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Toxic Effects of Diazinon on Adult Individuals of *Drosophila melanogaster*

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ABSTRACT

In this study, the effects of diazinon on morphology and sex ratio in adult individuals of *Drosophila melanogaster* were investigated. Diazinon solution was applied to *Drosophila melanogaster* by means of nutrition. In the F_1 and F_2 generation, quite a lot phenotypic abnormalities were seen. Therefore, the inheritance of the mutations caused by diazinon were investigated. For this purpose, individuals with normal and abnormal phenotypes, which developed in culture medium containing diazinon, were crossed and a second F_2 generation was obtained. High level of phenotypic abnormality was found in this generation, too. Based on the data obtained as a result of this two-way test, it can be concluded that diazinon causes hereditary mutations. Although there were some differences in the data about sex ratio that arose due to the effect of diazinon, this situation did not appear to be statistically meaningful. However, female individuals are more sensitive to diazinon.

Key Words: Drosophila, Diazinon, Morphology, Mutation, Sex Ratio

INTRODUCTION

Nowadays, starvation is one of the most important problems [1]. Human beings are obliged to struggle with pests which cause loss of yield during growing and storing plants and animals that they use as a source of nutrition. As physical and biological pest controls are time consuming, difficult and expensive methods, chemical control is employed as the first choice [2- 6]. But besides this, because pesticides are widely used and most of them are persistent, they cause important problems for non-target living beings by means of food chain. Many researchers indicate that insecticides and degradation products have mutagenic and carcinogenic effects on humans and other living beings, as well. It is known that insecticides with organic phosphorus content change the DNA of cell and cause mutation in case of excess usage [7]. Both active contents and metabolites of these chemical materials disperse into the environment and harm living beings other than the target organism. Only a few insecticides are effective on target organisms that are specific to themselves. Thus, other related species are in danger [3,8,9].

Diazinon is an organic phosphorus insecticide. Pesticides with organic phosphorus content are widely used because they degrade and are eliminated quickly.

However, because of containing two alkyl roots they are alkylating agents and these compounds are potential mutagens [1]. Alkylating agents, comprising a large portion of carcinogens and mutagens, are classified among chemicals that are certainly carcinogenic for humans by International Agency for Research on Cancer (IARC) [10-12].

Although there are lots of investigations about morphological abnormalities that occur due to toxic effect of diazinon [8,9,13-15], it was not investigated whether these abnormalities were hereditary or not. Investigation of especially non-target organisms in this respect would be beneficial in terms of evaluating the risk under which other populations including human are. The genetic properties and reproduction of *Drosophila melanogaster* are known thoroughly and it has nearly all of the metabolic activation enzymes present in mammals. It is known that any material which can effect *Drosophila* DNA can create a similar effect on mammal and human DNA, too [16,17]. Therefore, it is an organism which can be beneficial in inspecting the effect of synthetic materials on phenotypic features and keeping track of these effects in subsequent generations. For this purpose, the effect of diazinon on morphological properties and sex-ratio of *Drosophila melanogaster* was investigated in this study.

MATERIALS AND METHODS

In the studies, wild-type Oregon-R strain of highly inbred *Drosophila melanogaster* (Diptera: Drosophilidae) was used. Culture media used in the experiments were incubated in a cooling incubator set to 25±1°C. Diazinon was applied to adult individuals by means of nutrition. Diazinon solutions were added to culture medium in experimental group and no application was made to control group.

Due to the fact that the same active content is used under different commercial names in pesticide manufacture, the active content of diazinon was used in this study. The active content of diazinon was obtained from Plant Protection Department of Syngenta Agriculture Industrial and Commercial Inc. Co.

In toxicology investigations, it has to be known at which concentration the material to be used is mutagenic. Therefore, LC_{50} values were calculated. As the amounts over this value have toxic effect, they don't allow investigating the other effects. Initially, a stock solution of 1000ppm was prepared and then diluted to required concentrations [18]. For the determination of lethal concentration, diazinon solutions at various concentrations were added to culture media and 40 adult individuals were placed in each culture medium. Death percentages were calculated considering dead and alive individual numbers after 24 hours. According to this, LC_{50} concentration was found to be between $6x10^{-4}$ ppm and $1x10^{-4}$ ppm. In the experiments, three concentrations below LC_{50}, i.e. $6x10^{-5}$, $6x10^{-6}$ and $6x10^{-7}$ ppm, were tested. In a study performed with *Gillia altilis,* which is a good indicator for environmental contamination, the lethal concentration value for diazinon was found to be between 11-93 ppm [19].

1 ml of diazinon solution ($6x10^{-5}$, $6x10^{-6}$ and $6x10^{-7}$ ppm) was mixed with 50ml of culture medium and added to culture media of the experimental group. 10 male and 10 female individuals were placed in both experimental and control group bottles. After pupa formation was observed, adult individuals were removed from the culture medium. Morphologies of adult individuals of F_1 generation were observed under dissection microscope, discriminating male and female individuals, for eight days starting from the first day of eclosion. The abnormalities observed were recorded.

In the next stage of the investigation, observations regarding F_2 generation were made in order to see whether the toxic effect continued in the next generations or not. For this purpose, individuals exposed to diazinon in F_1 generation were transferred to culture medium which did not contain diazinon (SDM), in accordance with the concentration in F_1 generation. 10 female and 10 male randomly selected individuals were crossed. The same procedures applied to F_1 generation were repeated during observation stage [4,20,21].

In another part of the investigation, it was researched whether the phenotypic abnormalities that arose due to the effect of diazinon were hereditary or not. For this purpose, a large number of culture media containing diazinon solution were prepared and crossing was performed in order to obtain enough number of individuals with abnormal phenotypes.

After a period of nearly 15 days, 10 male and 10 female individuals were transferred to culture medium that did not contain diazinon and they were crossed. At the end of 10 days, phenotypes of individuals of F_2 generation were observed.

On the last stage of the investigation, in order to see the effect of diazinon on sex-ratio, males and females obtained in both generations were recorded separately. Evaluating the data, it was investigated whether there was a change in sex-ratio.

The statistical method employed for statistical evaluation of data is the z-test of comparison of ratios. The ratios were converted to z-points, and the difference between the accounts of two groups were tested. $z = \frac{(p_1 - p_2)}{\sqrt{\frac{p_1 q_1}{n_1}} + \left(\frac{p_2 q_2}{n_2}\right)}$; $q_2 = 1 - p_1$. Minitab for Windows ver 13.0 statistics software was used in calculations.

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RESULTS

According to frequency of occurrence, the main abnormalities observed are wing, leg, thorax, and abdomen abnormalities. Abnormalities observed in wings, being in left or right wing or in both wings, are curly wing (Fig. 1), tassel shaped wing tip, lance shaped wing, atrophy in wing, wing stuck to the body and having form of a knob filled with liquid.

The abnormalities seen in legs were seen mostly in third pair and very rarely in second pair of legs. These abnormalities are atrophy in femur, atrophy in tarsus, atrophy in whole leg, hook structure formation in femur, tibia, and tarsus **(Fig. 2),** femur and tibia being fused, alpha leg structure, and decrease in number of segments of tarsus.

Figure 1. Wing abnormality formed due to diazinon effect

Figure 2. Leg abnormality formed due to diazinon effect

The abnormal individual ratio observed in all experimental groups of F_1 generation was found to be significant when compared to control group **(Table 1).** Besides, in accordance with increasing concentration, when experimental groups were compared among themselves (except G3–G4 groups) the statistical difference was found to be significant. Accordingly, it can be said that diazinon increased the ratio of individuals with abnormal phenotypes parallel to the increase in concentration. Abnormalities observed are mainly on wings or third pair of legs. Both structures are borne on the third segment of thorax. Besides, it is obviously seen in Table 1 that there is a decrease in adult individuals parallel to concentration increase.

Table 1. Phenotypic abnormalities formed due to diazinon effect

Generation	ppm	Number of normal individuals	Number of abnormal individuals	Percentage of abnormal individuals	Z score
F_1	0 (G1)	4064	73	1.77	(G1-G2) -12.86**
	$6x10^{-7}$ (G2)	3001	208	6.93	(G2-G3) -3.65** (G2-G4) -4.90**
	$6x10^{-6}$ (G3)	3076	263	8.55	(G1-G3) -15.64** (G3-G4) -1.44 n.s.
	$6x10^{-5}$ (G4)	2514	248	9.86	(G1-G4) -15.66**
F_2	0 (G5)	4347	77	1.77	(G5-G6) -5.79**
	$6x10^{-7}$ (G6)	2841	123	4.32	(G6-G7) -1.13 n.s. (G6-G8) -3.50**
	$6x10^{-6}$ (G7)	3150	159	5.04	(G5-G7) -7.20** (G7-G8) -2.43*
	$6x10^{-5}$ (G8)	3222	208	6.45	(G5-G8) -9.56**

** p>0.01 * p>0.5 n.s.: not significant

In the next stage, individuals developed in culture medium containing diazinon solution were transferred to normal culture medium in accordance with the initial concentration. Phenotypes of the F_2 generation obtained by this method were observed. The abnormality ratio was high as it was in the first generation (Table 1). Furthermore, the difference between experimental and control groups is statistically significant. The abnormality ratio observed in F_1 generation increased in direct proportion with concentration in the second generation, too. This situation bears the idea that abnormalities occurred can be hereditary because the individuals obtained from F_1 generation were transferred to culture medium that did not contain diazinon.

Nevertheless, the high ratio of individuals with abnormal phenotypes brings to the mind that these abnormalities stem from hereditary mutations. Moreover, investigation results were inspected with respect to male and female individuals separately. The abnormal individual ratio increased parallel to concentration (except $6x10^{-7}$ ppm in male individuals, in F_2 generation) for both and it was found to be statistically significant **(Table 2 and 3).** When it is considered that the abnormal individual ratio of female individuals is higher than that of male individuals, it can be said that females are more sensitive.

In the second part of the investigation it was researched whether the phenotypic abnormalities that arose due to the effect of diazinon were hereditary or not. For this purpose individuals with normal and abnormal phenotypes were crossed among themselves **(Table 4).** Both experimental groups consist of individuals exposed to diazinon in F_1 generation. The ratio of individuals with abnormal phenotypes in the F_2 generation obtained by crossing individuals with normal phenotypes was found not to be different from the control group (Table 4). However, the ratio of individuals with abnormal phenotypes was found to be higher than both the control group and the other experimental group in the F_2 generation, which was obtained by crossing the individuals with abnormal phenotypes. This result made us think that phenotypic abnormalities that arose due to the effect of diazinon were hereditary.

Therefore, it can be claimed that phenotypical abnormalities that arise due to the effect of diazinon cause hereditary mutations. Because, in addition to this, the ratio of individuals with phenotypic abnormality observed especially in F_2 generation is quite high, as can be seen in Table 1. Moreover, it can be based on hereditary mutations that although the individuals of F_2 generation were not exposed to diazinon, the ratio of abnormality was high and the abnormality ratio in the F_2 generation, obtained by crossing individuals with abnormal phenotypes (Table 4), was high relative to control group **(Table 1, 2, 3).**

Table 2. Effect of diazinon on male individuals

Generation	ppm	Number of normal individuals	Number of abnormal individuals	Percentage of abnormal individuals	Z score
F_1	0 (G1)	1919	26	1.33	(G1-G2) -5.26**
	$6x10^{-7}$(G2)	1583	71	4.29	(G2-G3) -2.64** (G2-G4) -2.30**
	$6x10^{-6}$(G3)	1433	98	6.40	(G1-G3) -7.47** (G3-G4) 0.20 n.s.
	$6x10^{-5}$(G4)	1206	80	6.22	(G1-G4) -6.76**
F_2	0 (G5)	2171	29	1.31	(G5-G6) -0.39 n.s.
	$6x10^{-7}$(G6)	1401	21	1.47	(G6-G7) -3.07** (G6-G8) -1.64 n.s.
	$6x10^{-6}$(G7)	1581	51	3.12	(G5-G7) -3.65** (G7-G8) 1.53 n.s.
	$6x10^{-5}$(G8)	1683	39	2.26	(G5-G8) -2.19*

** p>0.01 * p>0.5 n.s.:not significant

Table 3. Effect of diazinon on female individuals

Generatin	ppm	Number of normal individuals	Number of abnormal individuals	Percentage of abnormal individuals	Z score
F_1	0 (G1)	2145	47	2.14	(G1-G2) -8.52**
	$6x10^{-7}$(G2)	1418	137	8.81	(G2-G3) -0.32 n.s. (G2-G4) -2.35*
	$6x10^{-6}$(G3)	1643	165	9.12	(G1-G3) -9.38** (G3-G4) -2.11*
	$6x10^{-5}$(G4)	1308	168	12.84	(G1-G4) -10.47**
F_2	0 (G5)	2176	48	2.15	(G5-G6) -6.33**
	$6x10^{-7}$(G6)	1440	102	6.61	(G6-G7) 0.20 n.s. (G6-G8) -3.41**
	$6x10^{-6}$(G7)	1569	108	6.44	(G5-G7) -6.35** (G7-G8) -3.68**
	$6x10^{-5}$(G8)	1539	169	9.89	(G5-G8) -9.85**

** p>0.01 * p>0.5 n.s.:not significant

Table 4. Phenotypic abnormalities in F_2 generation of individuals with abnormal phenotypes

Concentrations	Number of normal individuals	Number of abnormal individuals	Percentage of abnormal individuals	Z score
Control (G1)	4347	77	1.74	(G1-G2) 0.73 n.s.
Normal x Normal (G2)	3447	69	1.96	(G2-G3) -6.62**
Abnormal x Abnormal (G3)	1946	117	5.67	(G1-G3) -7.20**

** p>0.01 n.s.:not significant

In the last stage of the investigation, the effect of diazinon on sex-ratio was observed (Table 5). In the F_1 generation, for both female and male individuals, the difference between control group and the experimental group containing $6x10^{-7}$ ppm diazinon is significant and the other experimental groups are not different from the control group. So, only at the lowest concentration ($6x10^{-7}$ ppm) the sex-ratio changed against female individuals. The ratio of male individuals was high in this concentration (Table 5). This brings to mind that some of the female individuals could not develop in this concentration and remained in some stage of development. For other concentrations ($6x10^{-6}$ ppm and $l6x10^{-5}$ ppm), although female individual ratio was higher than the male individuals this difference was not statistically significant.

In the F_2 generation, diazinon caused statistically significant deviation in sex-ratio for neither male nor female individuals (Table 5). Besides, no difference could be found when the experimental groups were compared among themselves. Therefore, difference could be observed in only one experimental group in F_1 generation. Thus, it can be thought that the sex showing more abnormal individual ratio is affected more, because the sex-ratio which does not change with respect to control group shows that none of the sexes doesn't remain in some stage of development due to diazinon effect.

Table 5. Effect of diazinon on sex ratio

Generation	ppm	Female	%	Z score	Male	%	Z score
F_1	0 (G1)	2192	52.98	(G1-G2)3.85**	1945	47.01	(G1-G2) 3.85**
	$6x10^{-7}$ (G2)	1555	48.45	(G2-G3)4.61** (G2-G4)-3.84**	1654	51.54	(G2-G3) 4.61** (G2-G4) 3.84**
	$6x10^{-6}$ (G3)	1808	54.14	(G1-G3) -1.00n.s. (G3-G4) 0.55 n.s.	1531	45.85	(G1-G3) 1.00 n.s (G3-G4) -0.55n.s.
	$6x10^{-5}$ (G4)	1476	53.43	(G1-G4) -0.37n.s.	1286	46.56	(G1-G4) 0.37 n.s.
F_2	0 (G5)	2224	50.27	(G5-G6) -1.48n.s.	2200	49.72	(G5-G6) 1.48n.s.
	$6x10^{-7}$ (G6)	1542	52.02	(G6-G7) 1.06n.s. (G6-G8) 1.78n.s.	1422	47.97	(G6-G7) -1.06n.s. (G6-G8) -1.78n.s.
	$6x10^{-6}$ (G7)	1677	50.67	(G5-G7) -0.36n.s. (G7-G8) 0.73n.s.	1632	49.32	(G5-G7) 0.36n.s. (G7-G8) -0.3n.s.
	$6x10^{-5}$ (G8)	1708	49.79	(G5-G8) 0.42n.s.	1722	50.20	(G5-G8) -0.42n.s.

** p>0.01 n.s.:not significant

In the offspring obtained by crossing individuals with abnormal phenotypes, no statistical difference was found between control and experimental groups in terms of sex-ratio (Table 6). In generations obtained from individuals with normal and abnormal phenotypes (G2 and G3), which completed their developments in culture medium containing diazinon, sex-ratio did not show a variation. When all the results are evaluated together it can be said that diazinon does not change sex-ratio but female individuals are more sensitive.

Table 6. Effect of diazinon on sex ratio in F_2 generation of individuals with abnormal phenotypes

ppm	Female	%	Z score	Male	%	Z score
0 (G1)	2224	50.27	(G1-G2) -0.36	2200	49.72	(G1-G2) 0.36 n.s.
NormalxNormal (G2)	1782	50.62	(G2-G3) -0.78	1734	49.31	(G2-G3) 0.78 n.s.
AbnormalxAbnormal (G3)	1068	51.76	(G1-G3) -1.12	995	48.23	(G1-G3) 1.12 n.s.

n.s.: not significant

DISCUSSION

Although DNA, the inheritance material in living beings, is quite well preserved and has a stable structure, it can be damaged by environmental effects or by itself in time. The modification in molecular structure of DNA can cause considerable problems for the living being. Mutagenic materials can not only create changes in the living being at chromosomal level but also cause point mutations at gene level. While numerical chromosome aberrations can be easily observed cytologically, point mutations can only be understood when there is a change in phenotype [22].

Phenotypic abnormalities observed in large numbers and various ways during our studies makes us think that these abnormalities take place due to the mutagenic effects of diazinon. Due to diazinon effect, phenotypic abnormalities were determined in both male and female individuals of F_1 and F_2 generations. There are various resources that support this result.

Genotoxicity of four different organophosphate insecticides (methyl parathion, azamethiphos, dichlorvos, and diazinon) on *Drosophila* mutants were researched. In this investigation, in which SMART test was employed, it was indicated that all these four insecticides caused mutation and the biggest genetoxic effect stemmed from diazinon [23]. There are lots of investigations which show that pesticides cause morphological abnormalities. For example, developmental abnormalities were found in the study which investigated the effect of diazinon on chicken embryo [24]. In a similar study, diazinon and parathione were applied to quail embryos and abnormalities arose in the skeleton. It was recorded that skeletal abnormalities occurred because the aforesaid pesticides caused the inhibition of AChE [25]. Chlorpyrifos, an organophosphate insecticide, was applied to mice and caused skeletal abnormalities in pups [26]. Also, when 400 microg/L and 10 microg/L diazinon was applied to *Bufo melanostictus* larvae tail abnormalities occurred [9]. In another investigation abnormalities such as defect in eyes, deformation in spine and tail, bubble formation in fins, helical deformation of body were seen in zebra fishes (*Danio rerio*) which were exposed to high concentrations of diazinon [8]. Besides, the effect of α-cypermethrin which is a pyrethroid insecticide on *Rana arvalis* tadpoleses was investigated. Morphological or behavioral abnormalities were observed in individuals which were exposed to diazinon for 48 hours in egg stage [27]. In another study, it was observed that malathione caused phenotypic abnormalities and reduced adult individual occurrence when applied to *Drosophila melanogaster* [28]. The findings of the aforesaid studies suit the findings we obtained in our study. Most of the abnormalities observed are on wings and third pair of legs and these structures are borne on the third segment of thorax. This result is interesting. Therefore, it can be thought that mutations took place which changed the operation of genes related to the development of third segment of thorax.

In addition, in the F_2 generation obtained by crossing F_1 generation individuals, the ratio of individuals with abnormal phenotypes was quite high (Table 1, 2, 3). This result made us think that the effect of diazinon could be hereditary. For this reason the results of crossing supported this thought (Table 4). In another investigation, it was found that ziram, which is a fungicide,was genotoxic for somatic and germ cells in *Drosophila melanogaster* [29]. In the aforesaid study, in which recessive sex dependent lethal genes carried by chromosomes number one and three were investigated, a statistical difference was observed in the frequency of these genes in the groups which were exposed to diazinon for a long time. It was claimed that this effect stemmed from the recombination occurred on wing discs. As a result, it was suggested that ziram was mutagenic or recombinogenic on female germ cells of *Drosophila* [29].

Therefore, it can be asserted that abnormality observed intensely in the second generation due to diazinon effect stems from hereditary mutation. In another research, the effects of dichlorvos (DDVP) on three different mutant strains of *Drosophila melanogaster* were investigated and phenotypic abnormalities were observed in F_2 generation individuals. The phenotypic abnormalities were based on mutations that were caused by inversions and translocations in female individuals during meiosis.

It was suggested that mutations which arose in this way were observed as phenotypic abnormality in individuals [12]. It can be contended that phenotypic abnormalities observed in F_1 generation in our study are based on somatic mutations while the phenotypic abnormalities observed in F_2 generation are based on hereditary mutations.

Statistical calculations of findings we got in the sex-ratio related part of our study shows that insecticides applied to *Drosophila melanogaster* don't cause variation in sex-ratio. In a study made by Kaya [21] two hormone herbicides, 2,4-D (2,4-Dicholorophenoksiacetic acid) and 4-CPA (4-chlorophenoksiacetic acid), were applied to *Drosophila melanogaster*. 2,4-D affected sex-ratio in the form of increase in female sex, only in the highest concentration of F_3 generation. 4-CPA did not effect sex-ratio in any of the concentrations. These results are in accordance with the sex-ratio data we obtained. As sex ratio did not change in both generations, it can be thought that it does not cause sex-dependent lethality or diazinon does not cause different toxic effect depending on the sex in developmental period. Naturally, since the phenotypic abnormality ratio of female individuals is higher, it can be thought that female individuals are more sensitive. In a study which supports our finding, diazinon was applied to mice and it was found that females were affected more than males. Nevertheless, there are studies in which sex-ratio changes as a result of material application. For example, it was observed that endosulphane caused sex-dependent recessive mutations in *Drosophila melanogaster* and as a result male individuals died and there was an increase in female sex-ratio [2]. It was found that malathione caused change in sex-ratio when applied to *Drosophila melanogaster* [28]. In another study, it was shown that DDVP caused infertility and deviations in sex-ratio in F_2 generation in some mutants [12]. Nadda et al. [30] investigated the effect of beta-cyfluthrin on *Drosophila melanogaster*. They found that males were more sensitive than females.

As a result, when applied to *Drosophila melanogaster* in adult period, diazinon caused quite high levels of phenotypic abnormalities in F_1 and F_2 generations. The structures where abnormalities are seen (wings and legs) are placed on the third segment of thorax. Therefore, it can be thought that there are mutations in the genes related to the development of this segment. In addition, because of the high ratio of abnormal individuals as a result of crossing individuals with abnormal phenotypes, it can be thought that diazinon causes hereditary mutations. When sex-ratio is considered, it was seen that diazinon did not cause deviation but female individuals were affected more compared to male individuals. To sum up, it can be concluded that diazinon paralyzes developmental program in non-target living beings and causes hereditary mutations.

It should be kept in mind that such effects can be risky as they could damage the demographic structure of the population in non-target species. To concentrate on studies investigating these effects can be beneficial for especially the species that are under risk.

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