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Effects of Zinc oxide Nanoparticles (ZnO NPs) on Hemocyte Types of *Galleria mellonella*

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Abstract

In this study, 70 nm-sized and nanorod-shaped ZnO NPs concentrations (0.5, 1, 2.5, 5, and 10 µg/10 µl) was force-fed to fourth instar (110 ± 20 mg) *Galleria mellonella* (Lepidoptera: Pyralidae) larvae. The effects of ZnO NPs on plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte numbers in hemolymph of *G. mellonella* larvae was determined. Results showed that treating *G. mellonella* with 10 µg/10 µl ZnO NPs significantly decreased spherulocytes numbers, whereas numbers of plasmatocyte, granulocyte, prohemocyte, oenocytoid, and coagulocyte numbers did not differ significantly when compared to the control group after 24 h force feeding treatment. There was a statistically significant difference between the experimental groups in the prohemocyte numbers of larvae that exposed to 1 and 5 µg/10 µl ZnO NPs.

Keywords: *Galleria mellonella*, Hemocyte Type, Nanoparticle, Zinc Oxide.

1. Introduction

Nanomaterials are material arrangements at a scale of about 1 to 100 nanometers in length that have unique features because of their size [1]. Different sizes and diameters of nanomaterials are frequently used in a variety of industrial fields because of the rapid development of nanotechnologies [2]. Zinc oxide Nanoparticles (ZnO NPs), because of their absorption of UV light, their catalytic, antimicrobial, semiconducting, the use of nanoparticles in consumer products is currently increasing [3]. Because of such intensive use of nanoparticles, reliable methods for predicting any associated toxicity are important [4]. Today, there are some studies on nanoparticle toxicity that have been carried out using model experimental organisms with force-feeding method [5-8]. With force-feeding method, the changes that occur in the larvae after the material used in the experiment can be reliable and timing of the changes taking place is possibly [9]. *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae are a good model for carrying out toxicity studies [10]. Additionally, *G. mellonella* is a very inexpensive insect species which can be produced in large numbers under laboratory conditions. In our previous study, we determined the lethal concentration values of ZnO NPs on the fourth instar *G. mellonella*, effects of different ZnO NPs concentrations (0.5, 1, 2.5, 5 µg/10 µl) on the hemocyte counts, and the percentage of dead cells of larvae with two different methods [5]. Also, transmission electron microscopy image, lethal concentration 50 (LC_{50}) values of ZnO NPs, and the percentage of dead cells of force fed *G. mellonella* larvae after 24 h the treatments have been described by us [5]. But the objective of the present study is to evaluate the effect of nanorod-spahed ZnO NPs on hemocyte types in *G. mellonella*. For this purpose, the effects of the ZnO NPs (0.5, 1, 2.5, 5, and 10 µg/10 µl/larva) was tested in the hemolymph of *G. mellonella* larvae after ZnO NPs exposure by the force-feeding method. In addition, a different ZnO NP (10 µg/10 µl ZnO NP) concentration was additionally tested for the first time with this study.

2. Materials and Methods

2.1. Insects

Different life stages (egg, larvae, and pupae) of *G. mellonella* were obtained from the infested midrib of the wax combs.

2.1.1. Insect diet

The collected samples were placed and reared with wax combs in jars (1 l capacity). The eggs laid by the adult moths who had emerged were also collected. The first stage larvae hatched from the eggs were again placed and reared with wax combs in jars (1 l capacity). The control (untreated) and experiment group larvae (NP treated) were reared in dark conditions at 27 ± 4 ºC with 60 ± 5% relative humidity. All insect rearing cultures and experimental studies of NPs were studied at Avanos Vocational School of Fine Arts Avanos, Nevşehir, Turkey. Fourth instar (110 ± 20 mg) *G. mellonella* larvae were used in all, force-feeding studies [5].

2.2. Chemicals and Materials

The nanoparticle used in this study is a commercial product of Alfa Aesar (Zinc oxide, NanoShield®, 70 nm, ZN-3008C, 50% in H_2O, colloidal dispersion with cationic dispersant, Karlsruhe, Germany). Other materials used in the experiment are distilled water, 29 gauge micro-fine insulin syringe, ultrasonic bath sonicator (Isolab, Turkey), 20 ml plastic containers, giemsa stain solution (Bertek Chemistry, Lot no: P260417), filter paper (Grade 4, Whatman, Dia. 150 mm), Swift SW380T microscope (China), and Swift microscope camera (China) [5].

2.3. Force-Feeding Treatment

Firstly, stock concentrations of ZnO NPs (0.5, 1, 2.5, 5, and 10 µg/µL) were prepared. Then, they were sonicated for 10 min of duration with a bath-type sonicator [5]. 110 ± 20 mg weighed larvae were force-fed with 10 µL of the homogenized ZnO NPs concentrations (0.5, 1, 2.5, 5, and 10 µg/µL) or 10 µL distilled water with a micro-fine insulin syringe (29 gauges) [5, 11]. Postforce-feeding treatment, each larva was kept in a sterile plastic box (20 ml) without natural wax in dark conditions at 26 ± 3 °C with 60 ± 5% relativel humidity. Hemocyte types and numbers in the hemolymphs of the control and experimental group larvae were determined by a giemsa staining method for 24 h post force-feeding treatment.

2.4. Use of Giemsa to Stain Hemocytes for Classification by Light Microscope

The dye-staining steps of the insect hemocytes were carried out at room temperature based on [7] with some modifications as follows. Giemsa stain was diluted 1:4 with distilled water. The diluted stain solution was filtered by whatman paper. Larvae were drilled from the first segment on the back of the head with a fine needle, and 5 µL hemolymph was taken with a micropipette. Hemolymph was smeared onto clean and dry slide immediately. Slides were incubated in 96% ethanol for 5 min. Afterwards, it was waited until the ethyl alcohol completely evaporated from the slides. Then a giemsa protocol was applied. The diluted giemsa solution was dropped onto the slides and the dye was allowed to stain the hemocytes for 15 min. The slides were washed in distilled water for 2 min. Finally, the slides dried for 15 min at room temperature, and then they mounted on a coverslip with entellan. 200 hemocytes were randomly selected in each slide. Each slide was scanned under a light microscope (Swift SW380T, China) at a magnification of 1000x with a Swift microscope camera (China). The types of these selected hemocytes (plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte) were classified (Figure 1) and counted separately according to [12, 13] Five larvae were examined for each experimental group and were replicated three times. A total of 18.000 hemocytes were examined one by one at random, and the type of each hemocyte was determined and counted. The significance levels of differences between the control and experimental groups were determined separately for each type of hemocytes.

2.5. Statistical Analysis

IBM-SPSS (Version 20.0) was used for mean numbers of plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte analysis of *G. mellonella.* The parametric Tukey test was used for when data were normally distributed [14].

3. Results and Discussion

3.1. Hemocyte Types

The hemocytes were classified into six morphotypes, as plasmatocyte (Figure 1.a), granulocyte (Figure 1.b), spherulocyte (Figure 1.c), prohemocyte (Figure 1.d), oenocytoid (Figure 1.e), and coagulocyte (Figure 1.f).

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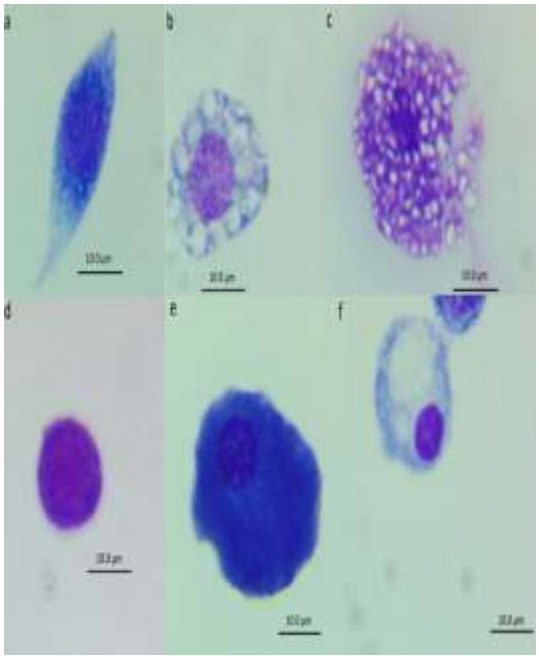

Figure 1. Types of hemocytes in *Galleria mellonella* larvae stained with Giemsa; a. plasmatocyte, b. granulocyte, c. spherulocyte, d. prohemocyte, e. oenocytoid, f. coagulocyte (measure bar: 10 µm; X100).

When we summarize the morphology and functions of the counted and typed hemocytes; plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocytes are types of hemocytes that can be found in *G. mellonella* and some insect species [12, 13]. Granulocytes and plasmatocytes are the only hemocytes adhering to foreign organisms, so they can participate in phagocytosis, encapsulation and nodulation [15, 16]. Plasmatocytes are usually spindle-shaped (10-20 µm) or round (about 10 µm in diameter) (Figure 1.a) [17]. Granulocytes are highly nonameboid, round or oval cells, 12-16 µm in diameter, with a central nucleus that is often masked by large numbers of granules, 1-1.5 µm in diameter (Figure 1.b) [17]. Spherulocytes are responsible for the synthesis and transportation of mucopolysaccharidic components of the cuticle. They are ovoid or round cells of varying sizes (9-25 µm in length and 5-10 µm in width) and usually larger than granulocytes (Figure 1.c) [15-17]. Prohemocytes are considered stem cells, capable of mitotic division and specialized hemocyte differentiation [15, 16]. Prohemocytes are small, round to oval cells with a thin cytoplasmic margin, about 6-13 m in diameter (Figure 1.d) [17]. Oenocytoids contain the cytoplasmic form of phenol oxidase, do not adhere to foreign bodies. They are small to large, thick, oval, spherical or elongated cells of highly variable sizes and shapes (16-54 µm or more) (Figure 1.e) [15, 16]. Coagulocytes generally range from small to large cells (3-30µm long), spherical, transparent, fragile, and combine the characteristics of granulocytes and oenocytoids [17, 18].

3.2. Effects of (ZnO NPs) on Hemocytes Types of *G. mellonella*

The effects of ZnO NPs on mean numbers of plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte in hemolymph of *G. mellonella* larvae was given in Table 1 below. Microscopic observations indicated that plasmatocytes were the most frequent hemocyte type. Also, plasmatocytes, granulocytes, and sphrerulocyte are the most numerous cells in the hemolymph of *G. mellonella* (Table 1). Coagulocytes were the least seen hemocyte type among hemocyte types. The mean numbers of six different hemocyte types according to the lowest and highest values in the table were as follows: 102.2 (1 µg/10 µl ZnO NPs) to 111.73 (5 µg/10 µl ZnO NPs) for plasmatocytes, 60.46 (5 µg/10 µl ZnO NPs) to 76.53 (10 µg/10 µl ZnO NPs) for granulocytes, 9.93 (10 µg/10 µl ZnO NPs) to 18.2 (control group) for spherulocytes, 4.73 (1 µg/10 µl ZnO NPs) to 11.13 (5 µg/10 µl ZnO NPs) for prohemocytes, 1.40 (10 µg/10 µl ZnO NPs) to 2.53 (1 µg/10 µl ZnO NPs) for oenocytoids, 0.46 (control and 0.5 µg/10 µl ZnO NPs) to 1.13 (10 µg/10 µl ZnO NPs) for coagulocytes. When evaluated in terms of the response of different hemocytes to the ZnO NPs exposure to the density of hemolymph, no statistically significant difference was observed between the control and experimental groups, except for the spherulocytes population (Tukey Test, Plasmatocyte: F: 0.534; df: 5; sig: 0.750, Granulocyte: F: 1.874; df: 5; sig: 0.108, Spherulocyte: F: 2.421; df: 5; sig: 0.042, Oenocytoid: F: 0.857; df: 5; sig: 0.514, Coagulocyte:

F: 0.981; df: 5; sig: 0.434) (Table 1). The mean number of spherulocytes significantly decreased 1.83 times in larvae exposed to the highest concentration of ZnO NPs (10 µg/10 µl) when compared to the control group. The mean number of *G. mellonella* larval prohemocytes that exposed to 5 µg/10 µl ZnO NPs was 2.35 times higher than the larvae exposed to 1 µg/10 µl ZnO NPs concentration (Table 1). This 2.35-fold increase in prohemocyte mean was statistically significant (Tukey Test, Prohemocyte: F: 3.069; df: 5; sig: 0.014) (Table 1).

Table 1. Effects of ZnO NPs on mean numbers of plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte in hemolymph of *Galleria mellonella* larvae (Mean ± Standard Error).

Concentrations of ZnO NPs (µg/10 µl)	Plasmatocyte (Mean[b] ± SE)[c]	Granulocyte (Mean[b] ± SE)[c]	Spherulocyte (Mean[b] ± SE)[c]	Prohemocyte (Mean[b] ± SE)[c]	Oenocytoid (Mean[b] ± SE)[c]	Coagulocyte (Mean[b] ± SE)[c]
0[a]	110.26 ± 4.22[a]	62.6 ± 4.86[a]	18.2 ± 2.49[a]	6.73 ± 0.98[ab]	1.73 ± 0.38[a]	0.46 ± 0.27[a]
0.5	107.33 ± 5.3[a]	70.73 ± 4.1[a]	11.33 ± 1.8[ab]	8.2 ± 1.81[ab]	1.93 ± 0.57[a]	0.46 ± 0.23[a]
1	102.2 ± 4.9[a]	76.4 ± 5.56[a]	13.13 ± 1.83[ab]	4.73 ± 0.86[a]	2.53 ± 0.47[a]	1 ± 0.35[a]
2.5	111.4 ± 5.27[a]	64.4 ± 5.15[a]	14.73 ± 1.65[ab]	6.33 ± 1.05[ab]	2.26 ± 0.50[a]	0.86 ± 0.37[a]
5	111.73 ± 5.18[a]	60.46 ± 6.37[a]	13.13 ± 1.85[ab]	11.13 ± 1.97[b]	2.33 ± 0.39[a]	1.20 ± 0.29[a]
10	106 ± 5.43[a]	76.53 ± 4.49[a]	9.93 ± 1.19[b]	5 ± 0.98[a]	1.40 ± 0.37[a]	1.13 ± 0.38[a]

a "0" control group. **b** Values are the means of three replicates with 5 larvae. **c** The difference between groups with different letters in the same column is statistically significant.

It has been reported by [19] that ZnO NPs cross the gut barrier in the lepidopter species *Bombyx mori* (Lepidoptera: Bombycidae). They demonstrated that ZnO NPs can reach the hemolymph and their subsequent interaction and/or uptake by the circulating hemocytes therein. Besides, they showed that a decrease in the percentage of prohemocyte and an increase in the percentage of granulocytes and plasmatocytes [19]. This may be the reason why the lowest mean prohemocyte number and the highest granulocyte mean number were observed at 1 and 10 µg/10 µl ZnO NPs concentrations in our study (Table 1). In a study tested with Nomolt insecticide on *Schistocerca gregaria* Forskal (Orthoptera: Acrididae), it was determined that spherulocytes were the most sensitive cells to the Nomolt whereas the oenocytoids showed the least affected cells [20]. Similarly, spherulocytes were significantly the most sensitive hemocyte type to nanorod-shaped 10 µg/10 µl ZnO NP concentration when compared to the control group in the present study for *G. mellonella* larvae (F: 2.421; df: 5; sig: 0.042) (Table 1) due to their fragile structure (Figure 1.c) [20]. In addition, the mean number of oenocytoids did not differ statistically between the control and experimental groups (Table 1). In other words, the mean number of the oenocytoids was not significantly affected by ZnO NPs. Plasmatocytes and granulocytes are the most predominant hemocytes in *G. mellonella* [21, 22]. In our study, firstly, plasmatocytes and then granulocytes were found to be denser in control and experimental group larvae (Table 1). And the mean number of the plasmatocyte and granulocyte did not differ statistically between the experimental and control group (Table 1). The effects of nanorod-shaped ZnO NPs on differential hemocyte count have not previously been studied. However, in another consistent study, [23] studied the effects of metyrapone on differential count in *Spodoptera littoralis* (Boisd) (Lepidoptera: Noctuidae). They observed that the proportion of plasmatocytes and granulocytes did not significantly change from days 4 to 7. As observed in our study, the fact that there is no significant change in plasmatocyte and granulocyte values of the insect immune response until the first 7 days after exposure to a foreign substance makes it more understandable. On a kind of stimulus, a labile cell resembling a platelet, the coagulocyte frees clotting factors in the surrounding hemolymph [24]. They are specialized for clotting [25]. [26] noted that the differential hemocyte counts (DHC) of coagulocytes can be affected by the titre of ecdysone in the last larval stage of *Heliothis armigera* Hübner (Lepidoptera: Noctuidae) [26, 27]. The reason why there was no significant increase or decrease in the number of coagulocytes at 24 h may be due to the ecdysone hormone level in fourth instar *G.mellonella* larvae (Table 1). Finally, the reason for the significant fluctuations seen in spherulocyte and prohemocytes in this study may have arisen from the toxic effects that occur as a result of the interaction of zinc oxide NPs with these hemocyte types in the hemolymph. Possible toxic interactions are summarized by [5, 28-30]. According to them, when compared to typical zinc forms, ZnO NPs have been shown to permeate into cells. After being taken in by the cells, they might

cause membrane architecture to deteriorate. The cytotoxicity in the cells is caused by their particle breakdown, zinc release into the media, or absorption by the cells [28-30].

Conclusion

The aim of this study was to determine the effects of nanorod-shaped ZnO NPs on hemocytes types of *G. mellonella* L. For this purpose, different concentrations of ZnO NPs were force-fed to fourth instar larvae. The toxic effects of ZnO NPs on plasmatocyte, granulocyte, spherulocyte, prohemocyte, oenocytoid, and coagulocyte numbers in hemolymph of *G. mellonella* larvae was studied. As a result of the study, it was understood that treating *G. mellonella* with 10 µg/10 µl ZnO NPs significantly decreased spherulocytes numbers. But mean numbers of plasmatocyte, granulocyte, prohemocyte, oenocytoid, and coagulocyte numbers did not differ significantly when compared to the control group after 24 h force-feeding treatments.

Author's Contributions

Ata Eskin: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ethics

There are no ethical issues after the publication of this manuscript.

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