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Alloxan effect on oxidative stress and hyperglycemic condition in *Drosophila melanogaster*

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Abstract
Chemicals used to induce metabolic diseases in animal models play a crucial part in the physiology, development, and survival of the organism. For current research, *Drosophila melanogaster* (*D.*melanogaster) was used to study the toxic effects of Alloxan on mortality rate, survival, circulating metabolites, and oxidative stress. Oregon-K strain of *D.melanogaster* was raised in control and Alloxan treated diets and were subjected for mortality rate, survival, circulating metabolites, and antioxidative stress enzymes (ROS, SOD, and CAT). Flies treated with Alloxan showed an increased mortality rate, increased levels of glucose, trehalose, triacylglycerol, and antioxidant enzymes ROS, SOD and CAT in comparison to control flies. Further Alloxan treated flies lived significantly lesser days than the control flies. Among Alloxan-treated flies mortality rate, circulating metabolites and antioxidant enzymes increased with increasing concentration of Alloxan whereas lifespan decreased with increasing concentration of Alloxan. Sub-lethal concentration for Alloxan was noticed at 3%. Thus these results in *D.melanogaster* suggest that Alloxan has a toxic effect on the above traits.

Keywords: *Drosophila melanogaster*, alloxan, circulating metabolites, antioxidant enzymes

Introduction

Experimental induction of diabetes mellitus in animal models is critical for furthering our knowledge and understanding of the disease's pathophysiology and, eventually, for the development of novel therapeutics and cures (Potenza *et.al*., 2011) [1]. The use of animals in experimental research has the significant benefit of removing characteristics such as ethnicity, economic and geographic variables, medication interactions, food, gender, and age disparities, all of which significantly restrict clinical research. Indeed, proper animal models have helped to deconstruct molecular pathways underlying the development, progression, and therapeutic management of diabetes by providing vital information on hereditary and environmental hazards (Suresh *et.al.*, 2012) [2]. Several strategies for inducing diabetes in experimental animals have been tried, with varying degrees of success and several challenges. Surgical removal of the pancreas is successful; however, at least 90-95% of the pancreatic must be removed to cause diabetes. (Rastellini *et.al.*, 1997) [3]**.**

The bulk of ethnopharmacology research published between 1996 and 2006 used a chemical-induced paradigm to investigate multifactorial effects on diabetes mellitus. Streptozotocin (STZ) 69% and alloxan 31% is effective chemicals often used for diabetes induction to study a variety of illness manifestations (Rydgren *et.al*., 2007) [4]. Parenteral administration of both the drugs has proven to have a diabetogenic (intravenously, intraperitoneally, or subcutaneously). The dose of these medicines necessary to induce diabetes varies depending on the animal species, delivery method, and nutritional state. (Mostafavinia *et.al.*, 2016) [5].

Alloxan, a well-known diabetogenic chemical, is commonly used in animal models to cause type 1 diabetes (Viana *et.al.*, 2004) [6]. With the generation of superoxide radicals, the chemical and its reduction product dialuric acid create a redox cycle. Dismutation of these radicals to hydrogen peroxide occurs and then the Fenton reaction produces highly reactive hydroxyl radicals. The combination of reactive oxygen species and a huge rise in cytosolic calcium concentration causes rapid destruction of β cells (Szkudelski *et.al.*, 2001) [7]. Diabetic condition caused by alloxan serves as a pathogenic biomodel for assessing a drug with antioxidant properties *in vivo*. (Bartosikova *et.al*., 2003) [8]. Pancreatic islets' DNA is one among the targets of reactive oxygen species. Its fragmentation happens in alloxan-exposed β cells (Takasu *et.al*., 1991) [9]. The diabetogenic agent alloxan is primarily responsible for the increase in oxygen free radicals. However, there is strong evidence that hyperglycemia is a potent inducer of oxidative stress damage. This is because the biochemical pathway activated by hyperglycemic condition causes glucose auto-oxidation, protein glycation, and advanced glycation end products (AGEs) formation, all of which can cause oxidative damage via free radical overproduction and antioxidant defense repairmen (Yim *et al*., 2001) [10]**.**

Efforts to treat this condition have been futile for the past 30 years. However, utilizing the mouse model to address this condition with oral anti-diabetic medications and nutritional therapy has showed some potential. (Frode *et al*., 2008; Karthikeyan *et al*., 2017; King *et al*., 2012; Eddouks *et al*., 2012) [11, 12, 13, 14]. Since the mouse model used to investigate metabolic diseases has a high care cost and a long developing period, scientists sought an appropriate model organism with a lower maintenance cost and a shorter developmental time. The neuroendocrine and metabolic architecture of animals has a remarkable degree of conservation. The Drosophila melanogaster is an excellent companion to mammalian models. Their short lifespan and strong genetic techniques allow for in-depth organ investigation in situ (Hales *et al*., 2015) [15]. While flies and mammals have significant differences, they also have significant resemblance. The fly genome, for example, has seven insulin-like peptide genes (Dilp 1–7) that activate the classical insulin pathway (Brogiolo *et.al*., 2001) [16]. The loss of insulin-producing cells mimicked various symptoms of type 1 diabetes. (Rufilson *et.al*., 2002) [17]**.** Furthermore, evidence suggests that Drosophila is sensitive to dietary-induced metabolic and cardiac organ dysfunctions similar to those seen in mammals. (Grandison *et.al*., 2009) [18].

These kind of evolutionary old and highly conserved mechanisms include insulin signalling and the storage of excess glucose in the form of glycogen and triglycerides (Musselman *et al*., 2011; Zhou *et al*., 2014) [19, 20]. *D. melanogaster* have comparable insulin signalling apparatus, as well as homologies between key transcription regulators of carbohydrate, protein, and lipid metabolism, such as FOXO TOR and PGC–K. (Baker and Thummel, 2007) [21], Because carbohydrates are the principal component of a fruit fly's natural meal, emerging evidence shows that has been extensively used to study Drosophila glucose metabolism in recent years. Although the effect of under and over feeding on physiological markers in flies have been investigated (Coogan, 2013; Morris *et al*., 2012) [22, 23], little research has been done on using drugs to cause hyperglycemia in Drosophila. Therefore, the current research has been attempted to comprehend whether or not Alloxan induces oxidative stress in *Drosophila*. If so, does Alloxan-induced oxidative stress induce hyperglycemic conditions in *Drosophila*?

Materials and Methods

Experimental Stocks

Experimental stocks used in the present study were unmated males and virgin females of Oregon-K obtained from the *Drosophila* stock center, the University of Mysore were cultured and flies were isolated within three hours of their eclosion. Twenty flies (20 males and females) were transferred to quarter-pint bottles (250 ml) containing 5 ml of the control diet (wheat cream agar media). Flies fed with the control diet were used for the control group. Each bottle was maintained with 20 male and 20 female flies at a temperature of 22°±1°C and relative humidity of 70%, with a 12:12- L: D photoperiod.

Experimental Diet (Alloxan treatment)

Experimental flies obtained as above were subjected to Alloxan treated diet for 10 days (wheat cream agar media + Alloxan) containing varying Alloxan concentrations (0.5%, 1%, 2%, 3%, 4%, 5%) keeping yeast constant. Flies obtained from control and Alloxan-treated diets were used for feeding behavior assay, metabolic assays, toxicity, and oxidative stress assays.

Feeding Behavior

Quantification of Food Intake in Flies Using Dye Method

Twenty flies obtained from control and Alloxan-treated diets were used to study feeding behavior. Flies were placed in a vial containing normal (wheat cream agar media) and Alloxan treated media (food containing normal media with varying Alloxan concentrations - 0.5%, 1%, 2%, 3%, 4%, 5%) along with 2.5% (w/v) blue food dye (FD & C Blue Dye no. 1). The flies were allowed to feed for 30 minutes. Then the flies were transferred to an Eppendorf tube and frozen. These frozen flies were homogenized by adding 200 µl of distilled water further 800 µl of distilled water was added. The absorbance was measured at 629 nm using a calorimeter. The flies which were not treated with blue dye were used as the blank. The amount of food taken was measured from the standard graph made from the serial dilution of a blue dye. Three replicates (twenty flies in each replicate) were taken separately and simultaneously for both the control and Alloxan-treated diets.

Metabolic Assay

Whole-Body Metabolite Measurements

For the study of metabolites, the procedure described by Coogan, 2013 [22] was used. Ten days old control and Alloxan treated twenty-five flies were decapitated anesthetized, weighed in groups, and transferred to chilled microcentrifuge tubes containing buffer A (20 mM HEPES, KOH pH7.5, 10 mMKCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors, 0.5 mM PMSF) and homogenized using a motorized micropestle. After centrifugation, supernatants were used to determine the metabolite levels. Glucose was determined by using the glucose detection kit (SIGMA). Trehalose and glycogen were determined after converting into glucose through the addition of trehalase (SIGMA, 0.2 U/ml) and amyloglucosidase (SIGMA, 0.1 U/ml) respectively, followed by glucose determination. Assays were measured using at least three biological replicates on a BioTek Synergy2 96- well plate reader. A total of three trials were performed separately for each of the control and different concentrations of alloxan-treated diets.

Percentage Mortality
The % mortality of alloxan was analyzed in control and Alloxan treated (10 days of treatment) flies of *Drosophila melanogaster*. For these studies, the varying concentration of alloxan was given along with the media. Twenty replicates were maintained for each of the control and treated media. Along with these experiments, LD50 was also calculated.

Biochemical Markers of Oxidative Stress
For these studies, ten days old control and alloxan-treated (10 days treatment) flies were subjected to cold anesthesia for 10 min. 50 fly heads were homogenized in 0.5 ml of respective assay buffers and centrifuged at 2500×g for 10 min at 4 °C. The supernatant was used to determine ROS and antioxidant enzymes (SOD and catalase) levels. For each assay, three replicates were tested with a total of 150 flies. Separate experiments were performed for each of the control and Alloxan-treated diet group.

Antioxidant Enzymes
Reactive Oxygen Species (ROS)
ROS was assayed using 2′7′-Dichlorofluorescin diacetate (DCFH-DA). The reaction involves the conversion of non-fluorescent DCFH-DA into a fluorescent product 2′7′-dichlorofluorescein (DCF) in the presence of ROS. Briefly, 100 µl of fly homogenate was incubated with 15 µl of 5 µM DCFH-DA and 85 µl of Tris-HCl buffer (pH 7.4) in microplate wells for 1 h at room temperature. The fluorescence was measured using a spectrofluorometer with an excitation wavelength of 488 nm and emission at 525 nm. ROS production was quantified using the DCF standard curve and expressed as µmoles of DCF formed/min/mg protein following the procedure described by Lebel *et al*., (1992) [24].

Superoxide dismutase (SOD)
SOD activity was determined by measuring the pyrogallol autoxidation procedure by Marklund and Marklund, (1974) [25]. Briefly, the reaction was started by adding 0.5 ml of pyrogallol (2 mM) to a reaction mixture containing 500 µl of homogenate, 0.5 ml of distilled water, and 2 ml of 0.1 M Tris-HCl buffer (pH 8.2). The change in absorbance was monitored for 3 min at 420 nm and the activity was expressed as enzyme units required to inhibit 50% pyrogallol autooxidation. Potassium cyanide (1 mM) was used to distinguish between Cu, Zn-SOD, and MnSOD since cyanide selectively inhibits the activity of Cu, and Zn-SOD, and allows the measurement of mitochondrial Mn-SOD activity.

Catalase (CAT)
Catalase activity was measured following the procedure by Aebi, (1984) [26]. The catalytic effect was studied by adding 50 µl of 1% (v/v) H2O2 to a 1 ml reaction mixture containing 50 µl homogenate and 950 µl of 0.05 M phosphate buffer (pH 7). The change in absorbance was monitored for 3 min at 240 nm and the activity was expressed as µmoles of H_2O_2 decomposed/min/mg protein.

Protein estimation
Lowry's method was used to estimate Protein content in the homogenate by using BSA (Sigma Chemical, St. Louis, MO, USA) as the standard (Lowry *et al*., 1951) [27].

Results
Feeding Rate
Figure 1, shows the feeding rate of control and Alloxan-treated flies. It was depicted from the figure that the feeding rate of control and Alloxan-treated flies were more or less the same suggesting the same quantity of food was taken by both control and Alloxan-treated flies. One-way ANOVA carried out on feeding rate data showed insignificant variation.

Survival Rate
Survival curve analysis of control and Alloxan-treated flies was presented in Figure 2. It was found that control flies survived significantly longer than those Alloxan-treated flies. Further lifespan among Alloxan-treated flies decreased with increasing concentration of Alloxan. The Kaplan Meier analysis for survival rate showed significant variation in lifespan between control and Alloxan-treated flies.

Alloxan Toxicity
It was noticed that the % mortality rate of control flies was significantly less compared to Alloxan-treated flies. Further, the % mortality rate increased with the increasing concentration of Alloxan in the treated flies. Sub-lethal concentration was noticed at 3% Alloxan treatment (Fig-3). Mortality data subjected to one-way ANOVA followed by Tuckey’s post hoc test showed a significant difference in mortality rate between control and Alloxan-treated flies.

Metabolite Levels

Mean value and analysis of data for whole-body glucose, glycogen, and trehalose levels in control and Alloxan treated flies of *D.melanogaster* were provided in Figure 4. Levels of glucose, glycogen, and trehalose in the whole body were least in control flies and highest in 5% Alloxan-treated flies. Further among Alloxan-treated flies, all these metabolites increased with increasing concentration of Alloxan in treated flies. The present findings demonstrated substantial variation in metabolite levels between control and variation concentrations of Alloxan-treated flies using one-way ANOVA and Tukey's post hoc test. When flies fed with alloxan were compared to the control group, there was a two-fold rise in glucose concentration and a one-fold increase in glycogen level.

Oxidative Stress

ROS, SOD, and CAT Enzyme Activity

Figure-5 A-C shows the mean value of ROS, SOD, and CAT antioxidant enzymes in control and Alloxan-treated flies of *D.melanogaster*. It was noticed that endogenous ROS, SOD, and CAT levels were lowest in control flies and highest in 5% Alloxan-treated flies. Among Alloxan-treated flies, these enzyme levels increased with the increasing concentration of Alloxan. In flies given Alloxan, the quantity of reactive oxygen species (ROS) surged, but the activity of antioxidant enzymes SOD and CAT was altered. The alloxan intake caused an increase in CAT activity and SOD activity when compared to the values found in the control group. One-way ANOVA followed by Tukey's post hoc test applied to the above data revealed significant variation in the ROS, SOD, and CAT levels between control and Alloxan-treated flies.

Discussion

The growing evidence shows that chemicals are frequently used to induce metabolism-related diseases such as diabetes in animal models; however, the survival rate associated with such studies is validated. Alloxan is one such chemical used to induce diabetes in animal models and this chemical is a product of bleaching of flour in the baking industry to produce some of the baking products which are consumed by people. In the present study, flies, *D. melanogaster* was used as a model organism for understanding the potentially toxic effects of consumption of Alloxan on oxidative stress and hyperglycemic condition.

In the present study, the fly fed with the Alloxan diet had elevated levels of whole-body glucose, trehalose content, and triacylglycerols levels in their hemolymph (Figure-4) suggesting induction of hyperglycemic condition in *Drosophila* which resulted in inducing phenotype similar to type 1 DM via (oxidative destruction of IPC's) insulin signaling dysregulation characteristics of type 1 DM. Further, in the present study, the expression of hyperglycemic condition was noticed in flies fed with 3% Alloxan and it increased with the increasing Alloxan concentration (Fig-4). Our results also confirm the diabetogenic effects of Alloxan in *Drosophila*. This study also confirms the diabetogenic effects of alloxan in other animal models too (Karthikeyan *et al*., 2017) [12]. Studies in *Drosophila* have also pointed out that the high sucrose diet also produced hyperglycemic conditions (Harshavardana and Krishna, 2019) [28].

In the present study, feeding rate was also studied to understand feeding rate variation in control and Alloxan treated flies, to rule out a difference in the feeding rate could be the basis for the observed variation in glucose metabolism of control and experimental flies of *D.melanogaster*. Figure-1 with the feeding rate of *D.melanogaster* in control and Alloxan treated diets revealed that the quantity of food taken by control and alloxan treated flies were found to be the same quantity suggesting that the feeding rate was found to be the same in the control diet and Alloxan treated diet. In *Drosophila*, it was shown that a larval stage shows an inhibition threshold when consuming a new or foul-tasting food (Melcher *et al*., 2007) [29] however such inhibition threshold is not observed in larvae fed on a control diet or Alloxan treated diet. This confirms the studies of (Alwyn D'souza and Krishna, 2015) [30] who while working with *D. melanogaster* have also shown insignificant variation in larval feeding rate on Natural energy drinks and Synthetic energy drinks. They also found that inhibition threshold was not observed in larvae fed on Natural energy drinks when compared to Synthetic energy drinks as well as Normal media since the rate of larval feeding was highest among larvae fed on Natural energy drinks. In the present study, it was noticed that larvae fed on both control and Alloxan-treated diets at the same rate. Further food consumed by the larvae in Alloxan treated diet was unaltered compared to that of larvae feeding on the control diet.

Studies have also suggested that when chemicals or excess use of dietary components in the experimental animals first we have to rule out the toxic effects of such components on mortality rate therefore in the present study toxic effect of Alloxan on % mortality rate was also studied, it was noticed from the Figure-3 that % mortality rate increased with the increasing percentage of Alloxan concentration and LC50 was noticed at 3% Alloxan in *D.melanogaster*. Alloxan-induced mortality was also studied in other experimental organisms and found that the mortality rate induced by Alloxan was different in different animal models (Frode *et al*., 2008; King *et al*., 2012; Eddouks *et al*., 2012) [11, 13, 14].

Toxic effects of Alloxan also had its effects on the life span of the organism therefore Alloxan effects on life span have also been analyzed in *melanogaster*, it was noticed from survival curve analysis that lifespan decreased with increasing concentration of Alloxan suggesting toxic effects of Alloxan on lifespan in *Drosophila*. This study has also shown that the use of chemicals to induce metabolic disease has toxic effects on the life span of animal models (Rydgren *et.al*., 2007) [4]. In *Drosophila* both biotic and abiotic factors could

affect life span (Alwyn D'souza and Krishna,2017) [31], however in the present study except for the use of Alloxan all other biotic and abiotic factors were the same therefore the resulted variation in lifespan in the present study was due to the effect of Alloxan. Earlier studies of life span in *D.melanogaster* have pointed out the existence of sex differences in lifespan in *D. melanogaster* (Austad and Fischer, 2016) [32]. In addition, in laboratory species such as *C.elegans*, *D. melanogaster*, and *Mus musculus*, lifespan analysis has been undertaken to demonstrate variances between males and females. (Austad and Fischer, 2016) [32]. However, in the present study, we have used an equal number of males and females together in the lifespan analysis.

Studies have also suggested that the use of Alloxan in animal models is always associated with the development of a hyperglycemic state which resulted due to oxidative events induced by Alloxan (Bartosikova *et.al*., 2003) [8]. Further studies have suggested that alloxan is a stress inducer that is being used to produce reactive oxidative species (ROS) which triggers other oxidative events. The current study in *D.melanogaster* found that flies fed on Alloxan-treated diet had higher levels of oxidative stress marker enzymes (SOD and CAT), thereby confirming the hyperglycemic state being caused due to the increased oxidative stress. The harmful effects of eating a diet containing alloxan resulted in an excess of hydrogen peroxide and a loss of mitochondrial viability. In addition, the activity of antioxidant enzymes SOD and CAT differs among different populations (Fig-5 A, B, C). The variation in antioxidant enzymes SOD and CAT could be a compensatory response to the elevated levels of hydrogen peroxide in flies fed with alloxan. Thus, these studies in *D.melanogaster* suggest that alloxan treatment induces oxidative stress which in turn induces hyperglycemic condition, increased mortality rate, and decreased survival rate.

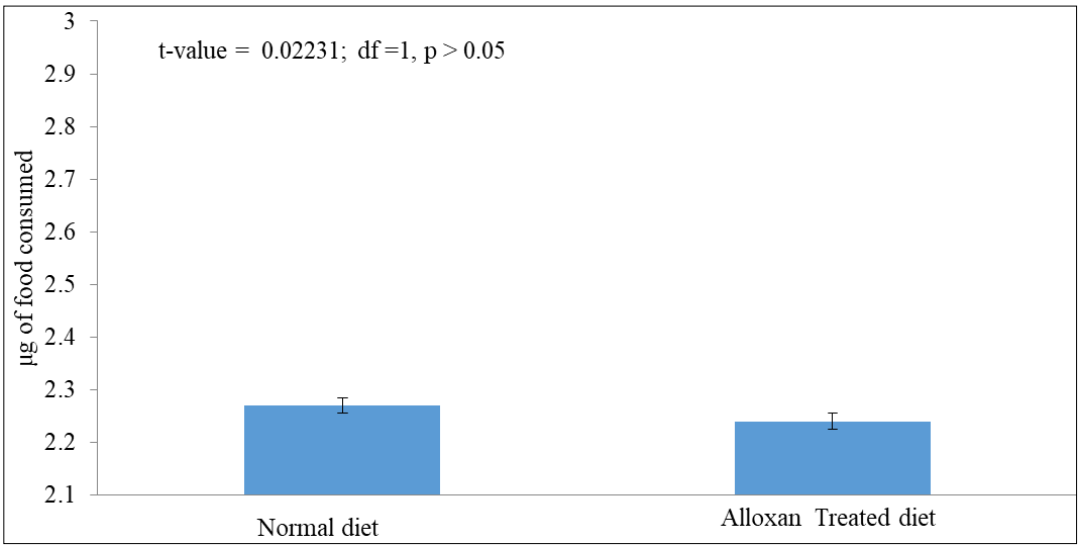

Fig 1: Alloxan effect on feeding behavior in normal and treated flies of *Drosophila melanogaster*.

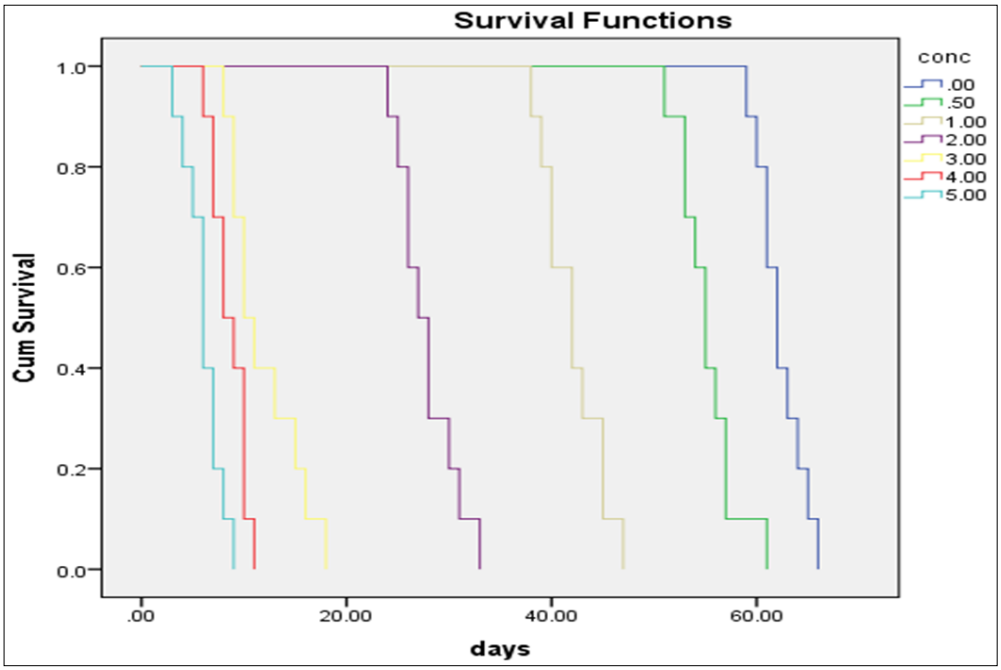

Fig 2: Survival curve of flies fed with different concentrations of alloxan(%). **[**Log Rank (Mantel-Cox) Chi-Square=150.452, df=6,p<0.0001]

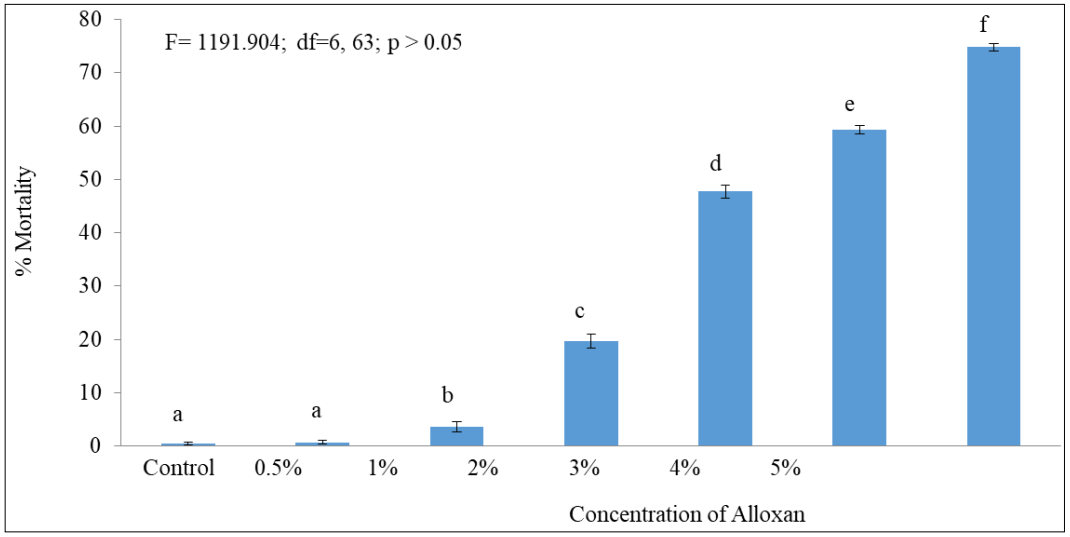

Fig 3: Effects of different concentrations of Alloxan on mortality of *Drosophila melanogaster*. [Different alphabet on the superscript of bar graph indicates significance at p<0.05].

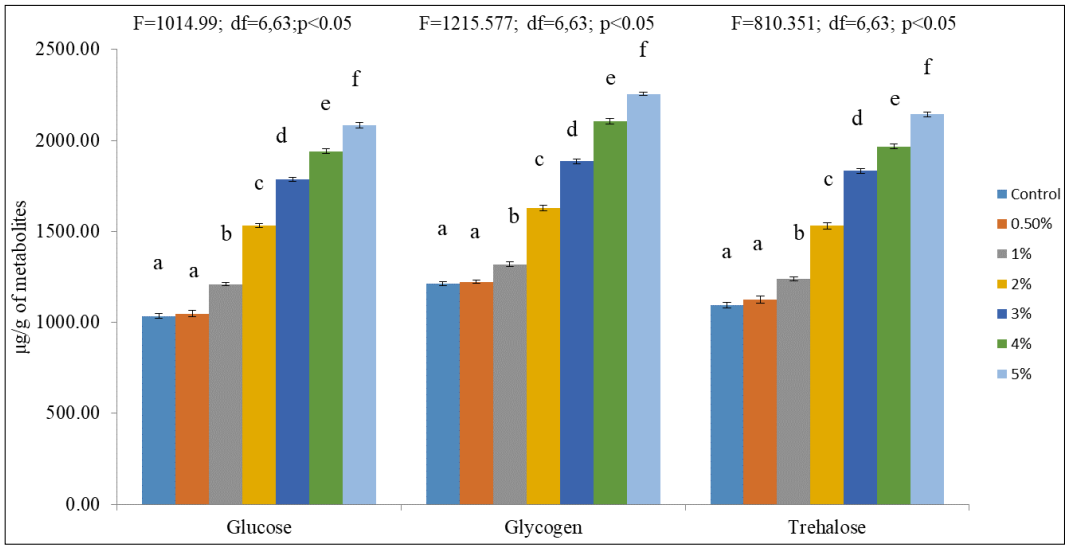

Fig 4: Alloxan effect on circulating metabolites (Glucose, Glycogen, Trehalose) in normal and treated flies of *Drosophila melanogaster*. [Different alphabet on the superscript of bar graph indicates significant at p<0.05].

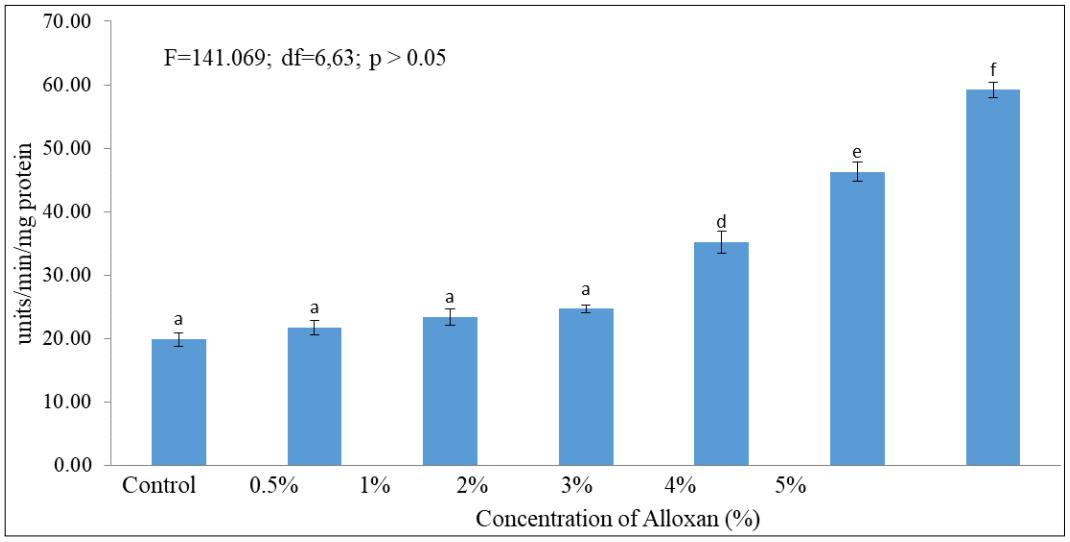

Fig 5: Alloxan effect on ROS levels in control and treated flies of *Drosophila melanogaster*. [Different alphabet on the superscript of bar graph indicates significance at p<0.05].

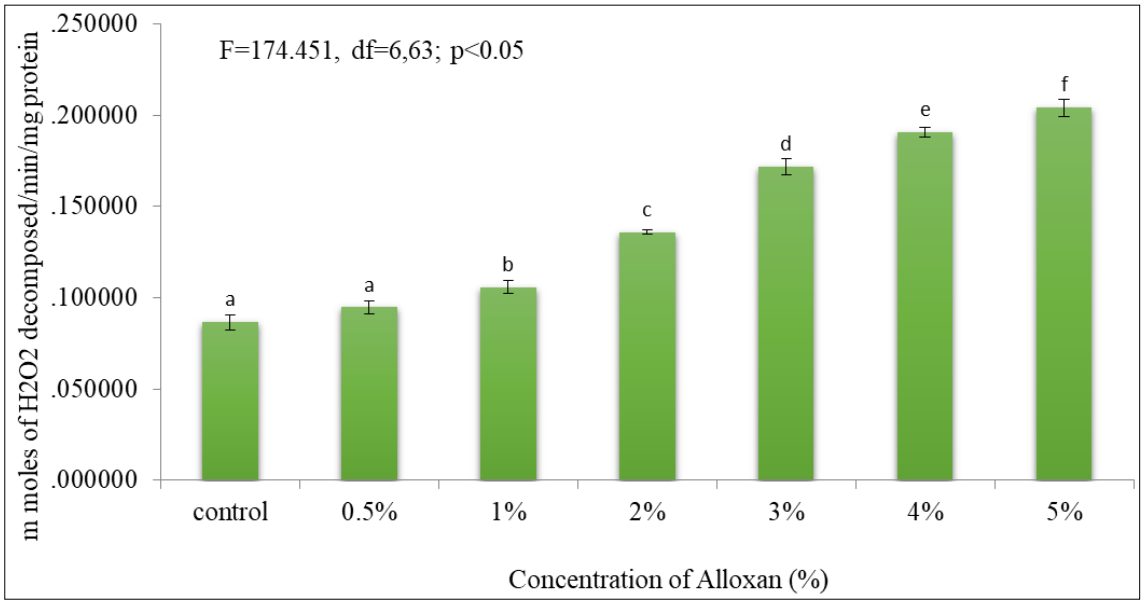

Fig 6: Alloxan effect on catalase activity in control and treated flies of *Drosophila melanogaster*. [Different alphabet on the superscript of bar graph indicates significant at p<0.05].

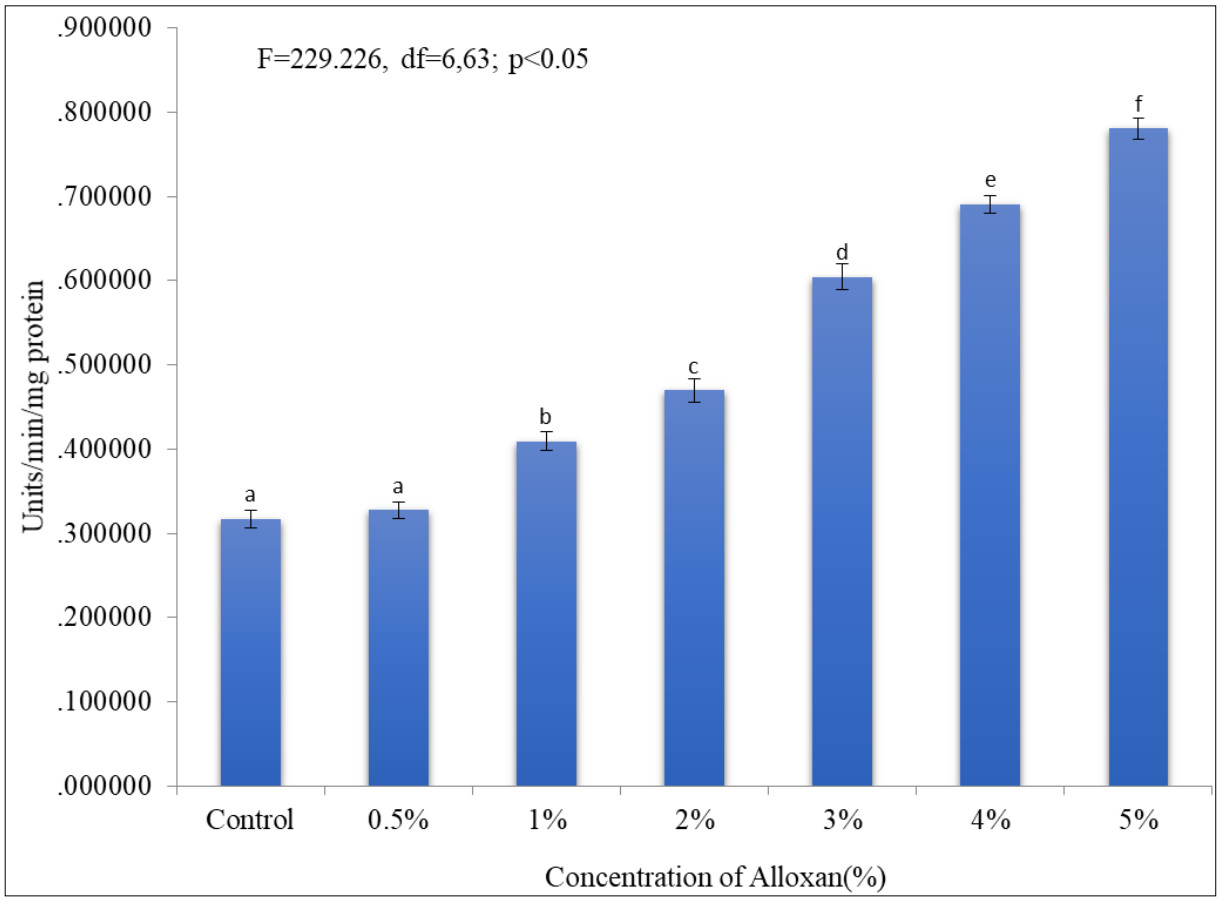

Fig 7: Alloxan effect on SOD activity in control and treated flies of *Drosophila melanogaster*. [Different alphabet on the superscript of bar graph indicates significance at p<0.05].

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Conflicts of Interest
The authors do not have any conflict of interest regarding the publication of this manuscript.

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