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THE EVALUATION OF TOXIC EFFECTS OF POLY (VINYLFERROCENIUM)-SUPPORTED PALLADIUM NPS (Pd/PVF^+) ON *APIS MELLIFERA* (HONEY BEE)

[1*]Yeşim Dağlıoğlu, [2]Şükrü önalan, [3]Mutlu sönmez çelebi [4]Dilek kabakçı, and [4]Gökhan akdeniz

[1]Biology Department, Faculty of Art and Sciences, Ordu University, Ordu, Turkey
[2]Aquaculture and Diseases of Department, Faculty of Aquaculture, Yüzüncü Yıl Universty, Van, Turkey
[3]Chemistry Department, Faculty of Art and Sciences, Ordu University, Ordu, Turkey
[4]Ministry of Food, Agriculture and Livestock, Apiculture Research Station Department, Ordu, Turkey

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ABSTRACT

The nanotechnology industry has made a rapid progress by introducing unusual properties to the substances at nano scales. When we look at LC_{50} values, since prolonged to 96 h than 48 h exposure time, toxic effect quantities of PVF^+, Pd/PVF^+ and K_2PdCl_4 suggest that the necessary amount to show the same toxic effect had diminished and its toxic effect increased quite a great deal in time. At the multiple comparison analysis; executed study the same concentration for PVF^+, Pd/PVF^+ and K_2PdCl_4, were found significant differences at the level $P<0.05$ and 0.01 between difference time groups and concentration. The test results of the differences in the mortality rates between different concentration and time groups of PVF^+, Pd/PVF^+ and K_2PdCl_4, which made the most difference in all the concentration and time groups were the 1 mg/L concentration and 96 h.

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INTRODUCTION

Nanotoxicology is referred to as a new branch of toxicology in which the adverse health effects caused by NPs are described (Donaldson *et al*., 2004). The nanotechnology industry has made a rapid progress by introducing unusual properties to the substances at nano scales. Such properties of nanopartcles (NPs) have also raised concerns about their potential toxicities and ecotoxicology. The varying quantum behaviors and the increase in their reactivities have made the manufactured NPs applicable in a number of practices in the industry; however, these substances can cause unexpected effects on the environment and living organisms (Handy *et al*., 2008). Besides, the NPs, thanks to their tiny sizes, prevent other xenobiotics from accessing into the living organism cells by passing through the membrane and the intercellular junctions by posing an obstacle to them. The increase in the reactivities of NPs can potentially cause harmful cellular effects and can harm the living organisms (Oberdörster *et al*., 2007, Crane *et al*., 2008, Griffitt *et al*., 2008, Kroll *et al*., 2009).

***Corresponding author: Yeşim özkan,** *Biology Department, Faculty of Art and Sciences, Ordu University, Ordu, Turkey*

Elements of platinum group are rarely found in nature and are spread around the environment through anthropogenic activities. Although commonly used in many fields of technology, medicine and catalytic converters in particular, the elements of platinum group accumulate around due to the environmental insufficiency towards disposal of wastes (Ek *et al*., 2004). Contamination through the elements of platinum group is primarily caused by the particles or substances in the air, and the dust by the roadside, soil, mud and water.

This contamination eventually results in the biological accumulation in living organisms (Ravindra *et al*., 2004). Palladium (Pd) and Platinum (Pt) are, today, the catalytic elements of automobile catalysts (Johnson, 2004). Besides, a prominent increase in the Pd levels has particularly been observed in the dust by the roadsides and in the soil (Whitely and Murry, 2003; Zereini, 2007; Leopold *et al*., 2008). In this study, the *Apis mellifera* (honey bee) was used for the hazards assessment of K_2PdCl_4 used as the source of Pd, Poly(vinylferrocenium) (PVF^+) and the poly(vinilferrosenyum) (PVF^+)-supported Pd NPs (Pd/PVF^+) which are the oxidized forms of the polymer. *Apis mellifera* is commonly used in such laboratory studies on toxicity. (Lethal

concentration: LC_{50}). We tried to find out where the NPs toxicity emerged from by comparing the data with one another, which was obtained as the result of studying the toxicity of the surface that the NPs was synthesized upon and the toxic effect of the NPs polymer compound.

MATERIALS AND METHODS

Bee Material

A month before the start of the experiment, the queen bee was caged on an embossed honey comb and was made to spawn, and then the spawning ground of the queen bee was recorded. No chemical pest control against diseases was performed within the colony used in the acute toxicity study. During the closed brood period, the honeycombs were taken to the grates and the young worker bees of the same age and the same race that emerged from the comb cells were randomly distributed into the cages. The worker bees taken into the cages were left hungry for 2 h prior to their treatment with the test groups. Before the start of the test, the worker bees that were about to die were replaced by the healthy ones.

Test Chemicals

Poly (vinylferrocenium) supported pd NPs were prepared according to the procedure described in the literature (Çelebi *et al*. 2008). K_2PdCl_4 (≥98.2%, Merck) was used as received.

The Preparation of Test Substances (Test Solutions)

In order to prepare the stock solutions at desired concentrations, the test substances, the PVF^+ supported Pd NPs (Pd/PVF^+), Poly(vinylferrocenium) (PVF^+) film and K_2PdCl_4 solid, were prepared in a deionized water through the medium of dispersion. Afterwards, this solution was vortexed for 20 seconds, and while the stock solutions of the NPs were being prepared, the ultrasonic water bath (Bandelin, sonorex) was used and was made sonicated for 30 minutes in order to increase the dispersion in water and ensure the maximum distribution of NPs in the water. The test concentrations determined in the wake of all of these stages were prepared from the stock solution via dilution. From the test solutions prepared, a sucrose solution at one-to-one proportion was also prepared for the purpose of feeding the bees throughout the testing period. This solution was prepared by adding 1 g of sugar into 1 ml of solution. The sucrose solution was prepared as fresh to feed the bees each day.

The Studies and Experimental Setup of Acute Toxicity

In order to shelter the bees for 96 h in vitro, 20x15 cm -long and 8 cm-wide self-covered plastic containers were utilized. Randomly selected 50 bees were put into each container. On one side of these containers, tiny holes were made to let the air in for the bees during the experiment, after which the sides of the cover were rubbed with emery in order not to let the cover open during the experiment. 2x1 cm hole was opened from the lower side of the plastic container to collect the bees that perished during the experiment, thanks to which the bees were easily collected. To feed the bees, on the other hand, 1 ml volume of droppers (pasteur pipettes) were used. The droppers were fixedly and vertically placed on the upper part of the containers, by means of which the bees were made to be fed easily from the droppers. The sucrose solution was put into the droppers with the help of a syringe. In this way, the groups exposed to the test were made to be fed with a test solution every four h. On the other hand, the control group members were fed with the sucrose solution prepared only with deionized water. The test room was conducted at 25±2ºC temperature and in the dark. The relative humidity which was normally 50-70% was recorded throughout the test. No behavioural disorder or mortality was observed in the control groups all through the test. The perished bees in the test groups were counted at the 24-h, 48-h, 72-h and 96-h. This study was carried out in 3 repetitions, independent of each other.

Statistical Analyses

All the tests/experiments were repeated three times independently, and the data were recorded on average by means of standard deviation. The LC_{50} value was calculated through the probit statistical analysis of EPA. The other analyses were performed through ANOVA and TUKEY multiple comparison analysis.

RESULTS AND DISCUSSION

Akut toxicity/ LC_{50} study

In this study where the lethal concentration against the Poly(vinylferrocenium) (PVF^+), K_2PdCl_4 and PVF^+ supported Pd (Pd/PVF^+) NPs on *Apis mellifera* is determined, the 48 and 96-hour- LC_{50} value was calculated through the probit analysis by taking the test results as the basis of the study. These values were shown in the following table along with the regression graphic in it. Throughout the test, no mortality or behavioral abnormalities were observed within the control group. Whether there was any difference depending on the concentration and the time in the mortality rate for these three substances performed at the same concentrations to be able to give the comparison was determined according to the statistical analyses. In tablo 1, when we evaluated comparatively the lethal concentration values determined for the 48-h and 96-h in the acute toxicity study carried out at the same concentrations for PVF^+, the LC_1, LC_5 values of PVF^+ for 96-h could not be calculated.

Table 1. 96 and 48 hour LC/EC values calculated for PVF^+

points	values of 48 H	values of 96 H
LC/EC 1.00	0.001	0.000
LC/EC 5.00	0.022	0.000
LC/EC 10.00	0.152	0.001
LC/EC 15.00	0.566	0.003
LC/EC 50.00	148.153	0.344
LC/EC 85.00	38771.102	34.135
LC/EC 90.00	144720.828	101.273
LC/EC 95.00	1018748.875	507.290
LC/EC 99.00	39599884.000	10416.576

Whereas the LC_{50} value at the 48-h hour was 148.153, this value at the 96-h hour regressed to 0.344, which suggests that the amount necessary for the PVF^+ to show the same toxic

effect had diminished and its toxic effect increased quite a great deal in time (Fig. 1,2). In tablo 2, when we evaluated comparatively the lethal concentration values determined for the 48-h and 96-h in the acute toxicity study carried out at the same concentrations for K_2PdCl_4, the LC_1, LC_5, LC_{10}, LC_{15} values of K_2PdCl_4 for 96-h could not be calculated. While the LC_{50} value at the 48-h was 20006.971, this value regressed to 4.339 at the 96-h, which suggests that that the amount necessary for the K_2PdCl_4 to show the same toxic effect had diminished and its toxic effect increased quite a great deal in time (Fig. 3,4). In tablo 3, the LC_1, LC_5, LC_{10}, LC_{15} values for the 96-h hour for Pd/PVF$^+$ could not be calculated. Whereas the LC_{50} value at the 48th hour was 148.150, this value at the 96-h regressed to 6.582. Again, these results, as in Pd/PVF$^+$, suggest that the amount necessary to show the same toxic effect had diminished and its toxic effect increased quite a great deal in time (Fig. 5,6).

Table 2. 96 and 48 hour LC/EC values calculated for K_2PdCl_4

Points	Values of 48 H	Values of 96 H
LC/EC 1.00	0.002	0.000
LC/EC 5.00	0.191	0.000
LC/EC 10.00	2.456	0.000
LC/EC 15.00	13.755	0.000
LC/EC 50.00	20006.971	4.339
LC/EC 85.00	29100156.000	19126826.000
LC/EC 90.00	162988608.000	713829952.000
LC/EC 95.00	2093220992.000	152290394112.000
LC/EC 99.00	251304837120.000	3556786.703

Table 3. 96 and 48 hour LC/EC values calculated for Pd/PVF

Points	Values of 48 H	Values of 96 H
LC/EC 1.00	0.001	0.000
LC/EC 5.00	0.022	0.000
LC/EC 10.00	0.152	0.000
LC/EC 15.00	0.566	0.000
LC/EC 50.00	148.150	6.582
LC/EC 85.00	38771.102	95244075008.000
LC/EC 90.00	144720.875	2413744.987
LC/EC 95.00	1018748.875	8797335.986
LC/EC 99.00	39599884.000	4212615.210

Multiple comparison analysis

In table 4, the group which made the most difference in all the concentration and time groups of PVF$^+$ were the 1 mg/L concentration and 96 h. According to the results of ANOVA in table 5, significant differences between 0.01 mg/L concentration and 0.1 mg/L concentration at $P<0.05$ level were found among the mortality rates at the end of the exposure period of PVF$^+$, and also significant differences at $P<0.01$ level were found among the 1 mg/L values. Significant differences were found between 1 mg/L concentration and 0* mg/L concentration along with 0.1 mg/L concentration and 0* mg/L concentration at $P< 0.01$ level. In addition, among the mortality rates of PVF$^+$, significant differences at $P< 0.01$ level were found between the time groups at the 24-h and 48-h and the time group at the 96-h

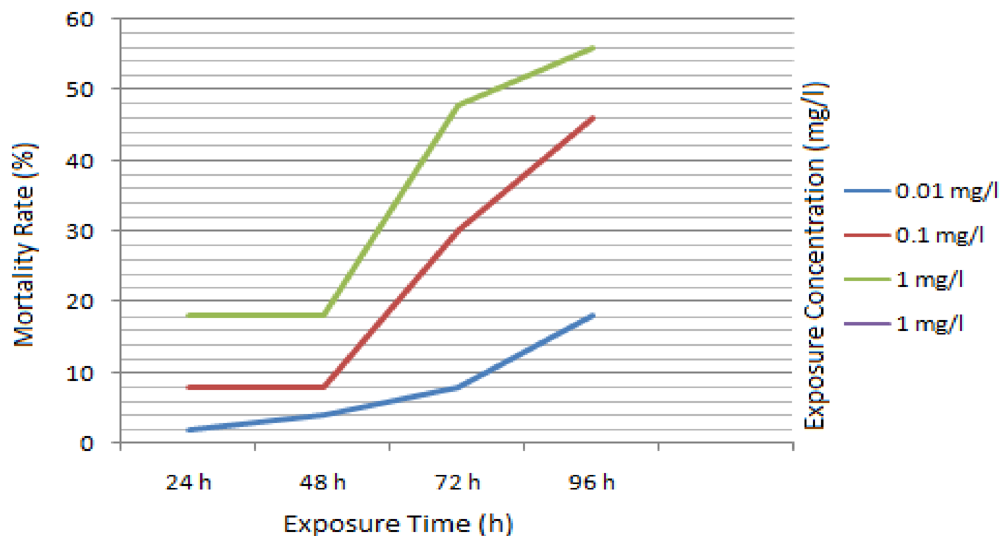

Fig. 1. The mortality rates regarding PVF$^+$ *Apis mellifera* according to the period of time and concentration

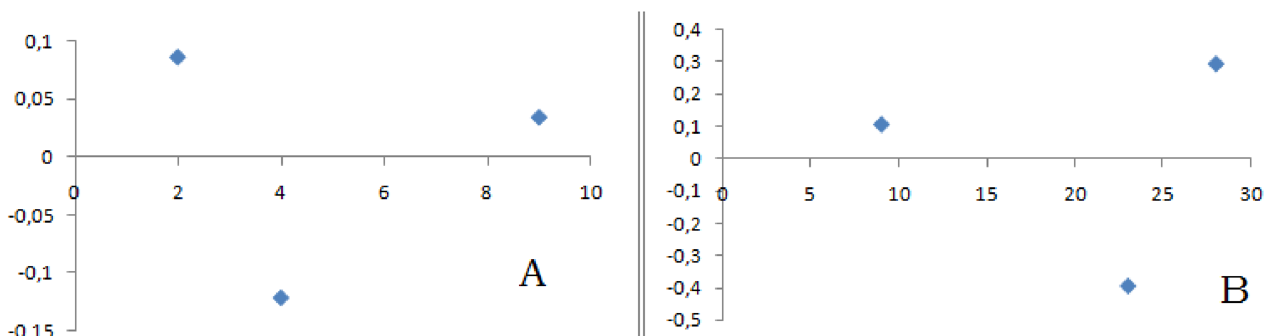

Fig. 2. Regression distributions for PVF$^+$ A) for 48 h B) for 96 h

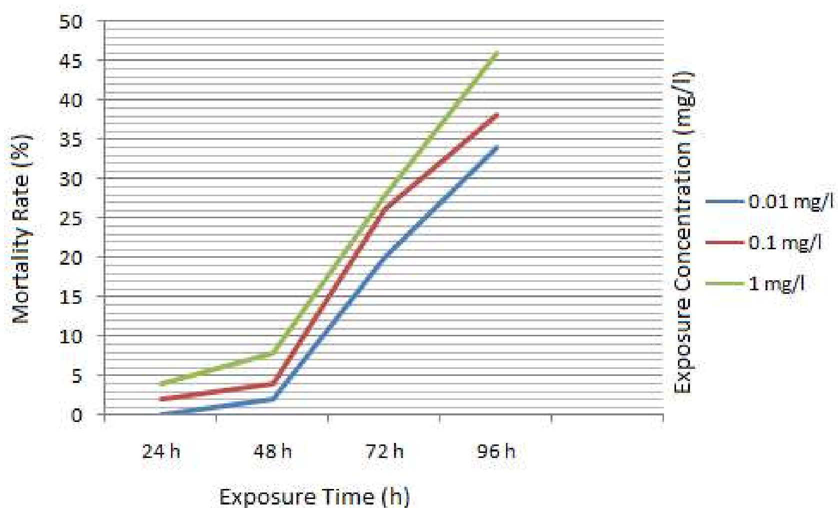

Fig. 3. The mortality rates regarding K_2PdCl_4 *Apis mellifera* according to the period of time and concentration

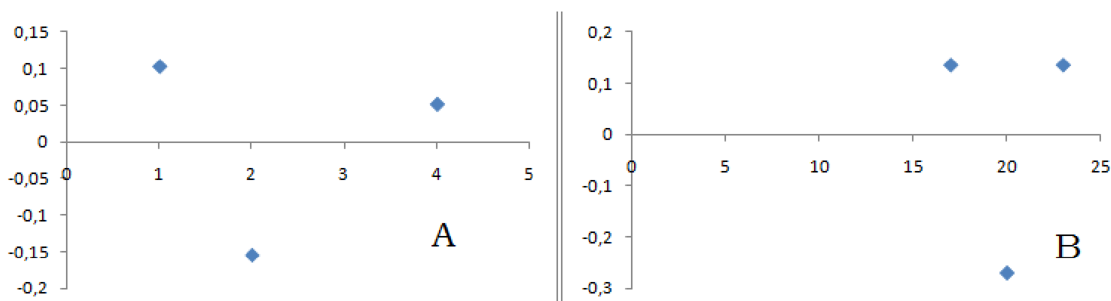

Fig. 4. Regression distributions for K_2PdCl_4 A) for 48 h B) for 96 h

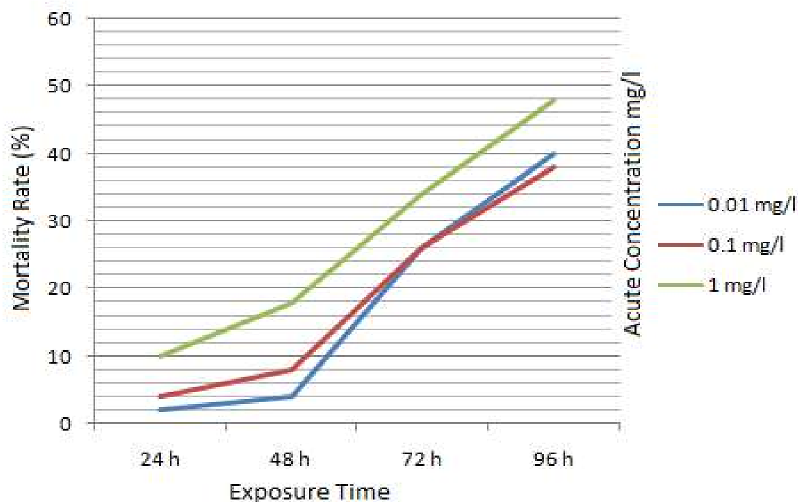

Fig. 5. The mortality rates regarding Pd/PVF^+ *Apis mellifera* according to the period of time and concentration

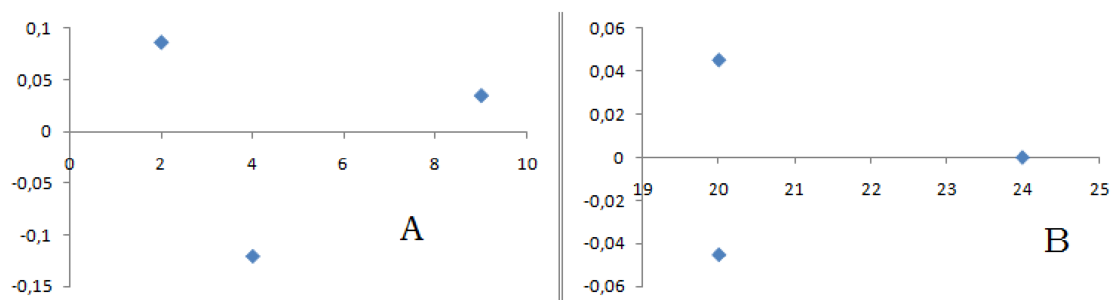

Fig. 6. Regression distributions for Pd/PVF^+ A) for 48 h B) for 96 h

Table 4. The differences in the mortality rates between different concentration and time groups of PVF^{+}

Concentration (mg/L)	N	Subset for alpha = 0.05		Time (h)	N	Subset For alpha = 0.05	
		1	2			1	2
0*	12	0.0000^{b}		24	12	0.0683^{b}	
0.01	12	0.0833^{b}		48	12	0.0750^{b}	
0.1	12		0.2300^{a}	72	12	0.2167^{ab}	0.2167^{ab}
1	12		0.3467^{a}	96	12		0.3000^{a}
Sig.		0.394	0.132	Sig.		0.118	0.580

0*:control group, Sig.: significance

Table 5. The Difference between the PVF^{+} concentration and time groups

Con.(I) (mg/L)	Con.(J) (mg/L)	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.01	1	-0.26333^{*}	0.05239	0.000	-0.4032	-0.1235
0.1	0.01	0.14667^{*}	0.05239	0.037	0.0068	0.2865
	0*	0.23000^{*}	0.05239	0.000	0.0901	0.3699
1	0*	0.34667^{*}	0.05239	0.000	0.2068	0.4865
Time (I) (h)	Time (J) (h)	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
24	96	-0.23167^{*}	0.06507	0.005	-0.4054	-0.0579
48	96	-0.22500^{*}	0.06507	0.006	-0.3987	-0.0513

Table 6. The differences in the mortality rates between different concentration and time groups of Pd/PVF^{+}

Time (h)	N	Subset for alpha = 0.05		Con. (mg/L)	N	Subset for alpha = 0.05	
		1	2			1	2
24	12	0.0383^{b}		0*	12	0.0000^{b}	
48	12	0.0717^{b}		0.01	12		0.1800^{a}
72	12		0.2133^{a}	0.1	12		0.1883^{a}
96	12		0.3200^{a}	1	12		0.2750^{a}
Sig.		0.915	0.175	Sig.		1.000	0.336

0*:control group, Con: concentration, sig.: significance

Table 7. The Difference between the Pd/PVF^{+} concentration and time groups

Con.(I) (mg/L)	Con.(J) (mg/L)	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Lower Bound
0.01	0*	-0.18000^{*}	0.05591	0.012	0.0307	0.3293
0*	0.01	-0.18000^{*}	0.05591	0.012	-0.3293	-0.0307
	0.1	-0.18833^{*}	0.05591	0.008	-0.3376	-0.0391
	1	-0.27500^{*}	0.05591	0.000	-0.4243	-0.1257
Time (I) (h)	Time (J) (h)	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
24	72	-0.17500^{*}	0.05123	0.007	-0.3118	-0.0382
	96	-0.28167^{*}	0.05123	0.000	-0.4185	-0.1449
48	72	-0.4167^{*}	0.05123	0.040	-0.2785	-0.0049
	96	-0.24833^{*}	0.05123	0.000	-0.3851	-0.1115

0:control group, con: concentration, sig.: significance*

Table 8. The differences in the mortality rates between different concentration and time groups of K_2PdCl_4

Time (h)	N	Subset for alpha = 0.05		Con. (mg/L)	N	Subset for alpha = 0.05	
		1	2			1	2
24	12	0.0133		0*	12	0.0000^{b}	
48	12	0.0367		0.01	12	0.1383^{ab}	0.1383^{ab}
72	12		0.1833	0.1	12		0.1750^{a}
96	12		0.2967	1	12		0.2167^{a}
Sig.		0.955	0.073	Sig.		0.087	0.522

0*:control group, con: concentration, sig.: significance

Table 9. The Differences between the $K_2PdCl_4^{+}$ concentration and time groups

(I) Con. (mg/L)	(J) Con. (mg/L)	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0*	0.1	-0.17500^{*}	0.05703	0.019	-0.3273	-0.0227
	1	-0.21667^{*}	0.05703	0.002	-0.3689	-0.0644
Time (I) (h)	Time (J) (h)	Mean Difference (I-J)	Standard Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
72	24	0.17000^{*}	0.04529	0.003	0.0491	0.2909
	48	0.14667^{*}	0.04529	0.012	0.0257	0.2676
96	24	0.28333^{*}	0.04529	0.000	0.1624	0.4043
	48	0.26000^{*}	0.04529	0.000	0.1391	0.3809

0*:control group, con: concentration, sig.: significance

In table 7, according to the results of ANOVA, the mean difference only between 0.01 mg/L concentration and 0* mg/L other concentrations of Pd/PVF$^+$ NPs was found to be at P<0.05 level, and there were also significant differences at P<0.01 level between 0* mg/L concentration and 0.1 mg/L and 1 mg/L concentrations. Among the mortality rates of Pd/PVF$^+$, significant differences were found at P< 0.05 level between the time groups at the 24th and 72th h, and also at P< 0.01 level between those at the 24th and 96th h, whereas the significant differences were observed at P< 0.05 level between the time groups at the 48th and 72nd h, and those differences were found to be at P< 0.01 level between the time groups at the 48-h and 96-h. In table 8, the group which made the most difference in all the concentration and time groups of K_2PdCl_4 was the 1 mg/L concentration and 96 h.

According to the results of ANOVA, among the mortality rates of K_2PdCl_4, the differences are significant at P<0.05 level between the 24-h and 72-h groups and between the 72-h and 48-h groups, and also between the 96-h and 24-h groups and 96-h and 48-h groups. Among the mortality rates of K_2PdCl_4, the mean difference is significant at P<0.05 level between 0* mg/L concentration and 1 mg/L and 0.1 mg/L concentrations (Table 9). In general, considering whether or not the mortality rates of PVF$^+$, Pd/PVF$^+$, and K_2PdCl_4 make any difference by depending on time (h) and concentrations, we can state that there are differences in the mortality rates, depending on both the time and the concentration. However, such a difference has occurred, for each three substance, in different ways between both the time and the concentration groups. According to the TUKEY test results, the difference caused by the mortality rate depending on the concentration is as follows: The mean difference between only 0* mg/L and other concentrations of Pd/PVF$^+$, and between 0* mg/L and 1 mg/L and 0.1 mg/L concentrations of K_2PdCl_4, and between 0.01 mg/L concentration and 0.1 mg/L and 1 mg/L, along with 0* mg/L and 0.1 mg/L and 1 mg/L concentrations of PVF$^+$ is significant at the P<0.05 level. According to the TUKEY test results, the difference indicated by the mortality rate depending on time is, on the other hand, as such: The mean difference is significant at P<0.05 level between the 24-h and 72-h and 96-h, 48-h and 72-h and 96-h groups for Pd/PVF$^+$, and between 24-h and 72-h and 96-h, 48-h and 72-h and 96-h groups for K_2PdCl_4 and also between 96-h and 24-h and 48-h groups for PVF$^+$.

Table 10. Values LC_{50} for 48 and 96 h of Pd/PVF$^+$ and PVF$^+$, K_2PdCl_4

Substances	LC_{50} / 48 H	LC_{50} / 96 H
K_2PdCl_4	20.006.971	4.339
Pd/PVF$^+$	5.467.546	6.582
PVF$^+$	148.153	0.344

0*:control group, LC50: lethal concentration

Table 11. The differences between concentration and time groups of mortality rate to Pd/PVF$^+$ and PVF$^+$, K_2PdCl_4

Substances	Concentration (mg/L)	Time (h)
PVF$^+$	0.01-0.1/ 0.01-1	24-96
	0.1-0*/1-0*	48-96
K_2PdCl_4	0*-0.1	24-72
	0*-1	48-72
Pd/PVF$^+$	0.0.1-0*	24-72/24-96
	0.1-0*	48-72/ 48-96

Conclusion

When compared with classical (micro-scale) substances, the NPs may interact more efficiently with the biological systems generating serious toxicity due to their great surface area and nano-scales, since the nano-sized surface areas of NPs are associated with their several basic properties, such as surface characteristics (the fact that approximately 40-50% of NPs atoms exist on the surface), chemical reaction, physical absorption capacity, etc. as well as the sizes of NPs. It was stated that the most important factor among these were the size of the NPs, the impurity and the surface it was synthesized upon. In this study, we tried to find out where the NPs toxicity emerged from by comparing the data with one another, which was obtained as the result of studying the toxicity of the surface that the NPs was synthesized upon and the toxic effect of the NPs polymer compound. For this purpose, in this study, the 48 and 96-hlethal concentration values of Poly(vinylferrocenium) (PVF$^+$), K_2PdCl_4 and PVF-supported Pd (Pd/PVF$^+$) NPs, which were LC_1, LC_5, LC_{10}, LC_{15}, LC_{85}, LC_{90}, LC_{95}, LC_{99} and notably, LC_{50} were determined in the *Apis mellifera* (Honey Bee).

In the table 10, we see the LC_{50} values of these three substances which were recorded for 48-h and 96-h. Accordingly, when we compare the LC_{50} values of each of the three substances for 48-h and 96-h, we can say that the toxic effect of each of the three substances increased in the course of time. Of these three substances, the least toxic one for 48-h was K_2PdCl_4, then came Pd/PVF$^+$ NPs and the most toxic one proved to be PVF$^+$. Considering the values, when we compare PVF$^+$ with K_2PdCl_4, there is 135 times decrease in the amount of it, whereas when compared with Pd/PVF$^+$, there is 3.6 times decrease in the amount of it. For 96-h, the most toxic one is, again, PVF$^+$, then comes K_2PdCl_4 and then comes Pd/PVF$^+$ NPs as the least toxic one. Considering all of these results, it is suggested that when the exposure time rises from 48-h up to 9-h, the toxic amount of each of the three substances diminished at a considerable rate. In particular, we see that there is a great deal of decrease in the amount of K_2PdCl_4. Depending on these results, we can say that the toxic effect of each of the three substances increased according to the exposure time. Again, the toxic effect of Pd/PVF$^+$ NPs can be said to result from PVF.

Whether or not the mortality rate of Poly(vinylferrocenium) (PVF$^+$), K_2PdCl_4 and PVF$^+$ supported Pd (Pd/PVF$^+$) NPs showed any difference was investigated through the multiple comparison test by comparing each time and concentration group with the other time and concentration groups. According to the statistical results, for each of the three substances, significant differences were found in the time and concentration groups, depending on the mortality rate. All these differences are given in the following (Table 11). When we evaluated the difference caused by the mortality rate of PVF$^+$, K_2PdCl, and Pd/PVF$^+$ between the concentration groups comparatively with one another, most of the difference among these three substances was determined to have been caused by PVF$^+$. There is a significant level of difference among the four different concentration groups of PVF$^+$. In K_2PdCl_4 and Pd/PVF$^+$, however, two different concentration groups made that difference. Again, when we comparatively examine the

difference caused by the mortality rate among the time groups, we can see that Pd/PVF$^+$ NPs forms 4 different time groups, while K_2PdCl_4 and PVF$^+$ form 2 different groups. The fact that the toxic effect of these three substances increased according to the exposure period (time) was pointed out in the LC_{50} results. It was recorded that when this exposure time extended up to 96 h for PVF$^+$, 72-h for K_2PdCl_4 and from 24 up to 48, and then from 48-h up to 72-h for Pd/PVF$^+$ NPs, it showed its toxic effect; therefore, the exposure time for the toxicity caused by Pd/PVF$^+$ NPs in all the time groups was quite important. Consequently, K_2PdCl_4 -the main compound from which Pd NPs was obtained-, PVF$^+$- a surface polymer on which Pd NPs is synthesized- and Pd/PVF$^+$ NPs obtained by synthesizing Pd into PVF$^+$ were compared within themselves and with the other substances by studying them at the same concentrations and by taking into consideration the 48 and 96-hour-exposure time of the toxic effect of each of the three substances. According to the results of this study, it was reported that the most toxic substance among these three was PVF$^+$ for both 48 and 96 h of exposure period.

In particular, when we compared the lethal concentration amount of PVF$^+$ during the 24-hour-exposure period, it was reported to have decreased 135 times more than $K_2PdCl_4$and 3.6 times more than Pd/PVF$^+$. According to the multiple comparison test results, on the other hand, the mortality rate for each of the three substances made a significant difference among the concentration and time groups. It was reported that most of the difference in the mortality rate was caused by PVF$^+$ according to the concentration groups, whereas that difference was caused by Pd/PVF$^+$ NPs according to the time groups. The toxic effect of NPs vary due to various factors, such as the way of NPs synthesis, the surface material that it is supported by and the main compound it is obtained from. In this study where a research has been done like it has never been done before, the main compound that it NPs synthesized, the material it is supported by and the supported NPs were evaluated as factors altering the toxicity of NPs. Such a study has never been conducted before. Hence, it is considered that due to lack of sufficient literature on it, it will make contribution to the future studies with its basic toxicological quality.

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