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The Effects of Magnetic Iron oxide Nanoparticles (Fe_3O_4) on Some Biological Aspects of *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract

In this study, 18-38 nm-sized and spherical-shaped nanopowder Fe_3O_4 NPs concentrations (0.4, 2, 10, 50, 250 µg/10 µl) was force-fed to sixth instar (180 ± 20 mg) *Galleria mellonella* (Lepidoptera: Pyralidae) larvae under laboratory conditions. The effects of magnetic iron oxide nanoparticles (Fe_3O_4) on the pupal and adult developmental times, pupal and adult weights and adult longevity of *G. mellonella* were recorded. Results showed that treating *G. mellonella* with 250 µg/10 µl Fe_3O_4 NPs significantly increased pupal weights. Additionally, while adult developmental time increased post 250 µg/10 µl Fe_3O_4 NPs treatment, it was observed that pupal developmental time, pupal and adult weights, and adult longevity were not statistically significantly different when compared to the control.

Keywords: Biology, *Galleria mellonella*, Iron Oxide, Nanoparticle.

* The data of the present study were obtained from master's thesis of the first author

1. Introduction

Nanotechnology is a technology for the development of functional materials, devices, and systems at the level of atomic and molecular structures [1]. Nanotechnology is also the science of particles measuring nanometers (usually 1-100 nm) [2]. Iron (III) oxide (Fe_2O_3) is a reddish- brown and an inorganic compound that is paramagnetic in nature. It is also one of the three main iron oxides. The other two of them are FeO and Fe_3O_4. Because of their very small size, magnetic properties, and biocompatibility, Fe_3O_4 NPs is used in various fields such as cancer, diabetes, atherosclerosis, inflammatory diseases, early diagnosis of contrast agents, drug magnetic resonance imaging, targeted drug delivery, hyperthermia, gene therapy, molecular/cellular tracking and in biomedical applications [3-4]. There are some studies on the use of Fe_3O_4 NPs iron oxide in terms of using as nutrients in agricultural studies [5-7]. During their widespread use, NPs come into contact with water. Subsequently, they are separated from the materials that are included in and pass into the water environment. As a result, they can turn into toxic substances [8]. *Galleria mellonella* L. (Lepidoptera: Pyralidae) is an important pest species for beekeepers and causes important problems in beekeeping activities by opening galleries on honeycombs. It can be produced in large numbers in a short time under laboratory conditions. Furthermore, *G. mellonella* is a very low costly insect species and is used as a model experimental organism in toxicological studies. Several previous studies reported that different chemical NPs caused adverse effects on hemocyte counts, biology and antioxidant system, bioaccumulation, antioxidant defense, and immune system on *G. mellonella* [9-12]. The force-feeding method is the forcible delivery of the calories, protein, macro and micro elements, and vitamins that a living thing needs to the body without free will. This method is generally used in toxicity studies [13]. The aim of this study is to obtain data on Fe_3O_4 NP toxicity on a model experimental organism's life cycle. For this, we investigated the effects of various concentrations (0.4-250 µg/10 µl) of Fe_3O_4 NPs on the pupal and adult developmental times, pupal and adult weights, and adult longevity of *G. mellonella* by the force-feeding method.

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 55 ± 5% relative humidity. All insect rearing cultures and experimental studies of NPs were studied at Avanos Vocational School of Higher Education, Avanos, Nevşehir, Turkey. Sixth instar (180 ± 20 mg) *G. mellonella* larvae were used in all force-feeding studies [10].

2.2. Chemicals and Materials

In this study, 18-38 nm-sized and spherical-shaped nanopowder Fe_3O_4 NPs (Nanokar, Istanbul/TURKEY) were used. Distilled water, 29 gauges micro-fine insulin syringe, 70% ethanol, ultrasonic bath sonicator (Isolab, Turkey), 20 ml plastic containers were formed the basic materials of the study.

2.3. Characterization of Fe_3O_4 NPs

The morphology of the Fe_3O_4 NPs was examined using field emission scanning electron microscopy (FESEM) at the Erciyes University Technology Research and Application Center (TAUM). The images of the Fe_3O_4 NPs were taken by using a Zeiss GEMINI 500 device which was connected to the FESEM detector at 25 kV [11]. X-ray diffraction (XRD) pattern of the Fe_3O_4 NPs was recorded at Marmara University, Faculty of Engineering, Metallurgical and Materials Engineering Department with reference code 98-001-7149. The diffractogram was compared with the standard powder diffraction card of the JCPDS iron file and previous studies.

2.4. Determination of Lethal Concentration 50 and 90 (LC_{50} and LC_{90}) Values of Fe_3O_4 NPs in *G. mellonella* Larvae

The toxicity test protocol was performed according to [10] and [11]. Fe_3O_4 NPs were added to distilled water and dissolved to prepare a stock solution of µg/10 µl. The concentrations of Fe_3O_4 NPs (1, 10, 50, 100, 200, 400 µg/10 µl/larva) were prepared to determine the lethal concentration 50 and 90 (LC_{50} and LC_{90}) values of Fe_3O_4 NPs in *G. mellonella* for 30 days (d). We used only distilled water as a control solution. The Np suspensions were homogenized by a bath-type sonicator for 10 min at 40 ºC. Sixty larvae (180 ± 20 mg) and three replicates, each replicate consisted of 20 larvae) were used for every treatment (control and for each experiment groups). The larvae, which were determined to be used in all experiments, were starved for 3 hours [10]. Then, larvae were force-fed with different Fe_3O_4 NPs concentrations (1, 10, 50, 100, 200, 400 µg/10 µl/larva) or only 10 µl distilled water (control group) with a micro-fine insulin syringe (29 gauges) [10]. Postforce-feeding treatment, each larva was maintained in a sterile plastic container (20 ml, with 20 pinhole holes on the top cover) without any diet in dark conditions at 27 ± 4 ºC with 55 ± 5% relative humidity. The numbers of dead larvae and the numbers of viable larvae were counted in 30 d for probit analysis. The lethal concentrations (LC_{50} and LC_{90} values) of Fe_3O_4 NPs on the sixth instar *G. mellonella* larvae were determined by probit analysis using IBM-SPSS (2011) software on the 30th d [14]. According to LC_{50} values of Fe_3O_4 NPs, (0.4, 2, 10, 50, 250 µg/10 µl) Fe_3O_4 NP concentrations were determined as experimental (treated) concentrations for all *G. mellonella* life cycle studies.

2.5. The Determination of Effects of the Magnetic Iron oxide NPs (Fe_3O_4) on Some Biological Aspects of *Galleria mellonella*

For bioassays, larvae (180 ± 20 mg) were force-fed with different Fe_3O_4 NPs concentrations (0.4, 2, 10, 50, 250 µg/10 µl). Each of these concentrations formed the experimental groups of the study. Only 10 µl of distilled water was given to the control group larvae. These studies were carried out under the stereomicroscope with using a micro-fine insulin syringe (29 gauges) [10]. Then, *G. mellonella* larvae were transferred individually into each in a sterile plastic container. The development of sixth instar larvae was monitored daily until the pupation to determine the pupal developmental time. Each pupa was weighed on an analytical scale to investigate the effect of Fe_3O_4 NPs on the pupal weight of *G. mellonella*. The individuals in both experimental and control groups were observed daily and adult developmental times were recorded. Each adult was weighed on an analytical scale with high sensitivity (1 mg to 500 g) to investigate the effect of Fe_3O_4 NPs on adult weight. Adult longevity time (day) was also recorded in treated with different doses of Fe_3O_4 NPs and untreated groups. Each biological assay was replicated three times with 20 sixth instar larvae.

2.6. Statistical Analysis

IBM-SPSS (Version 20.0) was used for the probit and pupal and adult developmental times, pupal and adult weights, and adult longevity data analysis of *G. mellonella.* Nonparametric Kruskal–Wallis test was

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used for biological assays of the insect when data were not normally distributed [14].

3. Results and Discussion

3.1. Characterization of Fe_3O_4 NPs

Figure 1 display the FESEM image of Fe_3O_4 NP powder. The FESEM image showed that Fe_3O_4 NPs were in a spherical morphology (Figure 1).

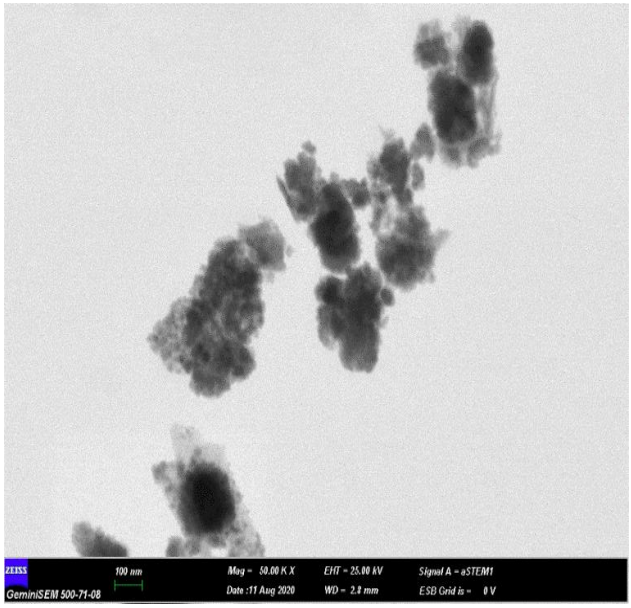

Figure 1. FESEM image of Fe_3O_4 NP powder (50.000x). The scale bar shows 100nm.

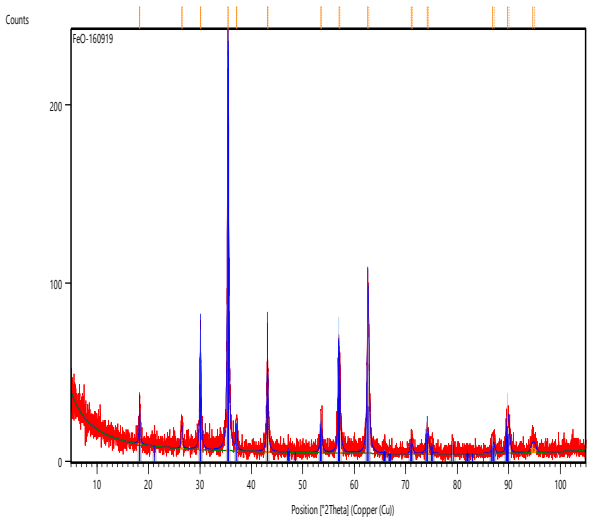

Figure 2. The XRD difractogram of Fe_3O_4 NPs.

The XRD results showed that Fe_3O_4 NPs matched by [15] with magnetite low property (Table 1). All peaks in the XRD patterns are well matched to the standard PDF Cards for Fe_3O_4 NP (ICSD Number: 98-001-7149) (Figure 2). Peaks for Fe_3O_4 NPs appear at 2θ= 30.09 (220), 35.45 (311), 43.10 (400), 53.50 (422), 57.02 (511), and 62.62 (440) respectively (Table 1 and Figure 2) [16]. The peaks confirmed that the studied nanomaterial is Fe_3O_4 NP and has a low magnetic property.

Table 1. XRD peak list of Fe_3O_4 NPs

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]	Tip Width	Matched by
18.27(1)	14(2)	0.23(6)	4.85134	8.42	0.2763	98-001-7149
30.091(7)	41(3)	0.30(3)	2.96744	24.86	0.3582	98-001-7149
35.453(3)	163(6)	0.27(2)	2.52994	100	0.3265	98-001-7149
37.08(2)	10(1)	0.28(5)	2.42286	5.92	0.3368	98-001-7149
43.109(7)	36(3)	0.30(4)	2.09671	22.31	0.3576	98-001-7149
53.50(2)	13(2)	0.36(9)	1.71152	8.09	0.4313	98-001-7149
57.022(7)	43(3)	0.38(4)	1.61376	26.27	0.4573	98-001-7149
62.625(6)	74(4)	0.35(3)	1.48218	45.16	0.4249	98-001-7149
71.05(4)	4(1)	0.6(1)	1.32576	2.47	0.7187	98-001-7149
74.17(2)	11(2)	0.5(1)	1.27749	6.77	0.5440	98-001-7149
86.88(4)	4(1)	0.6(1)	1.12025	2.74	0.7405	98-001-7149
89.75(1)	17(1)	0.57(6)	1.09172	10.67	0.6853	98-001-7149

3.2. LC_{50} and LC_{90} Values of Fe_3O_4 NPs

LC_{50} and LC_{90} values of Fe_3O_4 NPs were determined on *G. mellonella* larvae as 482.72 µg/10 µl and 1843.89 µg/10 µl (Probit, Chi-square= 30.383, df = 5, P = 0.00, y=0.65+1.09E-5*x) respectively for 30 d. Therefore, 0.4, 2, 10, 50, 250 µg/10 µl Fe_3O_4 NP concentrations were determined as experimental (treated) concentrations for all biological studies.

3.3. Effects of Iron Oxide Nanoparticles (Fe_3O_4) on Some Biological Aspects of *G. mellonella*

We tested the effects of Fe_3O_4 NPs on some life parameters (developmental time to pupal stage, pupal weight, developmental time to adult stage, adult weight, and adult longevity) of *G. mellonella*, to determine its sublethal toxicity on the insect by force-feeding method at concentrations of 0.4, 2, 10, 50, 250 µg/10 µl. Results were given in Table 2 below.

The mean of pupal developmental time increased significantly at 250 µg/10 µl concentration when compared with 2 and 50 µg/10 µl concentrations of Fe_3O_4 NPs (x^2= 30.294, df= 5, P= 0.00). But there were no significant differences between experimental groups and control group exist in the mean pupal developmental time. Under the influence of increasing concentrations of Fe_3O_4 NP, an increase in pupal weight was observed.

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Table 2. The effects of iron oxide nanoparticles on some life parameters of *G. mellonella*

Concentrations of Fe_3O_4 NPs (µg/10 µl)	Pupal developmental time (day) (Mean[b] ± SE)[c]	Pupal weight (mg) (Mean[b] ± SE)[c]	Adult developmental time (day) (Mean[b] ± SE)[c]	Adult weight (mg) (Mean[b] ± SE)[c]	Adult longevity time (day) (Mean[b] ± SE)[c]
0[a]	10.08 ± 0.63[ab]	110.61 ± 2.69[a]	14.08 ± 0.43[a]	65.29 ± 1.99[a]	7.61 ± 0.59[a]
0.4	10.42 ± 0.74[ab]	118.39 ± 2.69[ab]	15.65 ± 0.47[ab]	68.47 ± 2.04[a]	7.73 ± 1.00[a]
2	9.04 ± 0.39[a]	112.40 ± 1.75[ab]	15.43 ± 0.22[ab]	69.00 ± 1.85[a]	7.97 ± 0.65[a]
10	10.48 ± 0.44[ab]	112.00± 2.23[ab]	16.69 ± 0.37[b]	63.97 ± 1.82[a]	8.13 ± 0.45[a]
50	9.76 ± 0.44[a]	116.12 ± 2.20[ab]	14.97 ± 0.57[ab]	65.76 ± 2.03[a]	6.25 ± 0.31[a]
250	11.65 ± 0.63[b]	121.73 ± 3.64[b]	15.47 ± 0.39[ab]	69.82 ± 3.5[a]	7.00 ± 0.81[a]
SPSS test:	**Kruskal Wallis Test, P=0.00 < 0.05**	**Kruskal Wallis Test, P=0.008 < 0.05**	**Kruskal Wallis Test, P=0.000 < 0.05**	**Kruskal Wallis Test, P=0.340 > 0.05**	**Kruskal Wallis Test, P=0.178 > 0.05**

[a] "0" control group.

[b] Values are the means of three replicates with 20 larvae. Larvae who could not reach the adult stage were not included in the calculation.

[c] The difference between groups with different letters in the same column is statistically significant.

The pupa mean weight was significantly increased to 121.73 at 250 µg/10 µl Fe_3O_4 NP concentration (x^2= 15.585, df= 5, P= 0.008). Pupal weight in treated larvae ranged from 112 to 121.73 mg as compared with 110.61 mg in the control group (Table 2). We found that adult developmental time significantly increased at 10 µg/10 µl concentration of Fe_3O_4 NP when compared with the control group (x^2= 22.339, df= 5, P= 0.000). Mean adult weight did not differ significantly (x^2= 5.668, df= 5, P= 0.340) and ranged from 63.97 to 69.82 mg. Similarly, mean adult longevity was insignificant (x^2= 7.631, df= 5, P= 0.178) in all Fe_3O_4 NP treatments when compared to the control group and values ranged from 6.25 to 8.13 (d) (Table 2).

Currently, studies on the toxic effects of magnetic Fe_3O_4 NPs on *G. mellonella* are scarce. In this study, it was observed that mean of the pupal development time was prolonged at the highest Fe_3O_4 NPs concentration when compared to the lower concentrations of Fe_3O_4 NPs (Table 2). The reason why nanoparticles cause delays in the biological parameter of insects is explained as follows by [17-18]. According to them, after NPs entering the gut, they were able to induce apoptosis by crossing the peritrophic membrane [17-18]. So, this toxicity within the gut resulted in delays in development. There are some studies conducted with metal oxide NPs and showing that metal oxide NPs cause apoptosis in cell cellular systems of insects [11, 19-21]. We believe that there may be apoptotic mechanisms that occur as a result of NP stress in the larva, which prolongs the pupal development period in response to the increasing concentration of NPs. Changes in pup weights as a result of physiological stress resulting from exposure to silver NPs were obtained by [22] in two lepidopteran pests of the castor plant (*Ricinus communis* L.) namely Asian armyworm, *Spodoptera litura* F. (Lepidoptera: Noctuidae) and castor semi looper, *Achaea janata* L. (Lepidoptera: Noctuidae) larvae. They showed that larval and pupal body weights decreased along with the decrease in the concentrations of AgNPs and $AgNO_3$ in both the test insects [22]. However, in our study, the mean pup weight increased significantly at the highest Fe_3O_4 NP concentration (250 µg/10 µl) when compared to the control group. We speculate that the increase in pupal weights of *G. mellonella* may be related to the upregulation of expression of juvenile hormone-binding proteins (JHBPs) after treatment with Fe_3O_4 NPs as explained by [23]. Adult developmental time extended significantly at 10 µg/10 µl Fe_3O_4 NPs concentration when compared with control group (x^2= 22.339, df= 5, P= 0.00) (Table 2). Juvenile hormone is secreted from the corpus allatum, inhibits insect metamorphosis, and regulates development, reproduction, diapause, polyphenism, behavior throughout insect life [24]. Juvenile hormone metabolism may also affect the larval development time and can cause a prolongation in adult developmental time in *G. mellonella* [25]. So as a result, it is also thought that the increase adult developmental time in our study may be due to an irregularity in the balance of hormones associated with metamorphosis such as the juvenile hormone because of Fe_3O_4 NPs.

4. Conclusion

The purpose of this work was to determine the effects of Fe_3O_4 NPs on some biological aspects of *G. mellonella*. In this context, different concentrations of nanopowder Fe_3O_4 were force-fed to sixth instar larvae. The toxic effects of Fe_3O_4 NPs on the pupal and adult developmental times, pupal and adult weights, and adult longevity of *G. mellonella* were studied. Also, the morphology of the Fe_3O_4 NPs was examined using field emission scanning electron microscopy (FESEM). Finally, X-ray diffraction (XRD) pattern of the Fe_3O_4 NPs was recorded. As a result of these analyzes, it was understood that the Fe_3O_4 NPs have magnetite low property and were in a spherical morphology. Thus, the toxic effects of spherical-shaped and magnetite low

property Fe_3O_4 NPs on some biological stages of the model experimental organism *G. mellonella* were determined with this study.

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Author's Contributions

Ayşe Nazan Eskin: Carried out the toxicity studies. Read, wrote, and approved the final manuscript.
Şahlan Öztürk: Managed and coordinated the toxicity studies. Read, wrote, and approved the final manuscript.
Ata Eskin: Participated in the design of the study, interpreted the chemical analysis results and performed the statistical analysis. Read, wrote and approved the final manuscript.

Ethics

Ethical approval is not applicable, because this article does not contain any studies with human or animal subjects.

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