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Effects of lead on the development of *Drosophila melanogaster*

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Abstract

Background and Purpose: *Lead as a heavy metal and environmental pollutant causes sperm abnormalities, organogenesis, morphogenesis disorders and miscarriage. There are some similarities between mammals and* Drosophila melanogaster. *The present study is to investigate the lead-ion effects on some developmental aspects of Drosophila as a model.*

Materials and Methods: *Five pairs of three-day flies were added to the culture containing different concentrations of lead-ion to mate and lay eggs. Transformation rate of larvae to pupa, pupa to adult, the required time for insect development and morphometric changes as well as eggs hatching rate for insects that developed in culture containing lead-ion were studied.*

Results: *Culture-medium lead-ion (20-300 mg/L) increased larvae and pupae periods, but decreased the conversion rate of larvae to pupa, pupa to adult and eggs hatching. It also decreased the growth rate of larvae length/width, pupa length/width and adult length. The results show that eggs length/width did not change.*

Conclusion: *Factors such as the lead-ion interference with enzymes performances were involved in metamorphosis, reducing the mitochondrial cristae and ATP synthesis. In addition, the negative effect of lead on the production of growth hormones, metabolic enzymes and genes expression are the suggesting aspects for future study.*

INTRODUCTION

Environmental pollution with toxic and dangerous material causes serious problems for organisms, including man. Among these pollutants, heavy metals are the most important *(1).* Heavy metals tend to accumulate in the body of living organisms, and most of the ions are known as toxic or carcinogenic *(2).* Unlike organic pollutants, it cannot be broken down and change, so it is stable in the soil *(3).* Heavy metals are widespread in air, soil and water (4). Unfortunately, due to the increasing use of these metals in industrial activities, human exposure to the heavy metals has been increasing in recent decades, causing serious harm to human health *(5).* Given that the lead is accumulated in the bones, and it is possible that the lead is released in the blood during pregnancy and affects the growing embryo *(6).* Lead will easily cross the placenta, and since the blood-brain barrier is premature, there is no resistance against the entering of lead, so the developing fetal brain may be affected by this hazardous heavy metal *(7).* Lead causes diseases such

as high blood pressure *(8),* kidney disease *(9),* sperm abnormalities *(10-12),* damage to the nervous system *(13-16),* including cognitive impairment in children *(17-22)* and anemia *(23).* Lead toxicity can permanently damage the reproductive organs *(24-26).* Additionally, lead causes stillbirth, abortion, and decreased sexual desire *(24).* The presence of heavy metals such as lead and determining the effects of their toxicity is important to living organisms, especially during their development ontogeny. Furthermore, fruit fly (*Drosophila melanogaster*) has been employed as classical model for mutations and malformations investigations. Therefore, in this study the fruit fly was used as a developmental model organism. Fruit flies have simple requirements; short reproduction cycles and also produces a large number of offspring. The growth medium is inexpensive and in vivo tests are easily performed *(27).* Another important factor in choosing the fruit fly is the embryological similarities between fruit flies and mammals *(28).* Fruit fly models have great potential to study the effects of heavy metals and then generalize to other organisms, including mammals, because the protein Metallothioneins (MTs) are similar to those of mammals. Most animals have small, cysteine-rich proteins, which are called MTs. These proteins bind to heavy metals and neutralize their toxic effects. In addition, MTs can act as powerful destroyers of the radicals (29). Lead causes a concentration-dependent delay during the insect life, but no relationship between lead and the rate of pupa transforming adult has been reported. In this case there is no difference between males and females *(30).* In both mutant races of fruit fly, including Flare and Oregon, a direct correlation between increased concentration of lead nitrate, lead acetate and also insect mortality has been observed (27). Different concentrations of zinc, cadmium, lead and copper have had an effect on larval development in the *Drosophila melanogaster* and decreased the successful transformation percentage of larva to pupa and pupa to adult *(29).* Therefore, this study was carried out to investigate the effect of different concentrations of lead existing in medium in the development of the fruit fly (*Drosophila melanogaster*) using morphological quantitative and morphometric studies from egg laying, hatching, larvae of different ages, pupation and adults emerging.

MATERIALS AND METHODS

All stages of reproduction, breeding and experiments were conducted in Genetics laboratory, Department of Biology, Faculty of Science at Ferdowsi University of Mashhad. Wild-type flies were used in this study. They were kept in plates containing medium in an incubator at 25 °C in darkness. All experiments were performed in accordance with the NIH guidelines (NIH publication No. 80-23; revised 1978).

Testing the effects of lead

The test procedure was as following: Pb(NO3)2 solutions in six concentrations of 10, 20, 50, 100, 200 and 300 mg/L, with five repetitions in each concentration, using 50 ml beakers and 100 ml flat bottomed flasks. Each beaker was filled with 10 ml and flasks with 20 ml of medium containing different concentrations of lead ion. For the controlled sample, instead of the solution containing the determined concentration of metal salts, the beakers were filled with the medium containing distilled water. Then, five pairs of three-day flies (this number was determined based on screening) were added into the beakers and then have been exited after eight hours. Different stages of the development process from egg to adulthood were examined. After adult emergence, five pairs of three-day flies were added into the beakers containing normal medium and then have been removed after eight hours. Eggs were examined for assessing morphometric changes. Eggs of insects that were exposed to lead for eight hours were also used in order to observe the morphometric changes. A total of 20 pairs of three-day fruit flies were added to the flat-bottomed flask. After eight hours, allowing enough opportunity for mating and egg-laying, flies were removed and then sampled once every 12 hours until the pupa emerged. Larvae and pupae were examined for the morphometric changes. Beakers and flasks were kept in the incubator in complete darkness conditions, at 25 °C.

Quantitative morphological studies of developmental stages in fruit fly

In this study, we studied the effect of lead upon the length of the life cycle, the rate of larvae transforming into pupae and pupae to adult, and the rate of eggs hatching of insects that spent their embryonic and adult period in medium containing the lead ions. For this reason, the number of larvae in each beaker (molt stage III) were counted and moved to the next beaker, which had the same content as the first one. The number of pupae and emerged adults were counted and thus also the rate of larvae transforming pupae, pupa to adult, and larva to adult were examined. The method of larval counting was determined based on screening. At the same time, the life duration from egg laying until the emergence of pupae and from pupa to adult emergence was recorded. After that, emerged adults of the second generation were transferred to the beakers containing 10 ml of normal medium and then were released after eight hours. After releasing of adults, eggs in the medium were counted using a stereomicroscope and their number was recorded. The beakers were incubated and the larvae were counted in the stage of third instar larvae. Therefore, the hatching rate of eggs was determined (the method of egg counting was determined based on screening).

Methods for morphometric studies

The morphometric studies have been used to determine the size of the egg, larva, pupa and adult of fruit

TABLE 1

Results of quantitative morphological studies and the effect of lead concentration on the developmental stages of fruit fly.

$Pb(NO_3)_2$ concentration (mg/L)	Larval period (hour)	Pupal period (hour)	Conversion rate of larva to pupa %	Conversion rate of pupa to adult %	Eggs hatching %
0	108.9 ± 0.64	81.1 ± 0.6	98.9 ± 0.7	98.8 ± 0.8	79.1 ± 1.7
10	108.4 ± 1.1	84.4 ± 1.1	97.8 ± 1.4	96 ± 1.2	74.2 ± 1.7
20	120.9 ± 0.8***	84 ± 1.1	95.2 ± 1.6	85.5 ± 1.5***	68.4 ± 2.1***
50	138.5 ± 1.8***	89.3 ± 1**	90.6 ± 1.3**	80.1 ± 1.4***	55.6 ± 0.7***
100	159.8 ± 1.7***	99.4 ± 2***	88.4 ± 1.4***	82.1 ± 1.5***	45.4 ± 0.9***
200	193.3 ± 1.5***	98.8 ± 1.9***	72.8 ± 1.1***	58.8 ± 1.6***	33.0 ± 0.9***
300	262.2 ± 1.8***	103.4 ± 1.2***	49 ± 1.2***	34.7 ± 1.4***	22.6 ± 1.2***

The data were presented as mean±SEM. Compared with the control group, number of replicates, n=7 and the number of pairs of flies, m=5 (***p<0.001 and **p<0.01).

fly as well as the effect of different concentrations of lead on the development process of fruit fly. In this study, the length of larvae were measured based on the proposed method of Day and Wallman *(31).* The measurement of pupae and eggs was carried out from the most proximal to the most distal point of them. Adults' measurement was performed based on the proposed method of Santos *et al*. *(32).* Midpoint of the sample length was determined as the measured point of width (the point was determined according to the screening). Sampling of larvae was performed once each 12 hours in a determined period (12 hours after the release of adults until pupa emergence) and examined by stereo microscope. Boiling water was used to destroy the larvae and pupae. All samples were then fixed in 70% ethanol. Samples were placed on glass slides and then were photographed under a camera-equipped stereomicroscope. All photos were transferred to tpsDig software to carry out the necessary measurements. In each magnification using millimeter paper, photographs were taken and used as a scale in software.

Statistical Analysis

The results were presented as mean±SEM. Statistical analysis was carried out by GraphPad Prism 5 software. The significance of treatments in groups were examined by a One way ANOVA test, or necessarily by Two way ANOVA test and the average of data by T_{tukey} with the least significances of P<0.05. Graphs were plotted by Microsoft Office Excel 2007.

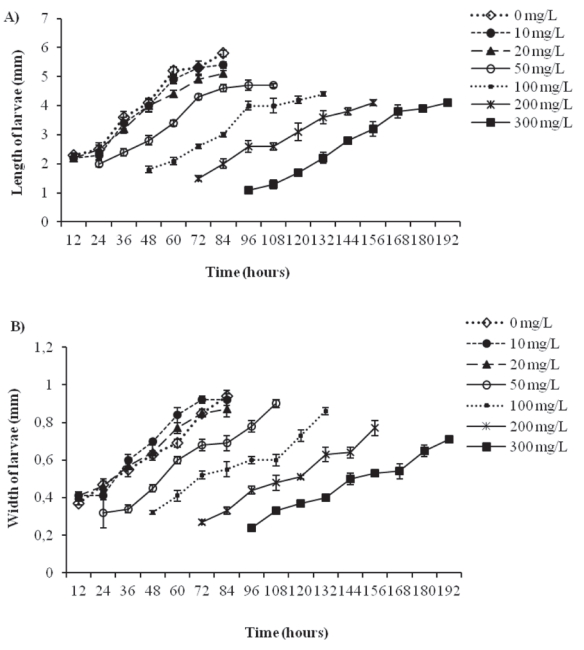

Figure 1. *(A) Comparison of larva length within the different concentrations of lead ions during larval growth. The arrows demonstrate the delay caused by increasing the concentration of lead ions in the medium at the beginning of sampling (p<0.0001, F(6,32)=18.203). (B) Comparison of larva width within the different concentrations of lead ions during larval growth. The arrows demonstrate the delay caused by increasing the concentration of lead ions in the medium at the beginning of sampling (p<0.0001, F(6,30)=20.298). The data were presented as mean±SEM (number of larvae, n=7 and the number of pairs of flies, m=20).*

RESULTS

Effect of lead concentration on the developmental stages of fruit fly

The results show that the presence of lead in the medium increased the larval period and also pupal period, but decreased the rate of larvae transforming into pupa, pupa to adult, and eggs hatching (Table 1).

Effect of lead concentrations on the length and width of larva

Average data of length and width of larva in each measurement were examined as a comparison between the controlled and test samples. Therefore, the combined effect of the concentration of lead ions in the medium on the growth of larvae was examined (Figure 1A and 1B). The data show a delayed elongation and $F(6,32)=18.203$ and transverse growth of larvae and $F(6,30)=20.298$, when the concentration of lead ions in the medium increased.

Effect of lead concentrations on the length and width of pupa

The results show that with increasing the metal concentration, the average length and width of the pupa is significantly reduced (Figure 2A and 2B).

Effect of lead concentrations on the size of the adult fruit fly

The results show that increasing the concentration of the metal, the average body length of adults significantly decreased (Figure 3A and 3B).

Effect of lead concentration on the length and width of egg

Comparison of the eggs' length and width of flies that spent their fetal and mature stages in the medium containing lead (from zero to 300 mg/L) with the eggs from the adult flies exposed to the same lead ion concentrations, shows that the concentrations of lead ions have no

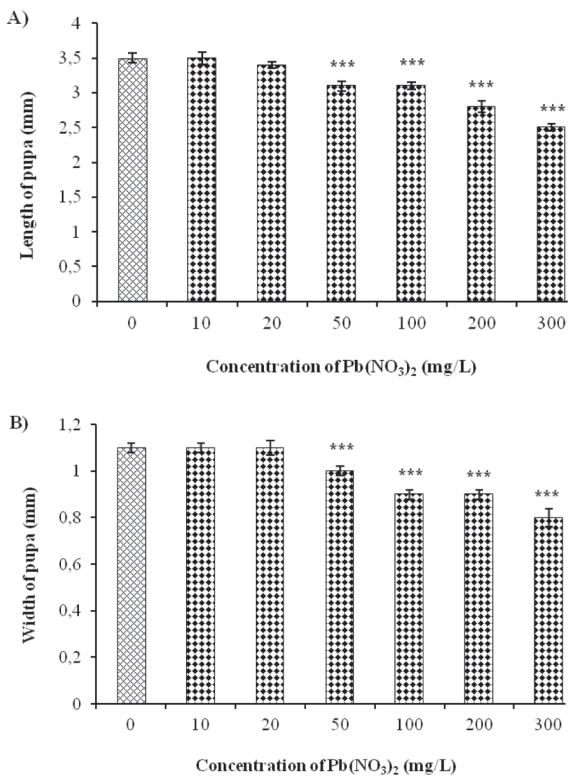

Figure 2. *(A) Average length of pupa in different concentration of lead. Increasing the concentration of this ion significantly reduces the average length of pupae (compared to control ***p<0.001). (B) Average width of pupa in different concentration of lead. Increasing the concentration of this ion significantly reduces the average width of pupae (compared to control ***p<0.001). The data were presented as mean±SEM (number of larvae, n=7 and the number of pairs of flies, m=20).*

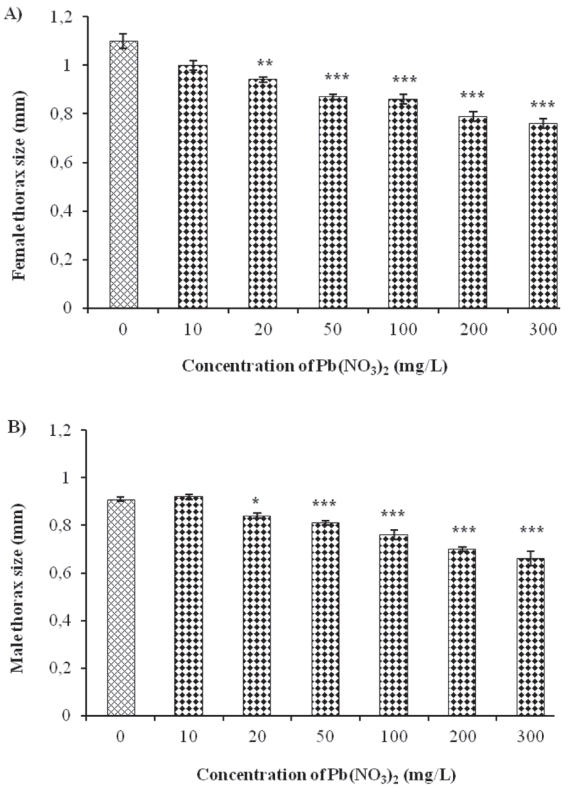

Figure 3. *(A) Average size of female adult in different concentrations of lead. Increasing the concentration of this ion significantly reduces the average length of male adult (compared with control **p<0.01 and ***p<0.001). (B) Average size of male adult in different concentrations of lead. Increasing the concentration of this ion significantly reduces the average length of male adult (compared with control *p<0.05 and ***p<0.001). The data were presented as mean±SEM (number of larvae, n=7 and the number of pairs of flies, m=20).*

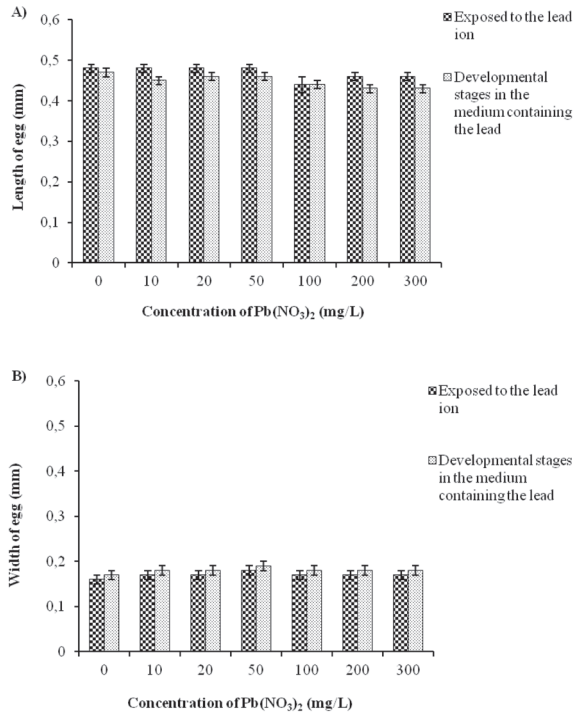

Figure 4. *(A) Comparison of the eggs' length of insects that spent their embryonic and adulthood in the mediums containing different lead concentrations (0-300 mg/L) with the eggs from the adult insects that were exposed to the same lead ions concentrations. (B) Comparison of the eggs' width of insects that spent their embryonic and adulthood in the mediums containing different lead concentrations (0-300 mg/L) with the eggs from the adult insects that were exposed to the same lead ions concentrations. No effect of lead on the length and width of the eggs were evident in either case. The data were presented as mean±SEM (number of larvae, n=7 and the number of pairs of flies, m=20).*

effect on the dimensions of the eggs, thus length and width of the eggs does not change significantly under the influence of the metal (Figure 4A and 4B).

DISCUSSION

In this study we used the fruit fly as a model for developmental studies. After reviewing the results, it can be seen that with increased concentration of lead, the rate of larvae transforming into pupae and pupa to adult reduced in a nearly direct correlation to the concentration of lead in the medium (Table 1). However, probably due to the insect's ability to detoxification of the metal at lower concentrations (20 mg/L), there is no discernable negative effect on the pupa. But in higher concentration, probably the MT protein is incapable of detoxification. Also, high concentrations of this metal can lead to impaired expression of MTs, resulting in the accumulation of large metal structures in the body and inducing deleterious effects on the body of the larva *(29)*. As a result, the negative effects of lead decrease the survival potential of larvae and then the rate of larva transforming to pupae decreases. Possible causes of reducing the rate of pupae becoming adults can be stated in which high concentrations of metals can interfere with the function of the essential enzymes needed for the production of hormones involved in metamorphosis *(29)*. To illustrate, it can be said that due to the tendency of lead to binding to sulphydryl groups, the function of enzyme related to the group affected by lead would be eliminated *(33)*. Hence, it can be said that the elimination of enzyme function causes a reduction or cessation in the production of hormones needed for metamorphosis. Therefore, the insect cannot succeed in passing this stage. In the present study, the increased concentrations of lead increases the developmental period of insect from egg to adult which increases both the larval and pupal periods (Table 1).

Probable cause of prolongation of the larval period is considered from two perspectives:

1 – Lead after being swallowed induces its toxic effects on the body of larva and decreases its' growth, delaying the development of the insect *(34)*. The authenticity of this claim is the significant difference of the average length of larvae between the control group and groups exposed to different concentrations of lead.

2 – Lead can also affect the fetus inside the egg and may cause delay in larva emergence from the eggs. To illustrate that, a significant lead concentration dependent delay, was occurred in the onset of larval sampling, as sampling for concentration of 300 mg/L was started when the control larvae had reached the stage of pupation (Figure 1A).

The possible reason for late emergence of larvae can be described in which probably the lead ion can enter into the egg coat or micropil, affects the developmental stages of embryo, delaying its growth. Moreover, the higher the concentration of the metal, the more this delay is observed and the later the larva emerges. The possible reason of elongation of pupal period can be explained as follows: Lead can reduce the cristae space of mitochondria which can result in the reduction of oxidative phosphorylation and ATP synthesis *(33)*. According to the fact that this insect requires energy in the pupal stage for morphogenesis and organogenesis, reducing the ability of ATP synthesis interferes with transformation. It is worthwhile to mention that, lead in this process interferes with releasing hormones required for transformation. Finally, we can say that once the larvae have reached the pupation stage, due to the existing problems, they need more time to pupate. The results derived from studying the eggs' hatching show that the presence of lead in the medium of fruit fly causes a reduction in the rate of eggs hatching in insects that spent their fetal and adult period in the medium containing lead (Table 1). The toxicity of the insect repellent of

diazinon reduces the eggs' hatching at all tested concentrations *(35).* Given that the insects were exposed to heavy metals, their fertility is altered *(36).* It can be claimed that probably the reduced egg hatching in these insects is due to the negative effects of lead metal accumulation during the development of male and female insects. After examining the results related to the morphological changes in larva, it has been observed that generally the higher the lead concentration, the smaller the larval size. It means that the average length and width in the examined samples compared to the control group has been reduced (Figure 1A, 1B). On the whole, in the initial samplings, only in concentrations of 200 and 300 mg/L, the size reduction is significant. However, the other concentrations have no significant difference with the controls. But gradually, with repeated sampling the difference becomes more evident and also the reduction in larvae size is significant in almost all concentrations, except 10 mg/L (Figure 1A, 1B). The possible reason for this finding is because the larva recently have emerged from the eggs and have not yet fed on the lead-containing medium. As a result, no change has been observed in the length of their body, but after feeding from the medium, metal enters into the body and gradually accumulates in body structures and induces toxic effects. Thus, further reduction in the size of the larvae comparing to a control is evident. The results show that, in general, with increasing the concentration of lead, pupa size decreases; moreover, the average length/width of pupa in different samples of lead metal decreases in comparison with the control (Figure 2A, 2B). Within the pupal stage, the body coat of third instar larvae became hard and colored and makes the coat for pupae. In fact, this is the third instar larvae which transforms to pupa. Obviously, a small larva will become a small pupa. Given the significant difference size of the control sample and the concentration of lead in the third molt, the pupae also have the same significant difference in the length and width. Studying the results indicate a negative correlation between the concentration of lead and adult size. In which, the more concentration increases, the less adult size become (Figure 3A, 3B). The size reduction of adults is probably caused by the negative effect of lead on the production of growth hormones, enzymes functions, metabolic genes and their expression *(29).* However lead has no effect on egg size, neither when the insect is only feeding from the medium containing lead for eight hours, nor when it is exposed to lead ions within the development period (Figure 4A, 4B).

In general, results show that the increased concentrations of lead critically affect the various stages of development in the fruit fly. The toxicity of lead also reduces the fertility and egg hatching. So it can be claimed that the toxicity of lead would result in a negative effect on the viability and development of the stages of the organism. It also can be said that the presence of 10 mg/L lead nitrate in the medium can have no effect on the developmental stages of this fly may be because of its ability for lead detoxification by their MTs or other ways. Given that fruit fly has a relatively high resistance to the lead; we cannot assume this insect as an indicator to assess the lead presence in the environment. However, this insect can be as an appropriate model to examine the effect of lead on the developmental stages of an organism.

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