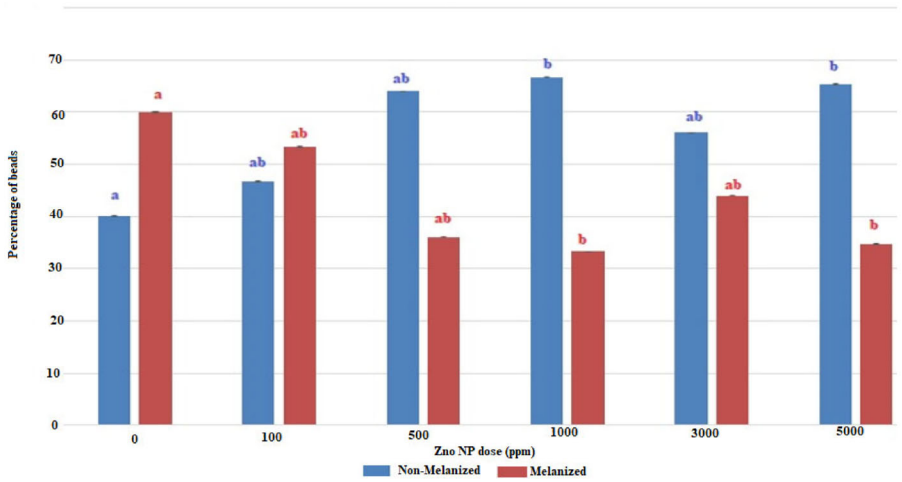

Figure 9. The effects of ZnO NPs on the melanization responses of *G. mellonella* in 24th-hour.

Table 2. 4th-hour results of the effects of ZnO NP on the encapsulation responses of *G. mellonella*.

	4th hour (number of beads (%±SE)*)			
ZnO NP dose (ppm)	Number of beads	No encapsulation (none)	Weak encapsulation	Strong encapsulation
0	150	58 (%38.66 ± 0.07)[a]	31 (%20.66 ± 0.07)[a]	61 (%40.66 ± 0.07)[a]
100	150	73 (%48.67 ± 0.05)[a]	51 (%34 ± 0.05)[b]	26 (%17.33 ± 0.04)[a]
500	150	79 (%52.66 ± 0.04)[a]	43 (%28.66 ± 0.03)[a,b]	28 (%18.66 ± 0.05)[a]
1000	150	69 (%46 ± 0.03)[a]	49 (%32.66 ± 0.02)[b]	32 (%21.33 ± 0.04)[a]
3000	150	60 (%40 ± 0.03)[a]	47 (%31.33 ± 0.02)[b]	43 (%28.66 ± 0.02)[a]
5000	150	53 (%35.33 ± 0.02)[a]	65 (%43.33 ± 0.02)[b]	32 (%21.33 ± 0.06)[a]
		[a]Tamhane's T2, $p > 0.05$	[a]Tamhane's T2 < 0.05	[a]Tamhane's T2, $p > 0.05$

*The mean cell count is the result obtained from the total 15 larvae used in the three replicates.
**The difference between the groups with the same letter in the same column next to the mean cell counts is not statistically significant (a, b).

Table 3. 24th-hour results of the effects of ZnO NP on the encapsulation responses of *G. mellonella*.

	24th hour (number of beads (%±SE)*)			
ZnO NP dose (ppm)	Number of beads	No encapsulation (none)	Weak encapsulation	Strong encapsulation
0	150	21 (%14 ± 0.06)[a]	44 (%29.33 ± 0.07)[a]	85 (%56.66 ± 0.06)[a]
100	150	30 (%20 ± 0.06)[a]	50 (%33.33 ± 0.03)[a]	70 (%46.66 ± 0.04)[a]
500	150	35 (%23.33 ± 0.06)[a]	34 (%22.66 ± 0.06)[a]	81 (%54 ± 0.06)[a]
1000	150	23 (%15.33 ± 0.05)[a]	49 (%32.66 ± 0.04)[a]	78 (%52 ± 0.05)[a]
3000	150	52 (%34.67 ± 0.08)[a]	45 (%30 ± 0.07)[a]	53 (%35.33 ± 0.06)[a]
5000	150	36 (%24 ± 0.06)[a]	42 (%28 ± 0.03)[a]	72 (%48 ± 0.06)[a]
		[a]Tukey, $p > 0.05$	[a]Tamhane's T2, $p > 0.05$	[a]Tukey, $p > 0.05$

*The mean cell count is the result obtained from the total 15 larvae used in the 3 replicates.
**The difference between the groups with the same letter in the same column next to the mean cell counts is not statistically significant (a, b).

managing plant health and enhancing soil (Mittal *et al.* 2020). Nanotechnology created a variety of materials at the nanoscale. NPs are a broad category of materials that include particles with at least one dimension less than 100 nm (Khan *et al.* 2019). ZnO NPs are metal oxide NPs that provide fresh possibilities for medicinal applications ranging from diagnosis to treatment (Pushpalatha *et al.* 2022). Today, in many fields from food to packaging people are exposed to ZnO NPs during their daily lives. This exposure is low and usually harmless. However, ZnO NPs, which can easily enter the cell, can cause oxidative stress due to its large surface area and small size, which are one of their most important properties. Among the damages caused by ZnO NP, there are protein disorders, DNA damage, lipid peroxidation, inflammation, and organelle dysfunctions (Sruthi *et al.* 2018, Karagöz 2022). Moreover, numerous studies found that ZnO-NP use may result in cytotoxicity, apoptosis, immune suppression, cell cycle change, and DNA damage (Valdiglesias *et al.* 2013, Kim *et al.* 2014, Olejnik *et al.* 2021, Belal and Gad 2022).

The absorption of metals in the body and their physiological effects on the living systems can vary by species. The possible physiological effects depend on many factors such as sex, age, developmental stage, and seasonal differences. Insects have been reported to keep huge amounts of metals in their body (Hsu *et al.* 2006). That is why, insects are used as bioindicators for metal pollution (Nummelin *et al.* 2007, Kayis and Emre 2012). *G. mellonella* is a holometabolous insect and is popularly known as the 'greater wax moth' in colloquial speech. It causes great economic damage to the beekeeping sector (Özer 1961). *G. mellonella* is also one of the best model organisms in ecotoxicology tests (Zorlu *et al.* 2018).

In studies investigating the toxic effects of NPs on insects, Kool *et al.* (2011) investigated the chronic toxicity effects of

Table 4. The effects of ZnO NPs on the melanization responses of *G. mellonella* in 4th-hour (%-percent values).

4th hour (melanization (%±SE)*)			
ZnO NP dose (ppm)	Number of beads	None-melanized	Melanized
0	150	124 (%82.66 ± 0.03)[a]	26 (%17.33 ± 0.07)[a]
100	150	93 (%62 ± 0.05)[b]	57 (%38 ± 0.05)[b]
500	150	99 (%66 ± 0.03)[ab]	51 (%34 ± 0.03)[ab]
1000	150	89 (%59.33 ± 0.02)[b]	61 (%40.66 ± 0.02)[b]
3000	150	92 (%61.33 ± 0.04)[b]	58 (%38.66 ± 0.02)[b]
5000	150	98 (%65.33 ± 0.04)[a,b]	52 (%34.66 ± 0.02)[ab]

*The mean cell count is the result obtained from the total 15 larvae used in the 3 replicates.
**The difference between the groups with the same letter in the same column next to the mean cell counts is not statistically significant (a, b).
[a]Tukey HSD, $p < 0.05$.

Table 5. The effects of ZnO NPs on the melanization responses of *G. mellonella* in 24th-hour (%-percent values).

24th hour (melanization (%±SE)*)			
ZnO NP dose (ppm)	Number of beads	Non-melanized	Melanized
0	150	60 (%40 ± 0.05)[a]	90 (%60 ± 0.04)[a]
100	150	70 (%46.67 ± 0.05)[a,b]	80 (%53.33 ± 0.05)[a,b]
500	150	96 (%64 ± 0.03)[a,b]	54 (%36 ± 0.03)[a,b]
1000	150	100 (%66.67 ± 0.04)[b]	50 (%33.33 ± 0.05)[b]
3000	150	84 (%56 ± 0.06)[a,b]	66 (%44 ± 0.06)[a,b]
5000	150	98 (%65.33 ± 0.05)[b]	52 (%34.66 ± 0.06)[b]

*The mean cell count is the result obtained from the total 15 larvae used in the 3 replicates.
**The difference between the groups with the same letter in the same column next to the mean cell counts is not statistically significant (a, b).
[a]Tukey HSD, $p < 0.05$.

non-nano ZnO, non-nano zinc chloride, and ZnO NP on *Folsomia candida* (Collembola). In this study, they reported that there was no effect of these substances on the results of the survival rates of the organism in the 6400 mg zinc/kg dose trial of the mentioned species and there was a decrease in the reproductive power of the insect, and the toxic effects could be due to zinc ions released from NPs in the environment containing nano and non-nano ZnO.

In a study they conducted on *Spodoptera litura* (Lepidoptera: Noctuidae) with copper oxide NPs and ZnO NPs, Abd El-Wahab and Anwar (2014) found that an amount of 0.01 g increased mortality (100% in ZnO NP and 33.3% in the copper oxide NP) and caused disability and the disabled larvae then died by turning to a dark gray color. They also reported that the ascorbate peroxidase and superoxide dismutase (SOD) activities were lower compared to the control group, and the number of apoptotic cells in hemocytes increased within 24 hours (at a dose of 1000 mg ZnO NP per kg).

Zorlu et al. (2018) investigated the developmental physiology, hemolymph total protein amount, antioxidant enzyme (SOD, glutathione S-transferase GST, and catalase (CAT)) activities, and hemolymph malondialdehyde (MDA) level of *G. mellonella* fed with diet containing TiO_2 NP at different doses (100, 500, 1000, 3000, and 5000 ppm) from the second instar stage. They found that larval and pupal development times, pupal and adult weights, male longevity, and the percentage of morphological abnormalities in adults increased in dose groups compared to the control group due to the exposure to TiO_2 NP. In the same study, it was also found out that the MDA level increased at 100, 500, and 1000 ppm doses and the total protein amount increased only at 1000 ppm dose while the SOD, CAT, and GST activities varied dose-dependently.

In another study in which Fe_3O_4 NP concentrations (0.4, 2, 10, 50, and 250 μg/10 μL) of 18–38 nm-sized spherical nanopowders were applied to the sixth instar (180 ± 20 mg) *G. mellonella* larvae by force-feeding method, it was reported that the weight of the pupae evolving from the larvae exposed to 250 μg/10 μL Fe_3O_4 NPs and the adult developmental time increased significantly (Eskin *et al.* 2021b).

In this study, we investigated the effects of ZnO NPs on the THC, encapsulation rate, and melanization response of *G. mellonella* larvae. The THCs of *G. mellonella* larvae were dramatically reduced by ZnO NPs at doses of 1000, 3000, and 5000 ppm, according to the results (Figure 5 and Table 1). Total counts of hemocytes were similar to the results of the study with ZnO NPs on *Bombyx mori* (Lepidoptera: Bombycidae) by Belal and Gad (2022) (Table 1). They found that ZnO NPs at concentrations of 50 μg/mL and 100 μg/mL significantly decreased the THC (Belal and Gad 2022). As is well known, an increase in intracellular free Zn^{2+} is detrimental and can eventually result in apoptosis and a reduction in cell viability (Sakabe *et al.* 1998, Zhang *et al.* 2017, Eskin *et al.* 2019). The partial adverse effects of ZnO NPs in a high percentage of dead cells can be seen (10.01%) in hemocytes that were obtained at the 5 μg/10 μL ZnO NPs dose in our previous study (Eskin *et al.* 2019). In brief, the decrease in THC may be due to apoptotic hemocytes in the insect's hemolymph resulting in decreased cell viability (Table 1). In another study, ZnO NP treatment increased hemocyte density, encouraged hemocyte aggregation, improved hemocyte phagocytosis, and stimulated phenoloxidase activity in *G. mellonella* larvae (Xu *et al.* 2021). The researchers explained the reason for the different effects of the same NPs on the same larva species as follows. 'This might be due to the difference in the size of ZnO-NPs, the modes of administration, the larval size and age, and culture conditions' (Xu *et al.* 2021).

The most vital defense systems in insects are encapsulation and nodulation, which enable the immune system to be directed to the site of injury and swiftly eliminate the invader (Dubovskiy *et al.* 2008). In Lepidoptera, encapsulation is started when granulocytes bind to form a layer of cells that enclose a pathogen. This layer is subsequently surrounded by many layers of plasmatocytes, and this is followed by the binding of other granulocytes (Hillyer 2016). Melanization is an important component of wound healing in arthropods and the dark coloration of the clots is due to melanin deposition (Figures 1(C) and 2). Melanin plays a significant part in a variety of physiological processes in insects, such as cuticle tanning, immunity, and wound healing (Bilandžija *et al.* 2017).

According to the results of the 4th-hour encapsulation response, only in the weak encapsulation response was found to be statistically significant in all doses except the larvae exposed to 500 ppm ZnO NP. There were no statistically

significant results were obtained in the 24th hour for all doses (Figures 6 and 7; Tables 2 and 3). The same ZnO NP used in this study was force-fed to fourth instar *G. mellonella* larvae with 0.5, 1, 2.5, 5, and 10 μg/10 μL concentration by Eskin (2022). In the study, the effects of ZnO NPs on plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte numbers in hemolymph of *G. mellonella* larvae were determined after 24 h force feeding treatment (Eskin 2022). Results showed that the numbers of plasmatocyte and granulocyte did not differ significantly when compared to the control group (Eskin 2022). The reason why a significant encapsulation response generally did not occur in the insect in our study may be that there was no significant change in plasmatocyte and granulocyte density in the larval hemolymph, as in the Eskin (2022) (Figures 6 and 7; Tables 2 and 3).

However, in terms of melanization, the number of melanized beads increased significantly at 100, 1000, and 3000 ppm doses after the short (4-h) post-injection (Figure 8 and Table 4). The number of melanized beads decreased significantly at 1000 and 5000 ppm doses after the long-term (24-h) post-injection (Figure 9 and Table 5). Studies have reported that free radicals are cytotoxic to melanocytes and inhibit the tyrosinase enzyme (Shameer *et al.* 2005, Bagherani *et al.* 2011). In a study that resulted in melanin loss and was performed with silver NP, the results were interpreted in terms of decreased tyrosinase enzyme activity, and melanin loss and tyrosinase enzyme activity associated with each other (Armstrong *et al.* 2013). Tyrosine must react with the tyrosinase enzyme so that DOPA synthesis can take place. The reason for the decrease in beads undergoing melanization in our study may be the fact that NPs increase in number in the hemolymph of the insect due to the free radicals formed in *G. mellonella* hemocytes with the consumption of ZnO NP. The tyrosinase enzyme may be inhibited in the hemocytes of the insect (Figure 9 and Table 5). In addition, the decrease in melanization depending on ZnO NP doses may be due to the decreased activity of phenol oxidases in the hemolymph after ZnO NP treatment because melanization reactions are a process related to phenoloxidase released from hemocytes and enzymes associated with the termination of melanization cascades. Finally, Thomaz *et al.* (2020) reported that low levels of oenocytes might explain the low phenoloxidase activity in *G. mellonella* larvae after exposure to Ag NPs. This may be another reason for the decrease in the melanization response (Figure 9 and Table 5).

Consequently, the toxic effects of ZnO NPs on the THC and hemocyte-mediated immune responses of *G. mellonella* were determined for the first time with this study. We think that this research will offer preliminary information for future research into the harmful effects of ZnO NPs in a living system.

Conclusions

In conclusion, the immune system of *G. mellonella* was altered by ZnO NPs by changes in innate immune parameters. Moreover, we found that experimental doses of ZnO NPs exhibited similar effects on the hemocyte counts and in the percentage of melanized beads in the 4th-hour. Our study suggests that ZnO NPs cause alterations to the THC, and cause significant changes on the melanization responses of *G. mellonella* in the 4th and 24th hours.

ZnO NPs' immunomodulatory potentials make it especially appealing for use in medical applications (Xu *et al.* 2021). The results of our study may be useful for researchers working on the antifungal effects of ZnO NPs and investigating how immune systems interact with NPs will make it easier to create and use novel nanomaterials (Li *et al.* 2017).

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Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Author contributions

A.E.: carried out the toxicity studies. Read, wrote, and approved the final manuscript. Z.U.N.: managed and coordinated the toxicity studies. Read, wrote, and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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