



## MALATHION-INDUCED CELL INJURY AND CELL DEATH IN THE NERVOUS SYSTEM VIA OXIDATIVE-STRESS-INDUCED CYTOTOXICITY IN *DROSOPHILA MELANOGASTER*

### Zoology

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### ABSTRACT

In the present study we have investigated the effect of Malathion on the nervous system of *Drosophila melanogaster* (DM). Our study evaluated the effect of sub chronic exposure of Malathion on the neuropathology, genotoxicity and antioxidant activity. We have also studied the effect of Malathion by validating its cytotoxic effects. Our study shows dose dependent Malathion induced neuropathology in the brain tissue of *D. melanogaster* as evidenced by silver nitrate staining, transmission electron microscopy and decreased super oxidatase specific activity induced Malathion treated *D. melanogaster*. We have also employed its significant association with oxidative DNA damage as observed by alkaline comet assay. Additionally we have also evaluated the cytotoxic damage induced by Malathion in Dmel-2 cells suggesting the loss of neuronal cells viability. Thus our findings demonstrated that Malathion induced toxicity resulted in neuroanatomical changes with neurotoxic damages to the nervous system.

### KEYWORDS

*Drosophila melanogaster*; Malathion, neurotoxicity, DNA damage.

### Introduction

Pesticides are a family of compounds that have several benefits to mankind in various sectors such as agricultural, industrial, and health, but their toxicities in both humans and animals have always been a concern [1]. At present, organophosphorus pesticides (OPs) are the most commonly utilized pesticides in the world. OP toxicity is considered a great problem in developed as well as poor countries [2]. Conversely, their chronic exposure has been associated with the other toxic effects viz., delayed neurotoxicity, developmental neurotoxicity and toxicities in different organs [3].

Malathion (O, O-dimethyl di-thiophosphate of diethyl mercaptosuccinate) is one of the Ops with elusive toxic effects. It is remarkable that, various researches demonstrated the neurotoxic effects of Malathion both in humans and experimental animal models [4, 5]. It was introduced in 1950's and one of the most prehistoric organophosphate [6]. It is used widely for agricultural, residential and public health purposes; providing enhancement in production of food and defense from disease vectors [7]. About 60% of this total is utilized for several insect eradication programs [8]. It is broadly used in various areas for of its low constancy in the environment related to other organophosphorus insecticides. Malathion toxicity is associated with its metabolites and also governed by the purity of the product, route of exposure, quantity of protein in the diet and gender [9,10].

According to the World Health Organization, during the last ten years the occurrence of poisonings due to pesticides has increased because of their widespread usage [11]. Malathion toxicity can cause early signs or mild symptoms to serious adverse health problems. Some of the symptoms of exposure comprise; numbness, tingling sensation, headache, dizziness, difficulty breathing, weakness, skin irritation, exacerbation of asthma, abdominal cramps together with other correlated symptoms [8].

It has been showed that genotoxic evaluation in populations with Malathion exposure has noticeably showed a positive genotoxic effect in lymphocytes of human. [12]. Animal models have been in use for studying the mechanism of disease. In recent past *Drosophila*, have demonstrated to be an excellent model for studying human neurodegenerative diseases and have drawn interest to the researchers [13].

Clinical and experimental reports has increased the interest that

Malathion appears to be a promising tool to study the neuronal cell death *in vivo* and *in vitro* in *D. melanogaster*. Therefore, the present work was undertaken to study the usefulness of *D. melanogaster* as an *in vivo* and *in vitro* model for assessment of Malathion induced neuropathological, antioxidant activity and cytotoxic damage after sub lethal chronic exposure. Additionally, we sought to determine if Malathion-induced neuropathology is correlated with toxin-induced DNA damage in *Drosophila* brain cells. Furthermore, we also investigated the cytotoxic effect of Malathion on the *Drosophila* embryonic cell line (D-Mel2) to confirm the association between the neuropathological and genotoxic changes.

We anticipate that further exploration of the animal models will further add a layer of knowledge in our understanding of mechanisms of neurodegeneration and also open doors for the development of rational treatments for devastating degenerative diseases.

### Materials and Methods

#### Chemicals

Malathion; lactate dehydrogenase (LDH) assay kit were bought from Sigma-Aldrich Chemical Company. 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT), l-glutamate, antibiotics, Schneider's Medium, EDTA, potassium dihydrogen, and monohydrogen phosphate were purchased from Hi Media Laboratories Private Ltd, Mumbai, India. All other chemicals used in this study were of analytical grade.

#### *Drosophila* culture and cell-line maintenance

Wild-type Oregon R *D. melanogaster* was cultured on standard *Drosophila* food containing agar, corn meal, sugar, and yeast at 25°C (24 ± 1) in a light/dark cycle of 12:12 h [14]. *Drosophila* cell lines (D-Mel2) were maintained for *in vitro* cytotoxicity assessment of pesticides. D-Mel2 cell lines were obtained from National Centre For Cell Sciences, Pune, and maintained in Schneider's Medium and 10 µl/ml insulin medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc.,CA) and were grown in a refrigerated incubator at 25°C.

#### Drug preparation and treatment

Malathion doses and treatment to the flies were done as described previously [15] with slight modifications. Different doses of Malathion viz., 50, 100, 150, 200, and 500µM were prepared from 5mM/ml stock.

### Histological detection by silver nitrate staining

Silver nitrate staining was done according to the method done by Bielschowsky [16] with slight modifications as done earlier by Mehdi et al., [15].

### Histological preparation of *D. melanogaster* brain tissue for transmission electron microscopy

The entire head was explanted from control and treated *D. melanogaster* and fixed for 4–6 h in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH = 7.4). After that the tissue was kept in PBS (pH = 7.4) at 4°C for 2 h or overnight. The secondary fixation of the tissue was done in 1% osmium tetroxide in distilled water for 1 h at room temperature and then washed twice with distilled water, followed by upgraded ethanol series (50–100%) for 15 min each. After washing, tissue was kept in propylene oxide twice for 10 min, followed by propylene oxide: resin (1:1 mixture) for 1–2 h. The tissues were infiltrated with a resin before being placed in an embedding mould, which was then polymerized in an oven at 60°C. The sections were cut at 0.5–1.0  $\mu\text{m}$  thicknesses and were transferred on the slide. After drying, slides were stained with toluidine blue for 2–5 min. The sections were observed under microscope for precise location to cut for ultrathin sections. Ultrathin sections were cut at 60–90 nm thickness (silver-yellow color; Ultramicrotome, Model UC6, Reichert), and the sections were collected on to the grids. The sections were dried overnight before staining, and finally the grids were stained with uranyl acetate for 15 min and with lead acetate for 5 min. After staining, the sections were observed under transmission electron microscope (Model Morgagni 268D; FEI Company, Netherlands) at Sophisticated Analytical Instrument Facility for Electron Microscopy, Department of Anatomy, AIIMS, New Delhi, India.

### Comet assay

The assay was performed in alkaline condition in accordance with protocol of Singh et al., [23], with slight modifications as done earlier by Mehdi et al., [15].

### SOD assay

Total SOD activity was measured in *D. melanogaster* after 7 days of Malathion treatment. Extracts were prepared by homogenizing treated flies brain in 500  $\mu\text{l}$  of ice-cold homogenizing buffer (210mM mannitol, 70mM sucrose, and 1mM EDTA). Homogenates were centrifuged at 800  $\times$  g for 10 min at 4°C to pellet solids. SOD activity was measured as described previously. Briefly, the hypoxanthine and xanthine oxidase were used to generate superoxide radical and the substrate of superoxide dismutase, nitroblue tetrazolium was used as an indicator of superoxide, and the absorbance was taken at 546 nm. The specific activity was reported in units per milligram of protein.

### Cytotoxicity assessment of Malathion on *Drosophila* cells

D-Mel2 cell lines were treated with different doses of Malathion to assess the cell viability by MTT and LDH assay. Briefly, cells were exposed to different Malathion (100 $\mu\text{M}$  to 500 $\mu\text{M}$ ) concentrations for 24, 48, and 72 h. At the end of the treatment, the medium was removed, and cells were incubated with 20  $\mu\text{l}$  of MTT (5 mg/ml in PBS) in fresh medium for 4 h at 27°C followed by DMSO (150  $\mu\text{l}$ /well), and the absorbance was read at 570 nm after 10-min incubation on the Multiskan EX microplate reader (Thermo scientific, Germany). Cytotoxicity induced by Malathion was also assessed by LDH leakage into the culture medium. Similar to the MTT assay, following exposure to the Malathion, the culture medium was aspirated and centrifuged at 3000 rpm for 5 min in order to obtain a cell-free supernatant. The activity of LDH in the medium was determined using a commercially available Cytoscan-LDH Cytotoxicity Assay Kit according to the manufacturer's protocol.

### Statistical analysis

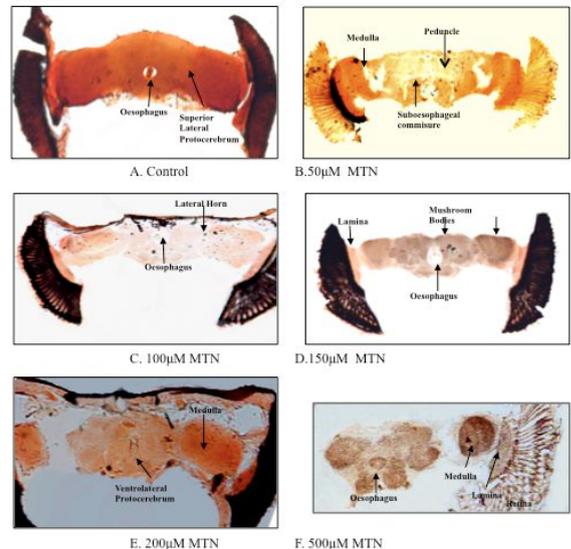
Statistical significance of above results was evaluated by one-way ANOVA using Graph Prism and SPSS (17.0 version). The probability of occurrence was selected at  $p < 0.05$ .

### Results

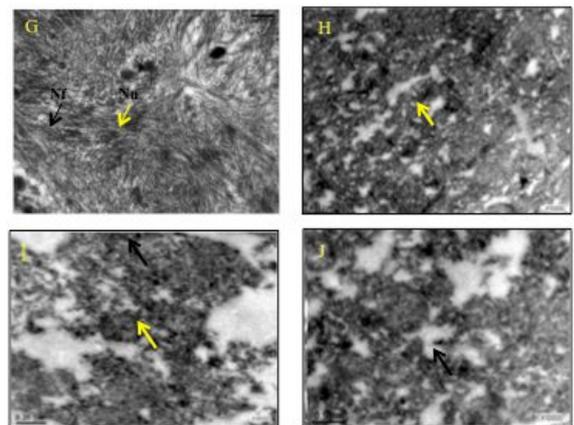
#### Malathion treatment revealed extensive neuropathological and ultrastructural changes in the nervous system of *D. melanogaster*.

Silver stained sections of *D. melanogaster* brain tissue were treated with different doses of Malathion showed differences in morphological features of brain tissue. Treated sections showed various degree of neuropathological damage in comparison to control. In treated sections the structural integrity is truly disrupted and the

optic lobe area is vacuolated. Marked disruption of structure in the medullar and laminar region at 200 $\mu\text{M}$  and 500 $\mu\text{M}$  doses (Fig 1) was observed. Vacuolar lesions and reduction in the thickness of the structure of medulla and lamina at all doses respectively were seen in comparison to control. Treated sections exhibit neurotoxic pathology in the neuron cell bodies resides in the lobular, laminar, ventrolateral protocerebrum and medullar region. In all treated sections changes in the structural integrity resulted in the increment of neuronal degeneration in the brain region. Whereas in, Transmission electron microscopic studies also revealed that Malathion exposure to *Drosophila* brain resulted in changes in neuronal mitochondria, including swelling and partial loss of cristae (Fig. 2, arrows indicate changes in mitochondria). Transmission electron microscopic study of *D. melanogaster* brain tissue also exhibit an overall vacuolar degeneration and rapid changes in the whole tissue structure compared with the untreated flies. This disruption is prominent in all sections. Vacuolar lesions are also prominent in all treated sections (Fig2).



**Fig 1:** Horizontal section of silver stained *D. melanogaster* brain shows different regions affected by different doses of malathion

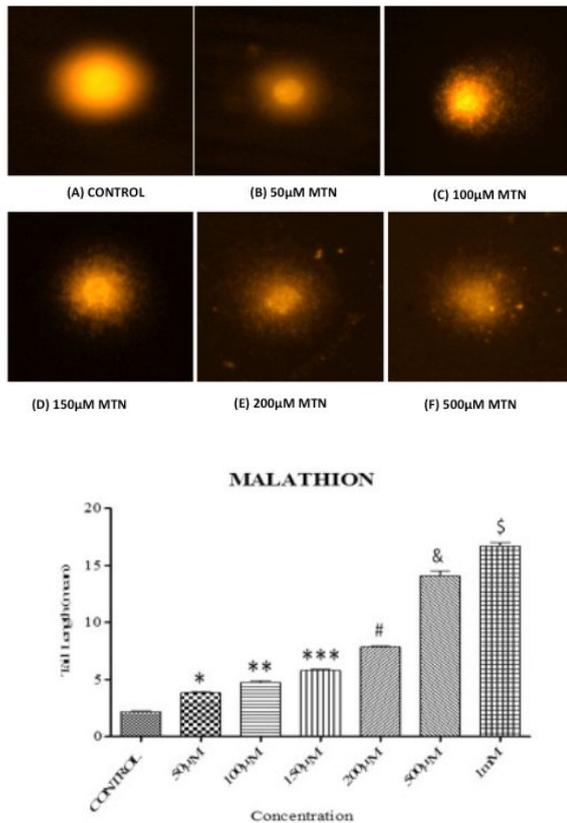


**Fig 2:** Ultra structure of Brain tissue of *D. melanogaster* treated with Different dose malathion (G-J) i.e G= Control H=100 $\mu\text{M}$ , I=200 $\mu\text{M}$  and J=500 $\mu\text{M}$ . Treated ultra structure displayed irregularity of shape with disintegration in all cellular structure and nerve fibers denoted by yellow arrow in all treated sections. Vacuolar lesions are denoted by black arrow in treated sections.

#### Exposure of Malathion resulted DNA damage in *D. melanogaster* Brain tissue

Comet assay was conducted to investigate the treatment incurred any noticeable nuclear damage in brain tissue of the treated *D. melanogaster*. *D. melanogaster* exposed to different doses of Malathion showed a dose dependent increase in DNA damage as evident by a statistically significant increase in comet parameter i.e. tail length. At 50 $\mu\text{M}$ - 500 $\mu\text{M}$  doses a significant increase in ( $P < 0.0001$ ) in the comet parameter were observed in the cells of

exposed organisms as compared with the respective control (Table 1). Malathion treatment caused increase in comet tail length by 72.98%, 113.71%, 161.99%, 254.61%, 538.00%, and 657.01% at 50µM/ml, 100µM/ml, 150µM/ml, 200µM/ml and 500µM/ml respectively in the brain tissue of *D. melanogaster*. Malathion treated group showed increment in the tail length in respect to increase concentration of dosage in the sequence. The increase in tail length in the tissue is clear indicator for genotoxic effect of Malathion (Fig 3). It means that Malathion were able to deteriorate the integrity of nuclear DNA in the tissue of treated *D. melanogaster* as evidenced by comets in nuclear DNA of the treated animals.



**Fig 3:** Representative comet picture of *D. melanogaster* brain cells treated with different doses of malathion under photoillumination are shown. Showing bars of average tail length of *D. melanogaster* brain cells against different doses of malathion. All the data have been represented as mean± SEM of three independent experiments. \*Significant at p≤ 0.0001 from the control, \*\*, \*\*\*, #, \$, ^Significant at p≤ 0.0001 from the control as well as all other groups.

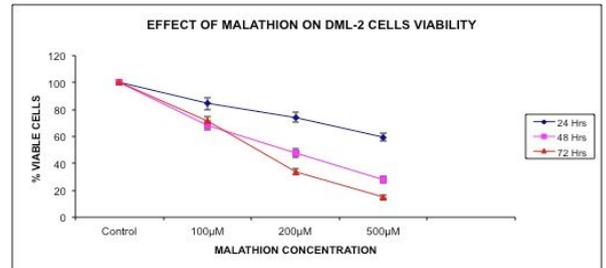
**Table 1:** Showing average comet length in *D. melanogaster* Brain cells treated with different doses of malathion. All the data has been represented in mean± SEM of three independent experiments. Significant at p≤ 0.0001 from the control.

Dose	Mean±SE	95% Confidence Limit		F	R <sup>2</sup>
		Lower	Upper		
Control	2.210±0.061	1.947	2.473	587.5***	0.996
50µM/ml	3.823±0.146	3.194	4.452		
100µM/ml	4.723±0.154	4.058	5.388		
150µM/ml	5.790±0.075	5.465	6.115		
200µM/ml	7.837±0.080	7.489	8.185		
500µM/ml	14.10±0.459	12.12	16.07		
1mM/ml	16.73±0.291	15.48	17.99		

**Malathion inhibited proliferation of D-Mel-2 (S2) cells by MTT method**

Malathion cytotoxicity revealed potent cytotoxic effect against D Mel-2 (S2) cell line. Fig. 4 shows cell viability after 24h, 48h and 72h of incubation in a medium containing 100µM-500µM of Malathion. These figures clearly show the inhibitory effect and sensitivity of Malathion on the D-Mel-2 (S2) cellular growth at different doses.

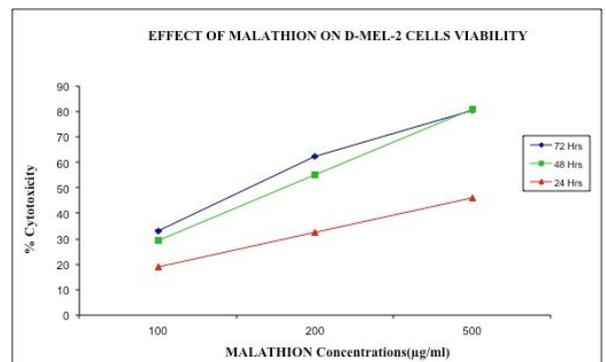
Malathion has cytotoxic effects *in vitro* at applied doses (IC50 values ≤ 200µM) by MTT method after 48h. After three different hours of exposure Malathion induced dose dependent cytotoxic effects in D-Mel-2 (S2) cell with IC50 values ≤ 53.5±3.2 after 48h respectively. Total cells viability of D-Mel-2 (S2) cells dropped down to 44.05% level due to acute toxicity of 1mM/ml of Malathion after 24h whereas after 48h viable cells were dropped down to 20.7% and after 72h it was dropped down to 13.4% level due to acute toxicity of 500µM/ml of Malathion. Results indicated both time dependent and dose dependent viability loss in the treated cell line. Further the result confirmed that in terms of sensitivity D-Mel-2 (S2) cell indicated only a low level of viability loss (approx 16%) upon treatment with 100 µM Malathion at 24h. Results obtained in the study were significant at p-value <0.05.



**Fig 4:** Comparison of Dose-dependent effect of malathion on D-Mel-2 (S2) cell proliferation. Cells were cultured in 10% FBS medium and treated with 100µM, 200µM and 500µM malathion for 24h, 48h and 72h and cytotoxicity was monitored by MTT assay. The percent cytotoxicity was calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD and the experiment was performed in triplicate (P<0.05).

**Cytotoxicity activity of Malathion on D-Mel-2 (S2) cells by LDH method**

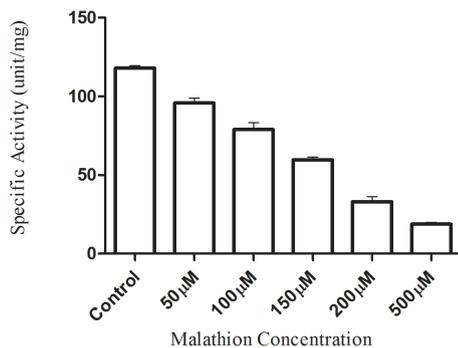
Testing the cytotoxicity activity of the Malathion revealed a potent cytotoxic activity against D-Mel-2 (S2) cells in concentration dependent manner with an IC50 values ≤ 200µM/ml after 48h. Figure 5 clearly shows the inhibitory effect of Malathion on the DMel- 2 (S2) cellular growth. The inhibition was demonstrated at four escalating doses of Malathion in a range of 100 µM/ml to 500µM/ml. Total cells cytotoxicity of D-Mel-2 (S2) cells increased to 61.55% due to acute toxicity of 1mM/ml of Malathion after 24h whereas after 48h cytotoxicity increased to 92.85 % and after 72h it was 96.85% level due to acute toxicity of 500µM/ml of Malathion. Results indicated both time dependent and dose dependent viability loss in the treated cell line. Further the result confirmed that in terms of sensitivity D-Mel-2 (S2) cells indicated only a low level of cytotoxicity (approx 18.89%) upon treatment with 100 µM Malathion at 24h. Results obtained in the study were significant at p-value <0.05 [Fig.5].



**Fig 5:** Comparison of Dose-dependent effect of malathion on D-Mel-2 (S2) cell proliferation. Cells were cultured in 10% FBS medium and treated with 100µM, 200µM and 500µM malathion for 24h, 48h and 72h and cytotoxicity was monitored by LDH assay. The percent cytotoxicity was calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD and the experiment was performed in triplicate (P<0.05).

**Malathion Exposure Results in the Decreased SOD Measurement**  
SOD specific activity was assayed in the brain samples of flies treated with various concentration of Malathion. From the result, it is evident that the decreased SOD specific activity is associated with the

increased Malathion doses. The SOD specific activity was reduced to 67.40% at 200 $\mu$ M of Malathion concentration. Following the same pattern, 500 $\mu$ M of Malathion was able to show the most decreased activity by 80.76%. These results clearly suggest the Malathion affected SOD activity in a dose dependent manner as demonstrated in [Fig.5].



**Fig 6:** Activity of antioxidant enzyme SOD in the brain tissue of *D. melanogaster* after 7 days of Malathion treatment. Significant at value ( $p < 0.05$ ).

### Discussion

In the present study neurotoxic effect of Malathion at different level of exposure and the relationship of pesticide exposure to neurological disease has been discussed. Chronic exposure of *D. melanogaster* to sub-lethal dose of Malathion, summarizes the major neurodegenerative disease symptoms viz., loss of cellular structures, loss in locomotor ability and changes in the structural integrity of the brain tissues. It has been demonstrated that Malathion administration causes toxicity in *D. melanogaster* both *in vivo* and *in vitro*. Nervous system is very sensitive to the toxicity of Malathion. Chronic exposure of *Drosophila* to sub-lethal doses of Malathion recapitulates the main symptomatic feature of neurological disease i.e. inducing locomotor deficits [15,17]. In this study, silver stained sections of brain tissue were visualized to study the neuronal connections, neurofibrils and neuronal bodies. After the exposure of Malathion on *D. melanogaster* brain tissue we observed distinguishes morphological changes in the structural integrity and neuropathological. The structural integrity is truly disrupted and the optic lobe area is vacuolated. From the result it is evident that Malathion exhibits marked damaged in the brain morphology of the *D. melanogaster*. This type of vacuolar neuropathology has been depicted in several other characterized neurodegeneration mutants identified in *D. melanogaster* and is a typical appearance of neurodegeneration in both flies and mammals [18]. Transmission Electron Microscopic observation also boost the silver nitrate staining morphology of the Malathion treated flies [19] have demonstrated the ability of OP insecticides to directly damage DNA in freshly isolated human lymphocytes *in vitro* utilizing the comet assay [20] investigated apoptosis and DNA damage inducing potential of chlorpyrifos in *D. melanogaster*. Previous studies also focus on the genotoxic effects of Malathion [21].

Present study revealed a dose dependent increase in genotoxicity level and DNA damage in the brain tissue of *D. melanogaster* and result indicated that Malathion is a potent genotoxic compound which has a higher potential to deteriorate the integrity of nuclear DNA in the tissue of treated *D. melanogaster*. We have found strong relationship between Malathion induced DNA damage in the neuronal system of *D. melanogaster* and the neuronal damage in *D. melanogaster* brain tissue (Figure No). DNA damage activates cellular signaling pathways that may ultimately lead to cell death, if the damage encountered is too great or not repaired [22].

Cytotoxic potential of any pesticide is necessary to study in order to relate the DNA damage caused by the pesticide [15]. Cytotoxicity can be defined as the ability of a compound to induce cell death. In the present study, the MTT assay was used to determine the cytotoxicity of Malathion in *Drosophila* cell line to determine the cytotoxic effect of Malathion which is the major environmental toxin used in the present time.

We have also found significant connection between the dose-dependent increases in cytotoxicity level in D-Mel2 cells and DNA damage in the brain tissue of *D. melanogaster*. Total Malathion induced cytotoxicity was also increased in a time- and dose-dependent manner. The results indicated that Malathion induced potential cytotoxic effects on cell viability.

### Conclusion

Our present investigation suggests that sub-lethal exposure of Malathion to *D. melanogaster* exhibit key aspects of neurodegeneration at different parameters which are mentioned above such as, neuropathology at various doses of Malathion, damage to DNA content in brain tissues in response to dose dependent treatment of Malathion. *In vitro* studies also suggested that cellular degradation in D-Mel-2 (S2) cells due to different doses of Malathion at different time exposure. More study should be needed on animal model to further validate the Malathion as a potential neuro toxic compound.

### Conflict of Interest: No

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