Paraquat-Induced Ultrastructural Changes and DNA Damage in the Nervous System Is Mediated via Oxidative-Stress-Induced Cytotoxicity in Drosophila melanogaster

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Received March 12, 2013; accepted May 7, 2013

Paraquat (PQ), a quaternary nitrogen herbicide, is commonly used as a pesticide despite of its high toxicity. Our study evaluated the effect of subchronic PQ exposure on the neuropathology, genotoxicity, and antioxidant activity on the nervous tissue of Drosophila melanogaster. We also explored the behavioral effect of PQ on D. melanogaster. Furthermore, we attempted to validate the mechanism by evaluating PQ-induced cytotoxicity on the D-Mel2 cell lines. The fruit fly D. melanogaster serves as a feasible model to understand the mechanism of neurodegenerative diseases. Our study shows a dose-dependent PQ-induced neuropathology in the brain tissue of D. melanogaster as evidenced by hematoxylin and eosin staining, silver nitrate staining, and transmission electron microscopy. Electron microscopic study of D. melanogaster brain tissue exhibited vacuolar degeneration and significant neuronal damage across the nervous tissue structure in comparison with control. Our findings also indicate a dose-dependent locomotor impairment and decreased superoxide dismutase (SOD) specific activity in PQ-treated D. melanogaster. These PQ-induced neuroanatomical changes and decreased SOD specific activity showed a significant association with oxidative DNA damage as observed by alkaline comet assay. Additionally, we show, for the first time, a dose-dependent PQ-induced cytotoxicity in the D-Mel2 cells suggesting loss of neuronal cell viability via cytotoxic damage. Our data suggest that PQ exposure results in neurodegeneration in D. melanogaster and that fruit fly is a suitable in vivo model for correlating the neuroanatomical changes with neurotoxic damages to nervous system.

Key Words: paraquat; Drosophila melanogaster; neuropathology; DNA damage; cytotoxicity.

Paraquat (PQ) is a widely used herbicide to control weeds in a huge variety of crops. Several epidemiological studies have linked the exposure of PQ to the risk of neurodegenerative diseases (Kamel et al., 2007). PQ especially destroys the dopaminergic neurons in mammals (Kuter et al., 2007; Li et al., 2005) and nonmammals (Chaudhuri et al., 2007). PQ also results in the marked neurotoxicity followed by neuronal degeneration and cell loss in rat brain (Calò et al., 1990). Studies have shown the behavioral and neuropathological effects of both systemic and intracranial injections of PQ in rats (Bagetta et al., 1992). A number of studies have shown that exposure of laboratory animals to PQ causes reduction in neurotransmitters in brain (Endo et al., 1988; Miranda-Contreras et al., 2005) that significantly disturbed or reduced motor activities including walking, drinking, rearing, and rotational activity (Chanyachkul et al., 2004). Numerous laboratory studies have demonstrated the possibility of PQ as a potential agent to cause the onset or accelerate the development of Parkinson’s disease (Hatcher et al., 2008). Drosophila melanogaster has served as a suitable model for dopaminergic neuronal damage caused by pesticides such as PQ in the last few decades and shows Parkinson’s type pathology when exposed to PQ and other neurotoxicant (Coulom and Birman, 2004).

Several researchers have found that PQ is a strong redox agent that yields a stable PQ radical that reacts with oxygen to generate superoxide anion, a reactive oxygen species (ROS) via NADPH-dependent reduction (Bus and Gibson, 1984). The accumulation of ROS creates an environment of oxidative stress (OS), where ROS can cause damage to lipids, proteins, and DNA (Lau et al., 2008). The induction of DNA damage may be through the generation of ROS in PQ-treated D. melanogaster. PQ is used extensively in the D. melanogaster model of OS (Legan et al., 2008; Strub et al., 2008). Superoxide dismutase (SOD) is one of the important parameters to assess antioxidation and oxidation levels related to ROS. It is an essential enzyme in a network of biological antioxidants, which is endogenously induced in order to eliminate superoxide radicals by converting superoxide radical into hydrogen peroxide and molecular oxygen (Barik et al., 2005).

This present study was therefore undertaken to evaluate the usefulness of D. melanogaster as an in vivo and in vitro model.
for assessment of PQ-induced neuropathological, behavioral, antioxidant activity and cytotoxic damage after sublethal chronic exposure. Our goal was to further validate the mechanisms involved in PQ-induced neuronal damage using D. melanogaster as a model. Additionally, we sought to determine if PQ-induced neuropathology is correlated with toxin-induced DNA damage in Drosophila brain cells. Furthermore, we also investigated the cytotoxic effect of PQ on the Drosophila embryonic cell line (D-Mel2) to confirm the association between the neuropathological and genotoxic changes.

MATERIALS AND METHODS

Chemicals. PQ; lactate dehydrogenase (LDH) assay kit was bought from Sigma-Aldrich Chemical Company. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), l-glutamate, antibiotics, Schneider’s Medium, EDTA, potassium dihydrogen, and monohydrogen phosphate were purchased from HiMedia 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 12:12h (Ravikumar and Muralidhara, 2009). 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 (GemiBio-Products, Inc., CA) and were grown in a refrigerated incubator at 25°C.

Drug preparation and treatment. PQ doses and treatment to the flies were done as described previously (Inamdar et al., 2012) with slight modifications. Five doses of PQ viz. 50, 100, 150, 200, and 500µM were prepared from 5mM/ml stock. Twenty flies from the stock culture were transferred to glass vials containing 50 µl of test food (sucrose + PQ) and incubated at 25°C for 7 days at different doses with a solution containing 0–500µM PQ.

Locomotor assay. Locomotor assay with negative geotaxis was determined as described previously (Le Bourg and Lints, 1992). Test flies were anesthetized and placed in a vertical glass column (standard length, 25 cm; diameter, 1.5 cm). After a brief recovery period, flies were gently tapped to bottom of the column. After 1 min, flies that reached the top of the column and flies that remained at the bottom were counted separately. Three trials were performed in each experiment at 1-min intervals. The scores are the mean of the number of flies at the top (ntop) and at the bottom (nbott), expressed as percentages of the total number of flies (nrot). Result was presented as mean ± SEM of the scores obtained in three independent experiments, which was calculated as 1/2[(nrot + ntop – nbott)/nrot] (Coulom and Birman, 2004).

Histological detection by hematoxylin and eosin staining. After 7 days of incubation, flies were anesthetized and fixed in 8% w/v paraformaldehyde for 8h. After fixation, flies were rinsed in PBS five times, each time for 10 min at room temperature. The tissues were dehydrated through an ethanol series to xylene. It was then kept in xylene + paraffin at 60°C in an oven. Tissues were finally embedded in paraffin wax and stored at 0°C for at least 24h. The sections were cut at 6µm thickness on a Yorco Rotary Microtome, and slides containing sections were collected on to the grids. The sections were cut at 0.5–1.0 µm 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 deparaffinized and washed with distilled water three times. Slides were then stained for 15 min 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., (1988) with few modifications in case of D. melanogaster. Briefly, slides were precoated with 1% normal melting agarose and allowed to set under coverslips on an ice-cooled chamber. After solidification, the slides were kept in lysis buffer solution for 2h at 4°C followed by unwinding for 30 min in alkaline electrophoretic running buffer (300mM NaOH + 1mM EDTA at pH 13) in electrophoretic tank. Then electrophoresis was performed for 35 min at 4°C with constant field strength of 0.74 V/cm and variable current strength under the range of 295–310 mA. Slides were drained and stained with 75 µl ethidium bromide (20 µg/ml) for 5 min in the dark. Finally slides were dipped in chilled distilled water, followed by placing coverslip on it, and stored in dark humidified chamber at 4°C prior to analysis. The slides were scored with the help of CXC41 fluorescent microscope (Olympus, Japan) coupled with an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, United Kingdom) attached with an integrated CC camera (Olympus, Japan) and an image analyzing system.

Histological detection by silver nitrate staining. Silver nitrate staining was done according to the method by Bielschowsky (1902) with slight modifications. Sections were deparaffinized and washed with distilled water three times. Slides were then kept in prewarmed 10% silver nitrate solution and stained for 15 min and again washed three times with distilled water. Slides were incubated in ammonium silver solution in oven at 40°C for 30 min. Then slides were directly placed in developer solution (20mL of formaldehyde, 0.5 gm citric acid, 50 µl nitric acid, and 100mL distilled water) for less than 1 min, followed by incubation in 1% ammonium hydroxide solution for 1 min, and then washed with distilled water three times. After washing, slides were placed in 5% sodium thiosulfate for 5 min and then again washed with distilled water three times. Slides were then dehydrated with ethanol series followed by xylene. Finally, the slides were mounted with resins medium and observed under Magnus Microscope (Model No-MLX-DX).

Histological preparation of D. melanogaster brain tissue for transmission electron microscopy. The whole head was explanated from control and treated D. melanogaster and were fixed for 4–6h in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH = 7.4). Then the tissue was kept in PBS (pH = 7.4) for 4°C for 2h or overnight. The secondary fixation of the tissue was done in 1% osmium tetroxide in distilled water for 1h 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 and then washed, followed by propylene oxide: resin (1:1 mixture) for 1–2h. The tissues were infiltrated with 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 µm 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, Reichet), 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.

SOD assay. Total SOD activity was measured in D. melanogaster after 7 days of PQ treatment. Extracts were prepared by homogenizing treated flies brain in 500 µl of ice-cold homogenizing buffer (210mM mannitol, 70mM sucrose, and 1mM EDTA). Homogenates were centrifuged at 800 x g for 10 min at 4°C to pellet solids. SOD activity was measured as described previously (Kovaceva et al., 2007). 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. One unit of SOD is defined as the amount of enzyme needed to clear 50% of the superoxide.

**Cytotoxicity assessment of PQ on Drosophila cells.** D-Mel2 cell lines were treated with different doses of PQ to assess the cell viability by MTT and LDH assay. Briefly, cells were seeded at a number of 2 × 10^5 per well onto 96-well plates (200 μl/well in triplicates, allowed to attach and grow for 24 h, and subsequently exposed to different PQ (100 μM to 1 mM) concentrations for 24, 48, and 72 h. At the end of the treatment, the medium was removed, and cells were incubated with 20 μl of MTT (5 mg/ml in PBS) in fresh medium for 4 h at 27°C. After 4 h, formazen crystals formed by mitochondrial reduction of MTT were solubilized in DMSO (150 μl/well), and the absorbance was read at 570 nm after 10-min incubation on the Multiskan EX microplate reader (Thermo scientific, Germany). Percent cell viability was calculated as a fraction of control (without PQ), and the cytotoxicity of PQ was expressed as IC_{50}. Cytotoxicity induced by PQ was also assessed by LDH leakage into the culture medium. Similar to the MTT assay, cells were seeded at a number of 2 × 10^5 per well onto 96-well plates (200 μl/well in triplicates, allowed to attach and grow for 24 h, and subsequently exposed to different PQ (100 μM to 1 mM) concentrations for 24, 48, and 72 h. At the end of the treatment, the 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. Percent inhibition of cytotoxicity was calculated as a fraction of control (without PQ), and the cytotoxicity of PQ was expressed as IC_{50}.

**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**

**Effect of PQ on Locomotor Activity of D. melanogaster**

To experimentally correlate the neurotoxic effect of PQ and *D. melanogaster* locomotory activity, behavioral assay was performed. Data obtained in the negative geotaxis assay revealed dose-dependent locomotor dysfunction in flies exposed to PQ for 7 days. In control groups, approximately 90% of the flies reached the top of the column in 1 min, whereas PQ-exposed flies group exhibited significant decrease in climbing ability. At higher doses of PQ, flies preferred to stay at the bottom and did not appear to coordinate their legs normally. The increasing dose of PQ clearly suggested the induction of locomotor deficits (Fig. 1). Different anomalous symptoms were also observed in flies such as abnormal movement in climbing, falling backward, and trembling on the floor. Results obtained in the study were significant at p < 0.05, suggesting a significant toxicity of PQ on locomotion behavior. Results indicated dose-dependent loss in the locomotory activity. As shown in Table 1, the mean value of the flies that remain at the top at 500 μM is low in comparison with control, whereas in the bottom the values are high in respect to control.

**Drosophila melanogaster Nervous Tissue Treated With Different Doses of PQ Revealed Extensive Neuropathological and Ultrastructural Changes in Neurons**

Hematoxylin and eosin (HE) staining of the sections of the *D. melanogaster* nervous tissue treated with different doses of PQ showed various degrees of neurological damage compared with the control. Neuronal damage was indicated by the appearance of lesions in the neuropile where most of the neuron cells reside. Significant numbers of neuronal lesions were observed in the neuropile area of PQ-treated flies compared with untreated *D. melanogaster* (Fig. 2). Furthermore, overall brain structure was preserved in control flies, which included outer chiasma (OC), inner medial protocerebrum, lamina (LA), and suboesophageal commissure. Treated brain sections showed moderate neuropathology including vacuole-like lesions scattered in the neuropile, ventrolateral protocerebrum, the areas that have most dopaminergic innervations similar to those found in humans (Inamdar et al., 2012), and medulla.

**FIG. 1.** Negative geotaxis assay of adult flies exposed to different PQ concentrations. Dose-dependent increase in negative geotaxis was observed compared with control. * indicates the significant value (p < 0.05) of negative geotaxis compared with the control and all the other groups.
(ME) regions located between inner chiasma and OC (yellow arrow). The pathology observed in PQ-treated flies was found to be more extensive and pronounced than that seen in control (Fig. 2).

Similarly, silver-stained horizontal sections of *D. melanogaster* brain tissue treated with different doses of PQ showed varying degrees of neuropathological changes in brain morphology. Brain morphology with dark stained area, especially lobula, ME, LA, and retina, was clearly visible, and their nerve fibers and neuropile area were not reduced in terms of thickness and size in untreated *D. melanogaster* section. However, in treated sections, the structural integrity was disrupted, and the optic lobe area was vacuolated (Fig. 3). There was a marked disruption of structure in the ME and LA regions at 200 and 500µM doses (Figs. 3E and 3F). Vacuolar lesions and reduction in thickness of the structure of ME and LA were observed at all doses, respectively. Extensive neurotoxic pathology in the neuronal cell bodies of the lobular and ME region was observed in PQ-treated flies compared with untreated. The protocerebral bridge, a component of the central body complex, exhibited change in the structural integrity, which resulted in the increment of neuronal degeneration in the brain region. Transmission electron microscopic studies also revealed that PQ exposure to *Drosophila* brain resulted in changes in neuronal mitochondria, including swelling and partial loss of cristae (Fig. 4, arrows indicate changes in mitochondria). In addition, partial chromatin dissolution and dispersed arrangement of the rough endoplasmic reticulum were observed in PQ-treated flies. At higher doses, the flies exposed to PQ had severely damaged mitochondria with loss of nearly all cristae. Transmission electron microscopic study of *D. melanogaster* brain tissue also exhibited an overall vacuolar degeneration and abrupt changes in the whole tissue structure compared with the untreated flies. PQ caused irregularity of shape with disintegration in all cellular structure, which is clearly visible in all nervous tissues of *D. melanogaster* treated with different doses (Fig. 4). In addition, dispersed arrangement of the rough endoplasmic reticulum was observed.

### Exposure of PQ Resulted in DNA Damage in *D. melanogaster* Brain Tissue

To evaluate whether the neuropathological damage caused by neurotoxic doses of PQ in *D. melanogaster* brain induce DNA damage, we performed Comet assay to quantify the total DNA damage in *D. melanogaster* brain tissue after PQ exposure. *Drosophila melanogaster* exposed to different doses of PQ showed a dose-dependent increase in DNA damage in nervous tissue as evident by a statistically significant increase in comet parameter, i.e., tail length. A significant increase in \( p < 0.0001 \) in the comet tail length was observed in the neuronal tissue of PQ-exposed flies compared with the respective control. PQ treatment resulted in increases in comet tail length by 53.8, 108.43, 175.27, 242.71, and 522.81% at 50, 100, 150, 200, and 500µM, respectively, in the brain tissue of *D. melanogaster*. Flies exposed to different doses exhibited significant increase in comet parameters in comparison with control (Table 2). This increase in comet tail length in the neuronal tissue is a clear indicator for genotoxic effect of PQ, suggesting significant damage to the integrity of DNA content in the brain tissue of PQ-treated *D. melanogaster* (Fig. 5).

### PQ Exposure Results in the Decreased SOD Measurement

SOD specific activity was assayed in the brain samples of flies treated with various concentration of PQ. From the result, it is evident that the decreased SOD specific activity is associated with the increased PQ doses. The SOD specific activity was reduced to 67.40% at 200µM of PQ concentration. Following the same pattern, 500µM of PQ was able to show the most decreased activity by 80.76%. These results clearly suggest the PQ-affected SOD activity in a dose-dependent manner as demonstrated in Figure 6.

### Cytotoxic Effect of PQ on *Drosophila* D-Mel2 Cells

In addition to *in vivo* effects of PQ treatment in *D. melanogaster* model, we also sought to investigate if PQ-exposure-induced cytotoxic effect has growth inhibitory properties *in vitro* in a
D-Mel2 cell line. Exposure of PQ resulted in inhibitory effects on cellular growth. Figure 7A shows the percent cell viability after 24, 48, and 72 h of incubation in a medium containing 100μm–1mM of PQ. D-Mel2 cells exhibited potent sensitivity to PQ at different doses. PQ has cytotoxic effects in vitro at applied doses (IC₅₀ values ≤ 200 and 100μM) by MTT method after 24 and 48 h, respectively. Similar kinds of results were also observed in LDH assay. PQ exposure resulted in potent cytotoxic activity against D-Mel2 cells in a dose-dependent manner with an IC₅₀ values ≤ 500μM and ≤ 200μM after 24 and 48 h, respectively. Figure 7B shows the percent cytotoxic effect of PQ on the D-Mel2 cells by LDH assay. The inhibition was demonstrated at four escalating doses of PQ in a range of 100μM–1mM. Results indicated both time- and dose-dependent loss of cell viability and cytotoxicity in the treated cell line, respectively (Fig. 7).

**DISCUSSION**

PQ, which is widely used as a cationic nonselective bipyridyl herbicide to control weeds and grasses in many agricultural...
areas, has emerged as a putative risk factor on the basis of its structural homology to 1-methyl-4-phenyl-pyridine, the active metabolite of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine, a neurotoxicant that induces Parkinson’s-like features in rodents, nonhuman primates, and humans (Langston and Ballard, 1984; Tanner and Ben-Shlomo, 1999). There is considerable evidence that PQ may cause the onset or accelerate the development of Parkinson’s disease (Hatcher et al., 2008). The evidence showed that the longer exposure extends the risk of development of the disease that may depend upon the time between exposure and development of symptoms. Earlier studies have shown that PQ produces symptoms of neurological disturbance such as decreased motor activity, lack of coordination, ataxia, and dragging of the hind limbs in rats at high doses (IPCS, 1984). Loss of motor coordination is a key symptom of Parkinson’s disease that is normally caused by selective loss of the dopaminergic neurons of the substantia nigra. In this present study, it was investigated that the exposure of *D. melanogaster* to subchronic doses of PQ exhibits the development of main features of neurodegeneration, inducing locomotion deficits. The locomotor deficits in the treated flies clearly indicated the toxic effects on the nervous system of *D. melanogaster* in comparison with the normal flies. Treated flies exhibited different traits of behavior and abnormal movement at different doses of PQ. Flies showed abnormality in movement at high doses of both pesticides as shown in Figure 1.

Although there is an uncertainty in the histopathological aspects of neurodegenerative disease mechanisms, yet it is well accepted that abruption in the pathology of the neuronal cells is one of the vital factors associated with these disorders. Bagetta et al. (1992) reported neuropathological and behavioral

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**FIG. 3.** Horizontal section (representative of at least 8–10 flies) of silver stained *D. melanogaster* brain shows different regions affected by different doses of PQ. (A) It represents control flies where no pathology or vacuolar lesions was observed. (B–F) represents nervous tissues of flies after 7 days of PQ exposure resulting in little or moderate pathology. βL, beta lobe; αL, alpha lobe. Scale bar: 1 μm.
Paraquat-induced neurotoxicity in a fly model

Coulom and Birman (2004) stated that chronic exposure of D. melanogaster to sublethal doses of rotenone recapitulates the main symptomatic feature of Parkinson’s disease: a selective loss of dopaminergic neurons inducing locomotor deficits. In this present study, we have observed several neuropathological differences in the PQ-treated D. melanogaster brain sections. Loss of neurons appeared as vacuolar lesions, which were apparent in the sections of PQ-treated flies. Disruption of cellular bodies resulted in neuronal dysfunction, which resulted in neurodegeneration in the treated flies and associated with disease conditions. This neurodegeneration in the exposed flies appears to be result of exposure to these PQ, paralleling the neuropathological onset of many progressive human-degenerative conditions. In PQ-treated sections, vacuoles are more distinct. Extensive neuropathology including loss of cellular structure is recorded in the neuropile, protocerebrum, area rich in dopaminergic innervations similar to those seen in humans and in ME regions.

Similar vacuolar neuropathy has been observed in several other characterized neurodegeneration mutants identified in D. melanogaster and is a typical manifestation of neurodegeneration in flies and mammals (Buchanan and Benzer, 1993; Kretzschmar et al., 1997; Min and Benzer, 1997; Palladino et al., 2002). According to Mitra et al. (2011), microglial-specific silver staining determines activation of microglial cells, as a primary event of neuroinflammation in mouse brain after PQ treatment. Fix et al. (1996) used silver-staining process to assess the morphologic integrity of central nervous system of rat and concluded that silver staining selectively impregnates degenerating neurons. Michael et al. (2002) reported that aluminum-treated brain tissue of rabbit offered a striking contrast with numerous pyramidal cell bodies because of prominent silver-stained profile. We also performed silver staining on the Drosophila brain tissue treated with the different concentration of PQ to observe the effect of PQ treatment on the neuronal

**TABLE 2**

Average Comet Length in D. melanogaster Brain Cells Treated With Different Doses of PQ

<table>
<thead>
<tr>
<th>Dose</th>
<th>Mean ± SE</th>
<th>Lower</th>
<th>Upper</th>
<th>F</th>
<th>R²</th>
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<tr>
<td>Control</td>
<td>2.063 ± 0.012</td>
<td>2.012</td>
<td>2.115</td>
<td>2619***; p &lt; 0.0001</td>
<td>0.9991*</td>
</tr>
<tr>
<td>50µM</td>
<td>3.177 ± 0.034</td>
<td>3.027</td>
<td>3.326</td>
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<td></td>
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<tr>
<td>100µM</td>
<td>4.300 ± 0.020</td>
<td>4.210</td>
<td>4.390</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150µM</td>
<td>5.670 ± 0.070</td>
<td>5.365</td>
<td>5.975</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200µM</td>
<td>7.060 ± 0.148</td>
<td>6.423</td>
<td>7.697</td>
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<tr>
<td>500µM</td>
<td>12.83 ± 0.072</td>
<td>12.52</td>
<td>13.14</td>
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Note. All the data have been represented in mean ± SEM of three independent experiments.

***Significant at p < 0.0001 from the control.

**FIG. 4.** (A) Ultrastructure of untreated brain tissue of D. melanogaster. Black arrow points correspond to cellular structure and a yellow arrow indicates the nerve fibers (NF). These structures appeared virtually identical in appearance. Cytoplasm is electron dense. Neuronal nuclei (Nu) were euchromatic with a regular or oval shape. (B–D) Brain tissue of D. melanogaster treated with different dose of PQ, i.e., 100, 200, and 500µM. Treated ultrastructure displayed irregularity of shape with disintegration in all cellular structure and nerve fibers denoted by yellow arrow in all treated sections. This disruption is prominent in all sections. Vacuolar lesions are also prominent in all treated sections displayed by bold brown arrow.
connections, neurofibrils, and neuronal bodies *D. melanogaster* brain tissue. We observed significant morphological changes in the structural integrity. Loss of neuronal network and cell body disintegration were recorded at different doses of PQ. Structural changes in the brain tissue marked changes in the neuron proliferation. In PQ-treated sections, the structural integrity was disrupted and vacuolated. Disruptions of structures were observed at high doses of PQ. Reduction in thickness of the structures in ME and LA was recorded at all doses of PQ. Our data suggest that PQ exposure resulted in marked damage in the morphology of the *D. melanogaster* brain. Transmission electron microscopic studies also strengthen the observations of HE and silver-staining morphological assessment of the brain tissues in PQ-treated *D. melanogaster*.

In the recent past, *D. melanogaster* system has been widely used as a model for environmental toxin-induced PD model (Feany and Bender, 2000). Several researches have indicated that the accumulation of the free radicals is closely associated with the neurodegeneration in specific brain regions has been proposed as causal factors in PD (Halliwel, 2006). This accumulation of oxidative free radicals and DNA damage might be due to the decreased activities of free radical defense enzymes such as SOD. In this study, we have measured the SOD specific activity in PQ-treated *D. melanogaster* and observed that decreased SOD specific activity is associated with the increased PQ doses. The lowest SOD specific activity was observed at highest concentration of the PQ (Fig. 6). Further, we thought to understand if the decreased SOD specific activity is associated with the DNA damage in PQ-treated flies and found the significant increase in the comet tail length in PQ-treated *D. melanogaster* along with the decreased SOD specific activity, which suggests significant correlation of increased OS to oxidative DNA damage *in vivo* (Figs. 5 and 6).

This present study is probably the first of its kind, which shows a strong correlation between PQ-induced DNA damage in the neuronal system of *D. melanogaster* and the neuronal damage in *D. melanogaster* brain tissue (Figs. 2, 3 and 5). Our study also reports significant correlation between the dose-dependent...

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**FIG. 5.** Figure shows the bars of average tail length of *D. melanogaster* brain cells against different doses of PQ. Representative comet picture of *D. melanogaster* brain cells treated with different doses of PQ under photoillumination are shown below the graph. All the data have been represented as mean ± SEM of three independent experiments. *Significant at $p \leq 0.0001$ from the control, **,***, *, *Significant at $p \leq 0.0001$ from the control and all other groups.
increase in cytotoxicity level in D-Mel2 cells and DNA damage in the brain tissue of *D. melanogaster* (Figs. 5 and 7). DNA damage activates cellular signaling pathways that may ultimately lead to cell death if the damage encountered is too great or not repaired. Cytotoxic potential of any pesticide is necessary to study in order to relate the DNA damage caused by the pesticide. Cell lines provide a useful tool to investigate the molecular mode of action of insecticides. Our study attempted to use *Drosophila* cell line to determine the cytotoxic effect of PQ where we observed a significant dose-dependent cytotoxic effect of PQ. Total PQ-induced cytotoxicity was also increased in a time- and dose-dependent manner. Our findings suggest that PQ is a potent cytotoxic compound giving higher potential to deteriorate the integrity of nuclear DNA in the tissue of treated *D. melanogaster*.

Our studies suggest that sublethal exposure of PQ to *D. melanogaster* resulted in extensive neurotoxic damage altering the neuronal morphology and resulting in neuronal

**FIG. 6.** Activity of antioxidant enzyme SOD in the brain tissue of *D. melanogaster* after 7 days of PQ treatment. * indicates the significant value (*p* < 0.05) of negative geotaxis compared with the control and all the other groups.

**FIG. 7.** Comparison of dose-dependent effect of PQ on D-Mel2 cell proliferation monitored by (A) MTT assay (B) LDH assay at different time points. The percent viability and toxicity was calculated in comparison with untreated cells taken as 100%. Values were expressed as mean ± SD, and the experiment was performed in triplicate (*p* < 0.05).
damage at cellular and organelle levels. This PQ-induced neuronal damage was associated with increased damage to neuronal DNA. Finally, our study confirms the cytotoxic nature of PQ-induced neurotoxicity.

FUNDING

The authors would like to acknowledge the University Grants Commission (India) for providing the financial assistance (Grant number: 39-631/2010 [SR]).

ACKNOWLEDGMENTS

The authors wish to thank Prof Iqbal Perwez, Department of Zoology, AMU, for his invaluable help with microscopy. The authors are highly thankful to the Sophisticated Analytical Instrument Facility for Electron Microscopy, Department of Anatomy, AIIMS, New Delhi, India, to carry out the transmission electron microscopy for this present study.

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