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Analysis of Pesticide Monocrotophos toxicity using *Drosophila melanogaster* model

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Abstract

Aim: Monocrotophos poisoning is a common medical emergency with high fatality in agricultural communities of Asia. Major cause of morbidity and mortality of poisoning is because it inhibits acetylcholinesterase and prolongs neuromuscular weakness. With this background present study was undertaken to analyze the toxicity and effect of Pesticide Monocrotophos on *Drosophila melanogaster* model.

Materials and Methods: Oxidative stress is implicated in mitochondrial dysfunction associated with neurodegeneration in various diseases. Depletion of the cellular antioxidant glutathione (GSH) resulting in oxidative stress is considered as an early event in degeneration of neurons and mitochondrial complex I (CI) is believed to be the central player to the mitochondrial dysfunction.

Results: The potential insults independently inhibit CI and other complexes of the electron transport chain, drop the proton motive force, and reduce ATP production, ultimately affecting the overall mitochondrial performance.

Conclusion: Here we tested the effect of Monocrotophos in drosophila model and showed that monocrotophos generates reactive species which decreased reduced glutathione (GSH) and concurrently increased oxidized glutathione (GSSG) levels, which in turn led to inhibition of mitochondrial complex I and complex II activities results in motor dysfunction and death of Drosophilaflies.

Key words: oxidative stress, neurodegeneration, pesticide toxicity, Monocrotophos, Drosophila

INTRODUCTION

Pesticides are class of biocides meant for attracting, seducing, destroying or mitigating any pest. It includes herbicide, insecticide, insect growth regulator, nematicide, termiticide, molluscicide, piscicide, avicide, rodenticide, predacide, bactericide, insect repellent, animal repellent, antimicrobial, fungicide, disinfectant (antimicrobial) and sanitizer [1, 2]**.** Most of the organophosphate pesticides are insecticides which were developed during the early 19th century, but later found out that their effects on insects are similar to their effects on humans. Monocrotophos [3hydroxy-N-methyl-cis-crotonamide dimethylphosphate] is anorganophosphorous insecticide that is widely used as an effective crop protectant. It has both systemic and contact properties and has been used against a wide range of insects including mites, bollworms, sucking insects, leaf eating beetles and other larvae on variety of crops. Monocrotophos is highly acutely toxic by all routes of exposure and it can be absorbed easily. Like most organophosphates, it can cause neurobehavioural problems and delayed neuropathy.

The toxicity of monocrotophos to mammals and insects is primarily attributed to their ability to inhibit acetyl cholinesterase [3, 4]. Few investigations have the reported effect of some pesticides on activity through phosphorylation of the active serine hydroxyl group situated in the active site of acetyl cholinesterase into acetic acid and hence making the enzyme non-available to hydrolyze acetylcholine into acetic acid and choline [5, 6]. Eventually results in accumulation of acetylcholine at all sites of cholinergictransmission, hence causing continuous stimulation of the muscle or nerve fiber, resulting in the exhaustion and tetany [7]. Studies have also reported the cardiotoxic effect of prolonged monocrotophos intake in rats and potent environmental cardiovascular risk factor.Acetylcholine acts through its receptor and increase the cytosolic Ca2+ released from the endoplasmic reticulum, which results in activation of calmodulin stimulating nitric oxide synthase and production of nitric oxide. Increased levels of nitric oxide are natural sequence to the inhibition of acetylcholinesterase by organophosphates. The earlier study conducted on Wistar rats examined nitric oxide signaling, activated in organophosphate poisoning, in modulating muscle mitochondrial function during the early phase of poisoning, as nitric oxide is known to modify mitochondrial proteins and disrupt mitochondrial function, including the ability to control calcium [8, 9].

Earlier research measured the effects of individual and combined exposure to low doses of atrazine and paraquat on climbing ability and longevity of *Drosophila melanogaster (D melanogaster).* These compounds interact with *D. melanogaster* and affect the climbing ability and longevity in different ways. Overall, their study shows that atrazine and paraquat can interact and that it is important to measure several traits when assessing the consequences of exposure to multiple stressors [10]. The goal of this study is to investigate the effects of Monocrotophos on nervous system of *D. melanogaster*. This system can serve as a model to help us better understands the consequences of exposure to Monocrotophos and how biochemical changes can affect mitochondria. In the current study we have employed Drosophila model tested toxicity approach to study the interdependence of mitochondrial dysfunction and GSH metabolism, and ultimate effect on behavioral defects and longevity.

MATERIALS AND METHODS:

Culturing drosophila and treatment with monocrotophos

Flies were maintained in bottles containing sufficient volume of media. Media was prepared by boiling900ml water contains 100g jiggery added 100gm Rava and 10gm agar by avoiding formation of clumps. Contents were boiled and stirred constantly until the medium is cooked and begins to thicken, then 7.5ml of Propionic acid and remaining 100ml of water was added and mixed well. The molten medium is poured into glass culture bottles (about 50ml/ bottle) using the funnels and allowed undisturbed for 30minutes and plugged the mouth with cotton.

Drosophila flies were exposed to various concentrations of monocrotophos (250nM, 500nM, 750nM and 1000nM). Flies were killed at 72 hours after exposure to monocrotophos and separated head from rest of the body for examine the biochemical changes.

Total glutathione (GSH+GSSG) estimation

Flies body and heads were subjected to total glutathione estimations by 5, 5'-dithio-bis- 2-nitrobenzoic acid (DTNB) recycling method, as described earlier [11]. Tissues were homogenized in PE buffer (100m*M* potassium phosphate buffer, pH 7.4, containing 1 m*M*EDTA), and total protein present in the homogenate was estimated by Bradford method. Eighty micro liters of the homogenate was acid precipitated with an equal volume of 2% sulfosalicylic acid (wt/vol). The mixture was centrifuged at 12,000 rpm (15 min), and the resultant supernatant was used for glutathione estimations. A 20-µl aliquot of the supernatant was incubated with assay buffer [PE buffer containing 0.8 m*M*DTNB and 0.32 U/ml of glutathione reductase (Sigma)] in a final reaction volume of 450 µl. The reaction was initiated by addition of 0.6 m*M*NADPH. The reaction kinetics of DTNB recycling, which was dependent on total glutathione levels, was monitored at 412 nm for 3 min. The glutathione amount in each sample was calculated based on the maximum reaction rate compared with GSSG standards (0–250 ng). All estimations were conducted in triplicate and normalized per protein.

Estimation of Reactive oxygen species (ROS)

Total ROS in drosophila flies were measured by a modified method of Ohashi et al. 2002 [12]. Briefly, the medium from treated or untreated samples were replaced with 1 ml of Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 5 mM HEPES, 2 mM CaCl2 and 10 mM Glucose, pH 7.4). Ten micromoles of dihydro-dichlorofluorescein diacetate (DCFDA) was then added and the samples were incubated at 37^0 C (10 min) in a CO2 incubator. The Locke's solution was then removed and the samples were harvested. Then pellet was washed with 1X phosphate buffered saline (PBS), pH 7.4, twice and reconstituted in lysis buffer (10 mM Tris–HCl containing 0.5% Tween-20). The lysate was centrifuged at 1000g (10 min) and the fluorescence of the supernatant was measured (Excitation - 480 nm; Emission - 530 nm).

Mitochondrial Complex I assay

Complex I (CI) is the first enzyme in the electron transport chain, which accepts electrons from $NADH^+$ and transfers them to the mobile electron carrier ubiquinone via a series of Fe-S clusters. The complex I assay was initiated by addition of aliquots of brain mitochondria to 50 mM potassiumphosphate/Tris-HCl (pH 7.4), 500 mM ethylenediamine tetraacetic acid (EDTA), 1% bovine serum albumin, 200 mM NADH, and 200 mMdecylubiquinone with and without 2 mM rotenone in the presence of potassium cyanide (KCN) with 0.002% dichloroindophenol as a secondary electron acceptor. The decrease in the absorbance at 600 nm was recorded as a measure of enzyme reaction rate at 37°C for 10 min, and specific activity was calculated. The results were plotted as relative rotenone-sensitive specific activity [13, 14].

Mitochondrial complex II assay

The citric acid cycle enzyme is succinate dehydrogenase, which generates $FADH_2$ with the oxidation of succinate to fumarate is part of the succinate-Q reductase complex (Complex II), an integral membrane protein of the inner mitochondrial membrane. $FADH_2$ transfers its electrons to Fe-S centers and then to Q for entry into the electron-transport chain. The complex II assay was initiated by addition of aliquots of tissue homogenates (15ug/10ul suspended in hypotonic buffer to 190ul of the reaction mix containing 10 mMTris-HCl pH 7.8, 2 µM EDTA, 10mM of succinate, 0.1% bovine serum albumin (BSA), 3µM rotenone, 1uM of antimycin, 0.3mM of KCN, 80uM of DCIP and just before the reaction 50 µM decyl-ubiquinone. The decrease in the absorbance at 600 nm was recorded as a measure of the rate of enzyme reaction at 37°C for 10 min and specific activity was calculated. The reaction was measured by spectrophotometer in a reaction volume of 200ul in an ELISA plate reader.

Negative Geotaxis assay

Geotaxis is generally measured for ten to twenty groups of ten individuals of the same genotype or treatment (100-200 flies total for each genotype/treatment). Groups of male flies on a CO_2 anesthesia apparatus were sorted and placed each group in a separate vial and allowed to rest for one hour so that flies will recover completely from anesthesia. Mean while climbing apparatus was prepared for each group, such that the alignment provides an even climbing surface for the flies. For the lower vial, measure a vertical distance of 10 cm above the bottom surface and made each vial by drawing a circle around the entire circumference of the vial. Then transferred flies into the lower vial carefully preventing the escape of any fly. Immediately covered the lower vial with the top vial and tapped securely near the contacting openings so that the flies acclimatize to new setup for 1 minute before conducting the assay. Then gently tapped the flies down to the bottom of the vial and measured the number of flies that can climb above the 10 cm mark in 10 seconds after the tap. This assay was repeated for the same group for 3 times, allowing 1 minute to rest between each trial. The number of flies per group that passed the 10cm mark was recorded as a percentage of total flies.

Survival assay

Mortality of the flies was noted every day, after exposing the flies to different concentrations of monocrotophos. Numbers of dead flies were noted every day, both in the control vials and in the treatment vials. The readings were taken at the interval of 24 hrs until the complete mortality is seen.

Statistical analysis

All quantitative data were accumulated from three independent experiments. The final data are expressed as mean with standard deviation differences between mean values were analyzed by one way analysis of variance (ANOVA).

RESULTS AND DISCUSSION:

Pesticide monocrotophos exposure decreases reduced glutathione (GSH) levels in head and body of *Drosophila Melanogaster*, which is shown in **Figure 1**.Drosophila flies were exposed to various concentrations of monocrotophos (250nM, 500nM, 750nM and 1000nM) one group was kept as control. Flies were killed at 72 hours after exposure to monocrotophos and separated head from rest of the body. GSH levels were estimated in head and body separately and are expressed as µg of GSH/mg protein. GSH levels were estimated for different aged flies such as 3, 10 and 30 days old flies. The results showed are in dose dependent manner. Pesticide monocrotophos exposure increased the oxidized form of glutathione (GSSG) levels in both head and body as shown in **figure 2.**Maleflies with different age groups such as3 days, 10 daysand 30 days old were exposed to various concentrations of monocrotophos (250nM, 500nM, 750nM and 1000nM) and one group without treatment served as control. Then files were killed and separated head from rest of the body, and estimated GSSG levels in head and body separately. GSSG levels are expressed as µg of GSH/mg protein. We can observe higher levels of GSSG in 1000nM treated drosophila group. GSH and GSSG data in **Figure 1** and **2** clearly shows that head are more responsive than rest of the body, and older flies are highly liable to monocrotophos toxicity. GSH decreased directly corrected with increased GSSG levels at different concentrations of monocrotophos and also on age dependent manner. This data indicating that nervous system cells are extremely susceptible to pesticide monocrotophos.

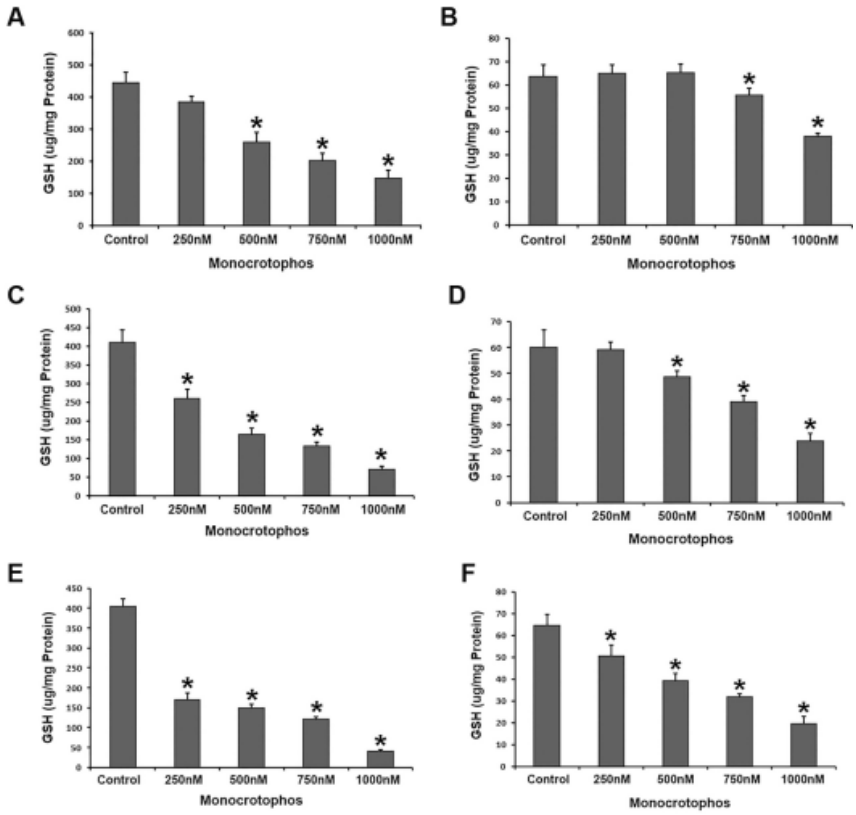

Fig. 1- Pesticide monocrotophos exposure decreases reduced glutathione (GSH) levels in head and body of *Drosophila Melanogaster*: 3 days (A and B), 10 days (C and D) and 30 days old (E and F) male Drosophila flies were exposed to various concentrations of monocrotophos (250nM, 500nM, 750nM and 1000nM) or vehicle alone (control). Flies were killed at 72 hours after exposure to monocrotophos and separated head from rest of the body. GSH levels were estimated in head (A, C and E) and body (B, D and F). GSH levels are expressed as µg of GSH/mg protein. Values are mean ± SD, n = 3 independent experiments. * indicate values significantly different from control flies with $p<0.05$.

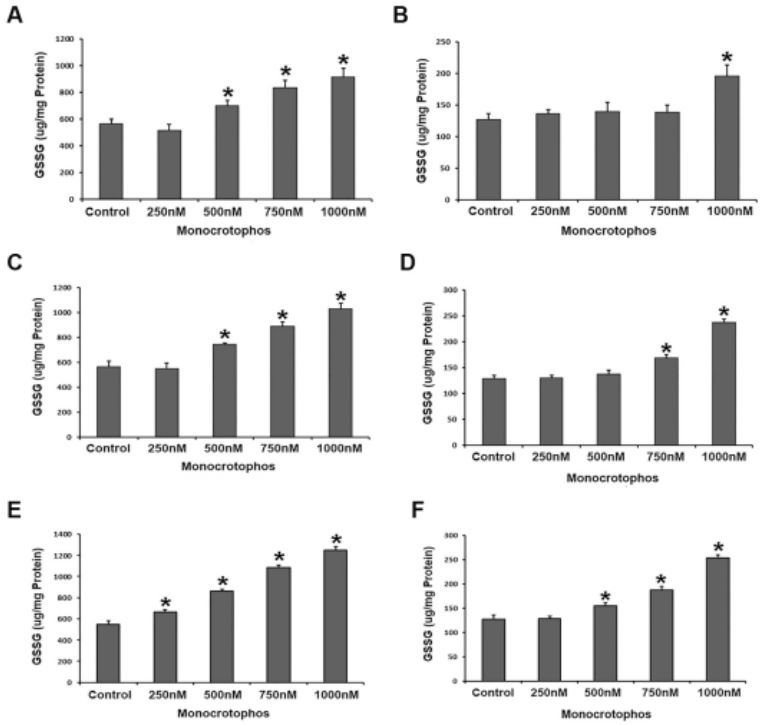

Fig. 2- Pesticide monocrotophos exposure increases oxidized form of glutathione (GSSG) levels in head and body of Drosophila Melanogaster: Maleflies with different age groups such as3 days (A and B), 10 days (C and D) and 30 days old (E and F) were exposed to various concentrations of monocrotophos (250nM, 500nM, 750nM and 1000nM) or vehicle alone (control) for 72 hr. Then files were killed and separated head from rest of the body, and estimated GSSG levels in head (A, C and E) and body (B, D and F). GSSG levels are expressed as µg of GSH/mg protein. Values are mean ± SD, n = 3 independent experiments and * indicate values significantly different from control flies with $p<0.05$.

It has been well know that GSH decrease is due to increased ROS levels, therefore, next we estimated the levels of ROS in head and body Drosophila melanogaster following exposure to monocrotophos. Similarly, ROS estimation indicated that compared to untreated controls, there was significant increase in monocrotophos treated drosophila group (**Figure 3**).After 72 hours flies were killed and ROS levels were estimated in head and body separately using a cell permeable fluorogenic dye (2',7' – dichlorofluorescin diacetate). It gets oxidized by ROS and produces florescent dichlorofluorescin, levels of which are monitored and expressed as fluorescent units/mg protein. Exposure of pesticide monocrotophos to Drosophila Melanogaster male flies generated reactive oxygen species in age dependent manner such as 3, 10 and 30 days old in response to different concentrations 250nM, 500nM, 750nM and 1000nM. In older files (30 days) we observed early response compared younger ones and brain cells are more susceptible than cells from rest of the body (**Figure 3**).

The mitochondrial machinery for ATP synthesis includes the electron transport chain comprising the respiratory complexes (CI, CIII and CIV) coupled with ATP synthase. Effect of monocrotophos exposure on mitochondrial complex I activity in head and body of Drosophila Melanogaster was estimated. 3, 10 and 30 days old male flies were treated with 250nM, 500nM, 750nM and 1000nM concentrations of monocrotophos for 72 hours. Flies were killed, heads and bodies were collected and mitochondria were isolated. Complex I activity was measured separately in head and body and is expressed as nmoles of NADH oxidized/min/mg protein, the data of which are as shown in **Figure 4**. Decreased Complex I activity was observed in both head and body of the flies with head being more responsive to lower concentrations of pesticide than body and older flies are more susceptible than younger ones (**Figure 4**).GSH (**Figure 1**) GSSG (**Figure 2**) and ROS (**Figure 3**) levels were altered at lower doses of monocrotophos Complex I activity was decreased at higher doses of monocrotophos, indicating and supporting that oxidative stress is up-stream of complex I inhibition.

The oxidation of high-energy compounds such as NADH generates free energy from the sequential transfer of electrons through the electron transport chain. This is to pump protons across the mitochondrial matrix to the inter-membrane space. The effect of monocrotophos exposure on mitochondrial complex II activity in head and body of *Drosophila Melanogaster* is shown in **Figure 5.** 3, 10 and 30 days old male Drosophila flies were exposed to 250nM, 500nM, 750nM and 1000nM concentrations of monocrotophos and killed 72 hours post exposure. Complex II activity was measured in mitochondria isolated from head and body of the flies, and activity is expressed as nmoles of 2, 6 dichloro phenol indophenol (DCIP) reduced/min/mg protein. Data shows that complex II inhibition is further down steam of effects on GSH, ROS and complex I, but follows same pattern as GSH, GSSG, ROS and complex I, means head are more susceptible than body.

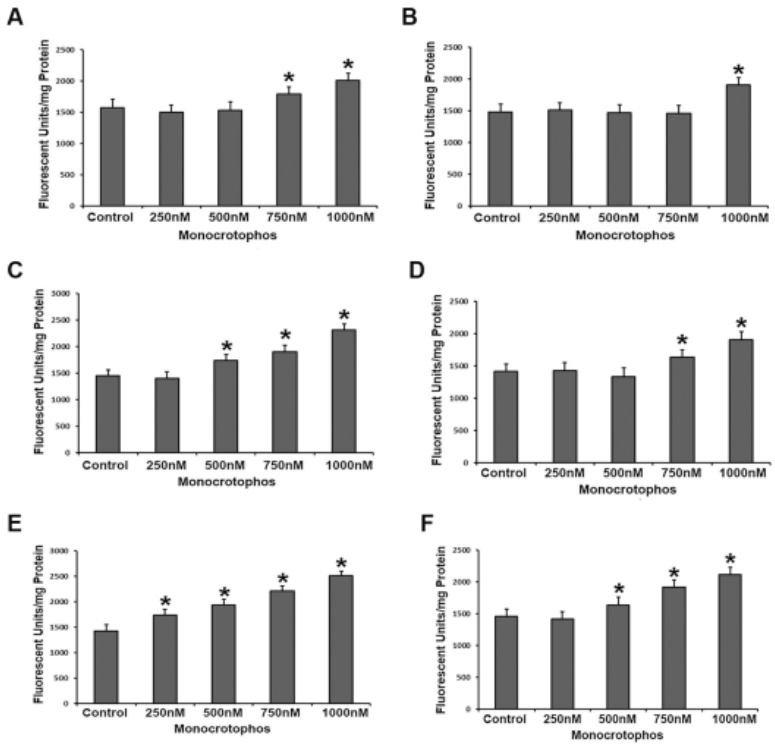

Fig. 3- Exposure of pesticide monocrotophos to *Drosophila Melanogaster* flies generates reactive oxygen species (ROS): 3 days (A and B), 10 days (C and D) and 30 days old (E and F) male flies were exposed to 250nM, 500nM, 750nM and 1000nM of pesticide monocrotophos or vehicle alone (control) for 72 hours. After 72 hours flies were killed separated head from rest of the body. ROS levels were estimated in head (A, C and E) and body (B, D and F) by using cell permeable 2',7' –dichlorofluorescin diacetate (**DCFDA)**, a fluorogenic dye which get oxidized by ROS and produces florescent DCF, DCF levels are monitored and expressed as Fluorescent units/mg protein. Values are mean ± SD, n = 3 independent experiments. * indicate values significantly different from control flies with $p<0.05$.

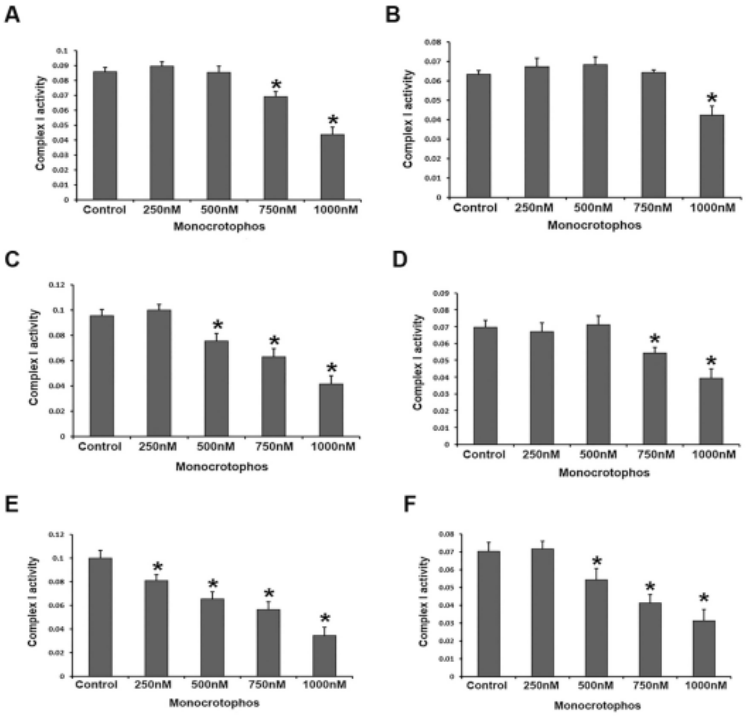

Fig.4- Effect of monocrotophos exposure on mitochondrial complex I activity in head and body of Drosophila Melanogaster: Vehicle (control) or250nM, 500nM, 750nM and 1000nM concentrations of monocrotophos were exposed to 3 days (A and B), 10 days (C and D) and 30 days (E and F) old male Drosophila flies for 72 hours. Flies were killed, heads and bodies were collected and mitochondria were isolated from both head and bodies. Complex I activity was measured in head (A, C and E) and body (B, D and F). Complex I activity is expressed as nmoles of NADH oxidized/min/mg protein. Data are mean ± SD, n = 3 independent experiments. * denotes significant difference compared to control with $p<0.05$.

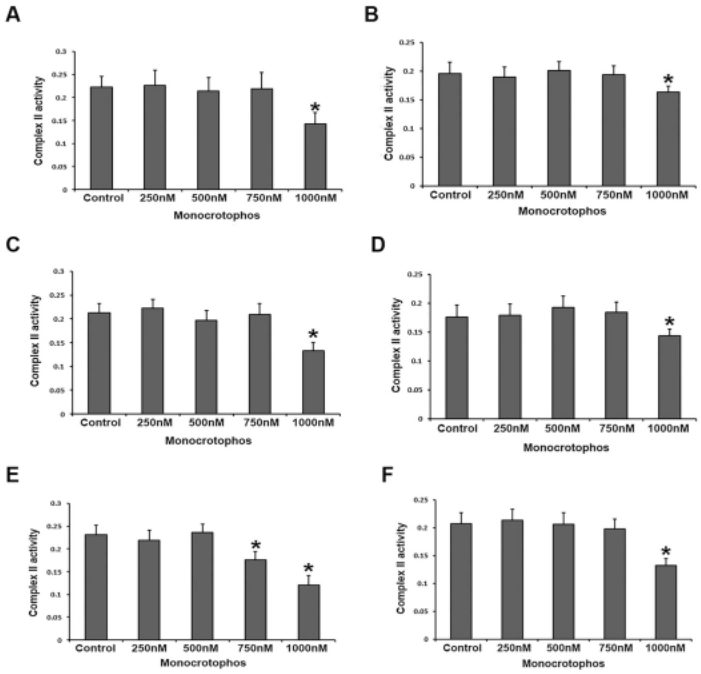

Fig. 5- Effect of monocrotophos exposure on mitochondrial complex II activity in head and body of Drosophila Melanogaster: Vehicle (control) or250nM, 500nM, 750nM and 1000nM concentrations of monocrotophos were exposed to 3 days (A and B), 10 days (C and D) and 30 days (E and F) old male Drosophila flies. 72 hours after exposure flies were killed, heads and bodies were collected and isolated mitochondria. Complex II activity was measured in mitochondria isolated from head (A, C and E) and body (B, D and F). Complex I activity is expressed as nmoles of 2, 6 dichloro phenol indophenol (DCIP) reduced/min/mg protein. Data are mean ± SD, n = 3 independent experiments. * denotes significant difference compared to control with $p<0.05$.

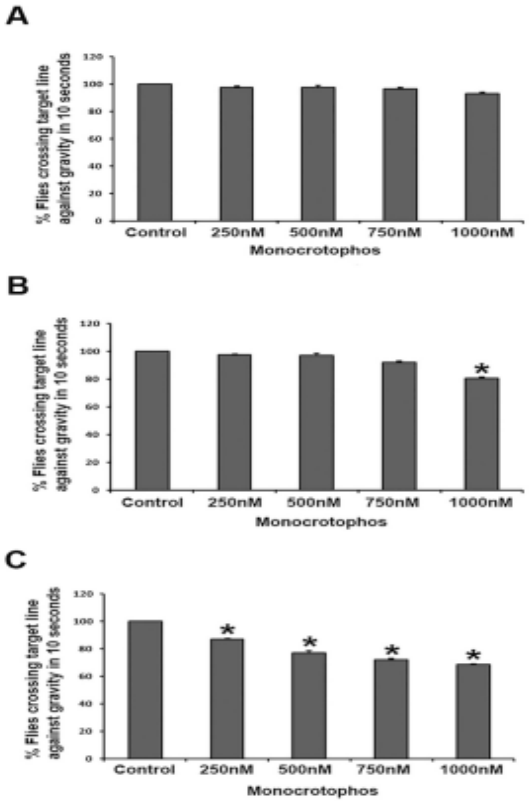

Fig. 6- Exposure of monocrotophos to Drosophila melanogaster male flies impacts climbing abilities: 3 days (A), 10 days (B) and 30 days (C) old flies were exposed to various concentrations of monocrotophos (250nM, 500nM, 750nM and 1000nM) or vehicle (control) for 72 hr. Then assayed for climbing ability with negative geotaxis assay and counted the number of flies climbs target line (above 10 cm length) in 10 seconds. Data is expressed as percent flies crossing target line against gravity in 10 seconds. Data are mean ± SD, n = 3 independent experiments. * denotes significant difference compared to control with $p<0.05$.

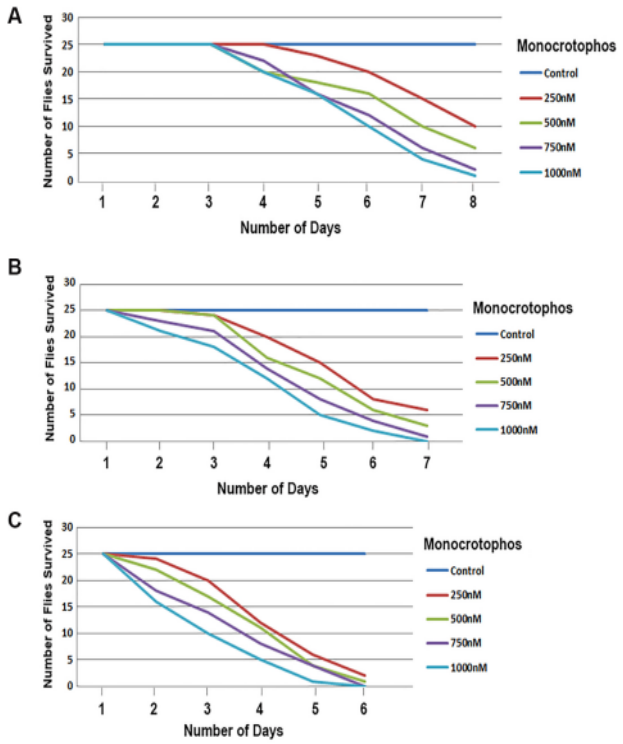

Fig. 7- Pesticide monocrotophos exposure induces mortality in Drosophila melanogaster male flies: 3 days (A), 10 days (B) and 30 days (C) old *Drosophila melanogaster* flies were exposed to 250nM, 500nM, 750nM and 1000nM or vehicle (control). 24 hours following exposure to monocrotophos started monitoring the flies for mortality and repeated this at 24 hours interval until complete morality is observed. Data are from 3 independent experiments and expressed as number of flies survived.

Negative Geotaxis assay was performed to analyze effect of monocrotophos on motor function and climbing potency of flies post treatment. Exposure of monocrotophos to *Drosophila melanogaster* male flies impacts climbing abilities**.**3, 10 and 30 day sold male flies were exposed to various concentrations of monocrotophos as explained before and then assayed for climbing ability. Climbing ability was counted by the number of flies which climbs the target line of above 10 cm length in 10 seconds. Data is expressed as percent flies crossing target line against gravity in 10 seconds as recorded in **Figure 6**. 3 days old young flies didn't show any signs of climbing defects; however, 7 days old flies had climbing defects at 1000nM concentration and 30 days old flies showed climbing defects even at 250nM of the pesticide, suggesting older nerve cells more susceptible to pesticide toxicity.

At the end we wanted to examine the effects of monocrotophos on survival of flies. Survival assay was conducted by exposing the flies to different concentrations of pesticide. It is observed that pesticide monocrotophos exposure induces mortality in *Drosophila melanogaster* of male flies. Time intervals and concentrations of toxin exposure were kept same for all experiments. 24 hours following exposure to monocrotophos started monitoring the flies for mortality and repeated this at 24 hours interval until complete morality is observed. Data is taken from 3 independent experiments and expressed as number of flies survived as noted in **Figure 7**, it clearly shows that older flies dies at low concentration and die faster than younger flies.

Increase in age produced more marked alterations to monocrotophos exposure. Similar results were obtained by various researchers in different experimental models. In a study, monocrotophos treated nuclear membrane was significantly lost in the necrotic hepatic cells resulting in the exclusion of nuclear material in the cytoplasm. Enormous infiltration of the cytoplasm with certain electron transparent vacuoles of unknown origin was also observed. The serum activity of acetylcholinesterase was found to be significantly inhibited whereas serum level of bilirubin was found to be increased [15]. Histopathological studies done on liver of fish by different workers also revealed nuclear pycnosis, mild to severe necrosis, disrupted hepatocytes, disorganized hepatic canaliculi, disintegrated blood vessels, ruptured central vein and vacuolation on exposure to monocrotophos and other organophosphates [16, 17 and 18]. Moreover, cessation of exposure to monocrotophos resulted in significant normalization of the inhibited levels of acetylcholinesterase. They speculate that it could be due to non-persistent nature and reversible inhibition of the organophosphates which by undergoing hydrolysis might lead to the restoration of active acetyl cholinesterase and hence might release the inhibitory effect on the various receptors from accumulated acetylcholine in thesynapses [19].

An another study demonstrated that muscle mitochondrial ATP synthase is inhibited in paralyzed muscle during the early phase of acute monocrotophos exposure an effect possibly mediated through nitric oxide. The inhibition of ATP synthase may be responsible for reduced calcium

uptake by the mitochondria, which in turn affect cellular calcium homeostasis and lead to the muscle damage [10].The catastrophic effect of toxicants in exposed aquatic animals like fish- *Chana punctatus* was histopathologically investigated and complete picture of toxicity on the life at cellular level is given [20, 21].Drosophila model tested toxicity of pesticide monocrotophos found the extent of harm in drosophila. Several key features of the model which are proved experimentally can be validated via *in vitro* and cell culture based experiments. Present study may be useful in providing insights on toxicology of monocrotophos in drosophila model and how it can be implied with human beings.

CONCLUSION

The results through light on few biochemical events leading to neurodegeneration. GSH depletion might be the first biochemical event followed by oxidative stress, CI inhibition, decreased ATP production, apoptotic signals etc and eventually leads to onset of the disease. The current outcome of our work does follow similar pattern of toxicity events. There might be other independent events that might impinge on mitochondrial physiology and GSH status that have not been elaborated here. Further research coupled with experimental studies to better understand how processes such as proteasome inhibition and protein aggregation might affect mitochondrial function and GSH metabolism is needed.

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