Aconitase and Developmental End Points as Early Indicators of Cellular Toxicity Induced by Xenobiotics in *Drosophila Melanogaster*

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ABSTRACT

Background: In this study, the toxicity of the different xenobiotics was tested on the fruit fly Drosophila melanogaster model system.

Methods: Fly larvae were raised on food supplemented with xenobiotics at different concentrations (sodium nitroprusside (0.1-1.5 mM), S-nitrosoglutathione (0.5-4 mM), and potassium ferrocyanide (1 mM)). Emergence of flies, food intake by larvae, and pupation height preference as well as aconitase activity (in 2-day old flies) were measured.

Results: Food supplementation with xenobiotics caused a developmental delay in the flies and decreased pupation height. Biochemical analyses of oxidative stress markers and activities of antioxidants and their associated enzymes were carried out on 2-day-old flies emerged from control larvae and larvae fed on food supplemented with chemicals. Larval exposure to chemicals resulted in lower activities of aconitase in flies of both sexes and perturbation in activities of antioxidant enzymes.

Conclusions: The results of this study showed that among a variety of parameters tested, aconitase activity, developmental endpoints, and pupation height may be used as reliable early indicators of toxicity caused by different chemicals.

Keywords: Aconitate Hydratase, Drosophila Melanogaster, Oxidative Stress, Xenobiotics.

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INTRODUCTION

Drosophila larvae stop feeding and initiate pupation at a very specific time after hatching. This can be used as a developmental transition point to evaluate growth pattern alterations [1]. Under optimal conditions, the length of pupal stage of *D. melanogaster* at 25°C is 8-9 days [2]. Recent studies have shown that xenobiotics can affect larval development and fly emergence [3, 4]. The findings corroborate the ideas of other investigators suggesting that exposure of *D. melanogaster* to organic chemicals, such as benzene, toluene or xylene [5, 6], silver nanoparticles [7], or the fungicide captan [8] in mixtures or individually, affects larvae development and emergence patterns. These studies report significant reductions in the number of flies emerged and a 2-day delay in the emergence pattern compared to controls. In addition, Singh *et al.* examined the expression of stress-related genes, generation of reactive oxygen species (ROS), and increase in levels of antioxidant stress markers under treatment and control conditions and concluded that all these end points were significantly altered in all treated groups [6]. In other studies, treatment with xenobiotics (sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), and potassium ferrocyanide (PFC)) significantly delayed pupation pattern and fly emergence and they suggested that this is closely connected with enhanced oxidative/nitrosative stress as a result of their metabolism, leading to disruption of larval development [3, 4]. In the present study, the possible relationships between developmental end points, xenobiotic influences, and aconitase activity are discussed.

MATERIALS AND METHODS

D. melanogaster stock and media

Mutant *D. melanogaster* w^{1118} and wild type Canton-S strains were used. Stock flies and larvae were reared on yeast-corn-molasses (regular) food with constant illumination at 25 ± 1°C. Nipagin 0.2% (methyl-p-hydroxybenzoate) was added to the medium to inhibit mold growth. During the

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course of larva growing up to pupa, the control fly group was fed by food contained 10% sucrose, 10% yeast and 1% of agar-agar, and the experimental groups were fed by the same food, but supplemented with different concentrations of xenobiotics (SNP, GSNO or PFC) [3, 4].

Pupation and emergence of flies

After egg laying for 18 h, the eggs were transferred to vials containing either regular food or food supplemented with different concentrations of chemicals (about 170 eggs per vial containing 20 ml of food). In these vials the eggs hatched and larvae developed until pupation. The number of pupated larvae was counted every day at 13:00 o'clock, according to Olcott *et al*. [9].

Third instar larvae, were raised for 90 ± 0.5 h on regular food and were transferred to vials (40 larvae per vial) containing regular food or food (20 ml) supplemented with chemicals in different concentrations. Larval development and pupation were allowed to continue. The number of flies emerging from different groups was recorded from day 9 until all flies emerged [6].

Food intake

Food intake was measured as described previously by Lushchak OV et al. [10]. Briefly, groups of 10 third instar larvae were reared on the abovementioned regular medium and then were placed for 24 h on the same food (control) or food containing different concentrations of xenobiotics with 0.5% dye FD&C Blue No.1 (Brilliant blue FCF) poured on Petri dishes. After 24 h, feeding larvae were homogenized and centrifuged at room temperature at 16000×g for 15 min. Supernatant samples were removed and absorbance was measured at 629 nm and compared to a calibration curve built with different concentrations of the dye.

Effect of test chemicals on larval behavior (pupation height preference)

Larval behaviour for tested chemicals was measured as pupation height preference. After 4-6 days, larvae were fully converted into pupae and reach a certain height. The height was measured from surface of food (pupation on food surface was considered zero). The height was measured in millimetres (mm) as described by Singh and Pandey [11].

Assay of enzymes activities

Aconitase (EC 4.2.1.3) activity was measured as the decrease in the substrate concentration using a modification of the method previously described by Andersson *et al*. [12]. The decrease in absorbance at 240 nm was followed for 2 min. The extinction coefficient used for calculations was 3.70l $M^{-1}cm^{-1}$ for cis-aconitate. The activities of superoxide dismutase, catalase, thioredoxin reductase, and glucose-6-dehydrogenase were measured as described previously [3, 4].

RESULTS

Pupation and emergence of flies: The highest concentrations of all investigated xenobiotics significantly lowered the pupation rate (up to 50%) and the number of emerged flies (3, 4).

Food intake: To asses the effects of SNP on food consumption, the food dye Brilliant Blue FCF was used. Maximum food consumption was observed in flies on the control diet and it was equal to 1050 ± 68 nl/larva. In the experimental diets, food consumption by larvae decreased and was significantly lower for the 1.0 and 1.5 mM SNP-treated groups at 874 ± 42 and 891 ± 7 nl/larva, respectively [3, 4, 10].

Effect of test chemicals on pupation height: Pupation height was measured in millimetres from the surface of food. Maximum pupation height was observed in the control groups. Meanwhile in groups that consumed SNP or PFC, remarkable decreases in pupation height were observed [3].

Assay of enzyme activities: The results revealed that aconitase activity decreased in a dose-dependent manner (the higher concentration of xenobiotic, the lower aconitase activity). Furthermore, a significant correlation was found between aconitase activity and adult fly emergence from larvae exposed to SNP (R^2=0.86 and R^2=0.93 for males and females, respectively) (Figure 1C, D); in other words, the higher the concentration of SNP, the fewer the number of flies that emerged. Also, a strong significant correlation was found between aconitase activity and the percentage of eggs that developed to pupa when exposed to GSNO (R^2=0.96 and R^2=0.97 for males and females, respectively) (Figures 1A, B); in other words, the greater GSNO exposure, the fewer the number of pupae that emerged.

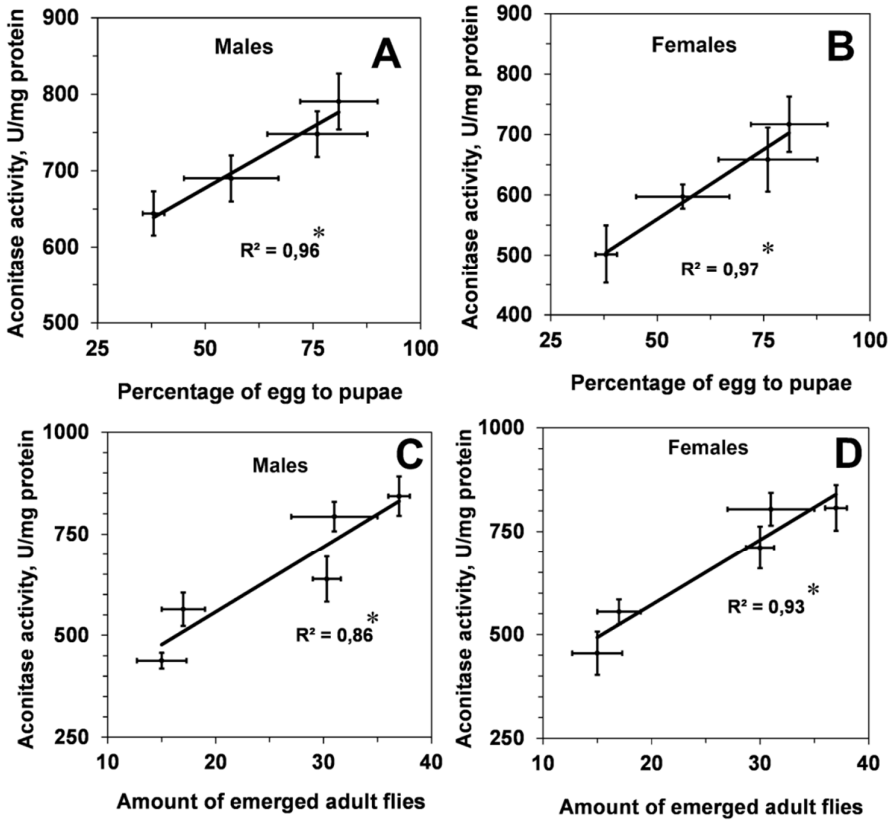

Figure 1. The relationship between aconitase activity in male (C) and female (D) adult flies and the percentage of egg to pupae exposed to GSNO; the relationship between aconitase activity in male (A) and female (B) adult flies and the number of adult flies that emerged from 40 larvae exposed to SNP. *Correlations were considered significant at $P<0.05$.

DISCUSSION

Food consumption: To assess the effects of SNP on food consumption by larvae, the food dye Brilliant Blue FCF was used. It was also shown that SNP decreased larval food consumption [3, 10]. A possible explanation for this might be that the decrease in food intake by SNP-treated larvae could be caused not only by SNP, but also by compounds released during SNP decomposition, particularly iron ions. This idea is supported by the findings of other investigations that showed that *Drosophila* larvae and adults avoided feeding on foods with high concentrations of heavy metals [13, 14]. Furthermore, reduced food intake by SNP treated larvae could be a reason for the developmental delay in pupation since the acquisition of a certain level of stored body fuel reserves may be required before pupation can start.

Pupation height as a marker of medium quality (toxicity): Pupation height was measured in millimeters from the surface of food. Maximum pupation height was observed in control groups. Meanwhile in the groups consuming SNP or PFC, some decrease in pupation height was observed. It is often assumed that the position at which a *Drosophila* larva pupates has some value in terms of individual fitness [15]. The position of pupation in *Drosophila* is partially under genetic control. Other biotic (sex, development time, and population density) and abiotic factors (food, moisture, and temperature) have also been shown to influence the pupation height of *Drosophila* [15, 16]. The results of this study were consistent with those of recent investigations. For example, it was shown that the number of emerged flies as well as pupation height of *Drosophila punjabiensis* larvae decreased with increasing chlorphyriphos

(organophosphate compound) in food medium [17]. The decrease in emergence of flies with increases in concentration of chlorphyriphos during study of Saxena *et al*. clearly correlated with behavior of pupation height. Saxena *et al*. proposed that this behavior pattern could be used for biological control of insect pests in agricultural fields [17]. Considering the observations and the results of the present study, it is suggested that pupation height as well as total number of pupae can be used as potential biomarkers of chemical toxicity.

The mechanism behind these results appears to be related to the development of *Drosophila* larvae in media treated with xenobiotics. It is a well-established fact that dipterian larvae need to achieve a certain threshold bodyweight to be transformed in viable pupae [18] and it is possible that the larvae pupate at the nearest location to the surface in order to avoid energetic costs [19] of migrating farther from resource. The results of the present study are consistent with the findings of Casares and Carracedo and Vandal *et al*. who suggested that the slowest developing larvae within treatments media pupated closer to the resource surface [20, 21].

Aconitase activity as a marker of xenobiotic toxicity: The active site of aconitase contains a cubic [4Fe-4S] cluster that is rather sensitive to oxidation, leading to its inactivation [12, 22-25]. Therefore, aconitase activity can be used as an efficient marker of oxidative/nitrosative stress. In this study, it was shown that exposure of larvae to xenobiotics leads to significantly lower aconitase activities in adult flies in a dose-dependent manner [3, 4]. Lower aconitase activities could be attributed to oxidation of the enzyme by different ROS/RNS released as a result of xenobitic metabolism in the flies. Previous studies have shown the high sensitivity of aconitase to ROS/RNS. Aconitase, either as a purified enzyme or in various cultured cells, was inactivated by superoxide anions through a mechanism that most likely involved the oxidation of the [4Fe-4S] cluster [12, 24]. The activity of aconitase also decreases by NO treatment, and superoxide anions together with NO can cause an even larger decrease in its activity. Since NO readily reacts with superoxide anions to form peroxynitrite [26], these results suggests that peroxynitrite might inactivate aconitase, probably via a reaction with the [4Fe-4S] cluster [24, 27]. Some studies have indicated that aconitase acts as a ‘‘circuit breaker’’ for cells exposed to oxidative and nitrosative stresses [22, 28]. Thus, it seems that superoxide anions and nitric oxide inactivate mitochondrial aconitase and potentially prevent further formation of ROS/RNS and reduction of electron flow in mitochondria [12].

Larval exposure to chemicals resulted in lower activities of aconitase in flies of both sexes and perturbation in activities of antioxidant enzymes[3, 4]. Hence, it can be argued that aconitase and developmental end points may be used as early indicators of cellular toxicity by SNP, GSNO, and other similar xenobiotics.

CONCLUSION

Exposure of *Drosophila* larvae to xenobiotics leads to a concentration-dependent delay in larval development and fly emergence. This indicates that xenobiotics have significant metabolic effects. These effects appear to result from oxidative and/or nitrosative stress caused by xenobiotics. The parameters examined suggest that aconitase activity and developmental end points may be useful early indicators of xenobiotics toxicity to flies. Noticing these observations and the findings of the present study, it can be suggested that pupation height as well as the total number of pupae can be used as simple reliable biomarkers for investigations of chemicals toxicity.

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