DISCUSSION
Poisoning by acute high-level exposure to certain pesticides has well known neurotoxic effects, but whether chronic exposure to moderate levels of pesticides is also neurotoxic is more controversial. Most studies of moderate pesticide exposure have found increased prevalence of neurologic symptoms and changes in neurobehavioral performance (Kamel, 2004). The best documented health effects due to pesticide exposure involve the dysfunction of nervous system which leads to ataxia, tremor, movement disorder, cognitive and memory loss. Exposure of pesticide is associated with range of symptoms including abnormalities in nerve function and deficits in performance of neurobehavioral activities (Keifer and Mahurin, 1977). Several studies have shown the possibility, of environmental toxins related development of neurological disorders (Norris et al., 2004; Landigran et al., 2005). In this present work, the neurotoxic effect of malathion and paraquat at different level of exposure are described, and the relationship of pesticide exposure to neurological disease is also discussed.

The effect of paraquat in inducing Parkinson’s disease or its symptoms is enhanced by synergistic interaction with the fungicide maneb even at low doses in animal studies (Thiruchelvum et al., 2000a, 2000b). Several animal based investigations have linked adult onset of Parkinson’s disease after neonatal exposure to paraquat. Neonatal exposure to paraquat even at low doses can induce permanent changes in brain function along with neuro-chemical and behavioral changes in the adult mouse, including reduced dopamine level (Fredriksson et al., 1993). Paraquat has not only been capable of crossing the blood brain barrier, but also resulted in adverse effect on the dopaminergic nigrostriatal system (Widdowson et al., 1996b and Corrasaniti et al., 1998).
In this study, we show that chronic exposure of *Drosophila melanogaster* to sub-lethal dose of paraquat and malathion, recapitulates the main neurodegenerative disease symptoms: loss of cellular structures, deficits in locomotor ability and changes in the structural integrity of the brain tissues. As shown in this work, paraquat and malathion treatment showed toxicity to *D. melanogaster* both *in vivo* and *in vitro*. The nervous system is very sensitive to the toxicity of these two pesticides. *D. melanogaster* is widely used as a model to understand the physiology of several neurodegenerative diseases. Several studies have shown that environmental toxin such as rotenone, maneb, paraquat and malathion induces neurological disease-like symptoms in experimental animals (Betarbet, 2002).

### 5.1 LOCOMOTION DYSFUNCTION DUE TO PESTICIDE TREATMENT:

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. Several studies have shown the effect of pesticide on *D. melanogaster* nervous system that mimic the Parkinson’s-like motor dysfunction in human. In *Drosophila*, the overexpression of WT and mutated (A30P, A53T) human α-synuclein causes the age-dependent loss of dorso-medial dopaminergic neurons, an accumulation of Lewy bodies (LB) like filamentous inclusions with α-synuclein immunoreactivity, and compromised locomotor activity such as (climbing ability) (Feany & Bender, 2000). Paraquat has been shown to cause dose-dependent loss of dopamine neurons, degeneration of the nigrostriatal dopamine system, aggregation of α-synuclein, formation of Lewy bodies, and decreased or altered locomotor activity (e.g. Liou *et al.*, 1996; Brooks *et al.*, 1999; Choi *et al.*, 2010; Songin *et al.*, 2011). Similarly, chronic exposure of *Drosophila* to sub-lethal doses of rotenone recapitulates the main
symptomatic feature of Parkinson’s disease: a selective loss of dopaminergic neurons inducing locomotor deficits. According to Betarbet et al., (2000) and Di Monte (2003) rotenone and other complex-I inhibitors are used as pesticides or herbicides which may contribute to the appearance of sporadic Parkinson’s disease in humans. Malathion, used as pesticide, is an environmental factor that may contribute to the appearance of Parkinson’s disease in humans. Studies have also shown that the Drosophila dopaminergic system is also involved in locomotor control (Yellman et al., 1997; Lima and Miesenboch, 2005) although the details of the underlying neural circuit(s) are unknown. It is therefore reasonable to assume that loss of dopaminergic neurons can affect locomotion in Drosophila comparable to the situation in PD.

In the present work, we show that exposure of sub lethal doses of paraquat and malathion on D.melanogaster resulted in the development of main features of neurodegeneration, inducing locomotion deficits. Locomotor dysfunction is the primary assayed behavior with respect to neurodegeneration. The locomotor deficits in the treated flies clearly indicated the toxic effects on the nervous system of D. melanogaster in comparison to the normal flies. Treated flies exhibited different traits of behavior and abnormal movement at different doses of paraquat and malathion. Flies showed abnormality in movement at high doses of both pesticides as shown in Fig 4.1 and Fig 4.2. At higher dose (500μM) frequency of the number of flies that remained at the bottom increases whereas in case of lower doses number varied depending on the extent of neuropathology as shown in the result section Fig 4.1 and Fig 4.2 . Indeed, loss of subsets of dopaminergic neurons in the brain as well as locomotion defects are the two principal parkinsonian-like phenotypes used to characterize fly models of Parkinson’s disease (Feany and Bender, 2000; Auluck et al., 2002; Yang et al., 2003; Haywood and Staveley, 2004 and 2006; Periquet et al.,
2007; Botella et al., 2008; Todd and Staveley, 2008; Seugnet et al., 2009; Wang D et al., 2006; Yang et al., 2008; Wang C et al., 2007; Sang et al., 2007; Lee et al., 2007; Liu et al., 2008; Imai et al., 2008; Ng Ch et al., 2009; Venderova et al., 2009).

Another study which substantiated present study revealed that after seven days of rotenone exposure flies presented major locomotor defects. After the rotenone treatment flies did not appear to co-ordinate their legs normally (Coulom and Birman, 2004).

5.2 HISTOLOGICAL ABNORMALITIES DUE TO PESTICIDE EXPOSURE:

Although there is an uncertainty in the histopathological aspects of neurodegenerative disease mechanisms, yet it is well accepted that neuropathology are vital factors associated with these disorders. In 1992, Bagetta et al., reported neuropathological and behavioral effects induced in rats by paraquat injections via systemic and intrahippocampal routes. However, studies have also shown that single dose of malathion (88% pure) up to 2,000 mg/kg caused no neuropathologic lesions in segments of the medulla, cervical and lumbar spinal cord, and branches of the tibial nerve, and cerebellum from rats sacrificed 21 days after dosing (Ehrich et al., 1993). Coulom and Birman (2004) stated that chronic exposure of Drosophila to sublethal doses of rotenone recapitulates the main symptomatic feature of Parkinson’s disease: a selective loss of dopaminergic neurons inducing locomotor deficits.

In the present study, we observed several histopathological differences in the treated and non-treated D. melanogaster brain sections. Loss of neurons appeared as vacuolar lesions which was apparent in the sections of paraquat and malathion 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 these flies appears to be result of exposure to these pesticides, paralleling the neuropathological onset of many progressive human degenerative conditions. At different doses of malathion, a widespread neuropathology including vacuolar like lesions were recorded. At 1mM dose of malathion the cellular disruption in the sub esophageal commisure is more extensive. Vacuoles are distinctly visible in all malathion treated sections. In paraquat treated sections vacuoles are more distinct in comparison to malathion treated sections. Extensive neuropathology including loss of cellular structure is recorded in the neuropile, protocerebrum and medulla regions. In connection with the above mentioned result some studies revealed that single or repeated brief seizure could produce neuronal death in flies (Sutula, 2003; Cendes, 2005). It was reported that distinct metabolic changes can result both in increased seizure susceptibility and neurodegeneration (Kunz, 2002) and impaired neuronal viability may be independent of actual seizures. Similar vacuolar neuropathology has been observed in several other characterized neurodegeneration mutants identified in Drosophila and is a typical manifestation of neurodegeneration in both flies and mammals (Buchanan and Benzer, 1993; Kretzschmar et al., 1997; Min and Benzer, 1997; Palladino et al., 2002). However, brain congestion, neuronal degeneration, and gliosis were seen in the brain of rats during the first few days after administration of a single dose of 1,950 mg/kg of malathion (Piramanayagam et al., 1996). Burgees et al. (1999) observed that a organophosphate insecticide reduced cholinesterase activity in birds. Taylor et al. (1999) stated that a sublethal dose of field grade malathion (0.01 mg/g) lowered brain cholinesterase levels by 22% and 17%, respectively. Parson et al. (2000) observed effect of organophosphate and carbamate on non-target wild animals and these pesticides inhibited cholinesterase activity.
In the above mentioned study, silver stained sections of brain tissue were visualized to study the neuronal connections, neurofibrils and neuronal bodies. After the exposure of pesticide on *D.melanogaster* brain tissue we observed distinguish morphological changes in the structural integrity. Loss of neuronal network and cell body disintegration were recorded at different doses of pesticide. Structural changes in the brain tissue marked changes in the neuron proliferation. In paraquat treated sections the structural integrity is disrupted and vacuolated. Disruption of structures in medullar and laminar region were observed at high doses of paraquat treated sections. Reduction in thickness of the structure of medulla and lamina were recorded at all doses of paraquat. The silver stained sections of *D. melanogaster* brain tissue treated with different doses of malathion showed various degree of neuropathological damage. In treated sections the structural integrity is truly disrupted and the optic lobe area is vacuolated. From the result it is evident that paraquat and malathion both exhibits marked damaged in the brain morphology of the *D. melanogaster*. According to Mitra, (2011) microglial specific silver staining determines activation of microglial cells, as a primary event of neuroinflation in mouse brain after paraquat treatment. Microscopical observation revealed numerous aggregations of microglial cells in CNS, compared to controls, and also changes in microglial morphology. Fix *et al.*, (1996) used silver staining process to assess the morphologic integrity of central nervous system of rat and concluded that silver stain selectively impregnates degenerating neurons. Siddiqui *et al.*, (2000) demonstrated monoclonal antibodies pattern with specific staining characteristics in the olfactory system of *D. melanogaster* by Bodian silver method. Michael, (2002) reported that aluminum treated brain tissue of rabbit offered a striking contrast with numerous pyramidal cell bodies because of prominent silver stained profile.
Electron microscopic study of *D. melanogaster* brain tissue exhibits vacuolar degeneration, and abrupt changes in the whole tissue structure in comparison to control in the present work. Both paraquat and malathion exhibit irregularity of shape with disintegration in all cellular structure. This abrupt change is clearly visible in all nervous tissue of *D. melanogaster* treated with different doses of paraquat and malathion. Some transmission electron microscopic, studies revealed that lead exposure to rat brain resulted in changes in neuronal mitochondria, including swelling and partial loss of cristae. In addition, partial chromatin dissolution and dispersed arrangement of the rough endoplasmic reticulum were observed in the animals exposed to lead. In contrast, the animals exposed to cadmium had severely damaged mitochondria with loss of nearly all cristae, as well as plasma membrane disorganization and Nissl body dissolution of endoplasmic reticulum were observed in the animals exposed to lead (Zhang et al., 2009).

5.3 DNA DAMAGE IN BRAIN TISSUE OF *D. melanogaster* DUE TO PESTICIDE EXPOSURE:

Pesticides are used in agriculture to protect crops but at the same time pose a potential risk to farmers and environment. Atherton *et al.* (2006) have demonstrated the ability of OP insecticides to directly damage DNA in freshly isolated human lymphocytes *in vitro* utilizing the comet assay. Rahman *et al.* (2002), in contrast to other reports, concluded that chlorpyrifos was able to cause DNA damage in mice leucocytes in a dose dependent manner, with DNA damage measured using the alkaline comet assay. Salazar-Arredondo *et al.* (2008) evaluated sperm DNA damage by several OPs including chlorpyrifos and chlorpyrifos oxon in human spermatozoa from healthy volunteers incubated with 50–750μM chlorpyrifos and chlorpyrifos-oxon. The study also noted that the DNA was repaired 48hrs after exposure. Gupta *et
al. (2010) investigated apoptosis and DNA damage inducing potential of chlorpyrifos in *D. melanogaster*. Third instar larvae of *Drosophila* were treated with different concentrations of chlorpyrifos (0.015–15.0 μg/L) for 2–48 h. Reactive oxygen species (ROS) generation, oxidative stress markers, DNA damage and apoptotic cell death end points was measured in them. A significant increase in DNA damage was associated with apoptotic mode of cell death in 15.0 μg/L Chlorpyrifos-treated organisms for 24 and 48 h. The study suggested that ROS might be involved in inducing apoptosis and DNA damage in larvae of *Drosophila* after exposure to chlorpyrifos. Studies have shown that paraquat also induces DNA damage by ROS (Ali et al., 1996). Furthermore, Yang et al. (2008) reported that paraquat produces apoptosis in human neurobasatoma SH-SY5Y cells through the mitochondrial intrinsic pathway associated with p53. Previous studies also focus on the genotoxic effects of malathion (Giri et al., 2002). Organophosphorus compounds show alkylating properties (Garrett et al., 1990; Wild, 1975) and the methyl esters have a higher alkylating potential than the ethyl esters (Garrett et al., 1990). Alkylating agents are known to cause DNA damage (Ferguson and Denny, 1995). These studies suggest that alkylating properties of malathion is supposed to induce DNA Damage.

The present study is aimed to investigate the potential of paraquat and malathion to induce DNA damage in the neuronal system of *D. melanogaster* using alkaline Comet assay. Paraquat treatment caused an increase in comet tail length by 53.8%, 108.43%, 175.27%, 242.71% and 522.81% at 50μM/ml, 100μM /ml, 150μM/ml, 200μM/ml and 500μM/ml respectively in the brain tissue of *D. melanogaster*. Whereas in malathion treatment comet tail length increased by 72.98%, 113.71%, 161.99%, 254.61%, 538.00%, and 657.01% at 50μM/ml, 100μM /ml, 150μM/ml, 200μM/ml, 500μM/ml and 1mM/ml respectively. This study revealed a
Discussion

dose dependent increase in genotoxicity level and DNA damage in the brain tissue of
*D.melanogaster*. This result also indicates that malathion is more potent genotoxic
compound in comparison to paraquat thus giving malathion higher potential to
deteriorate the integrity of nuclear DNA in the tissue of treated *D.melanogaster* in
comparison to malathion.

Along with DNA damage Gupta *et al.*, (2004) also reported apoptotic mode of
cell death in the *D. melanogaster*. A few in-vitro studies showed an apoptotic mode of
cell death in cisplatin exposed human monocyte cell line U937 (NAkadai *et al*., 2006)
and in human placental choriocarcinoma cells (Saulsbury *et al*., 2008).

Several studies on the effect of pesticides in different fish species have been
carried out using different genotoxicoty tools (Hai *et al*., 1997; Das & John, 1999;
Pena- Llopis *et al*., 2003). However, genotoxicity studies of pesticides on various
indigenous fish species of India are very limited. Banu *et al*. (2001) studied the
genotoxic effects of monocrotophos, one of the popular organophosphate pesticides
on the fish *Tilapia mossambica* using comet assay and found a dose-related increase
and time-related decrease of comet tail length. Pandey *et al*., (2006) evaluated the
genotoxic potential of Endosulphan in *Channa punctatus*. They exposed fish to
different doses of pesticides and assessed DNA damage in gill and kidney tissues by
comet assay. Zineb, a carbamate fungicide, has been reported to be mutagenic in both
somatic and germ-line cells in *Drosophila* (Tripathy *et al*., 1988). In another report,
the same research group has reported that the fungicide ziram is mutagenic in the
wing, eye and female germ-line mosaic assays, and in sex linked recessive lethal test
in *D.melanogaster* (Tripathy *et al*., 1989). In another study, Franekic *et al*., (1994)
reported that ziram, zineb and thiaram are mutagenic in a battery of bacterial test
systems. The thiocarbamate pesticide malinate and vernolate have been reported to

67
cause chromosomal changes like SCE and chromosomal aberrations in vitro and increased frequency of polychromatic erythrocytes in mouse bone marrow cells (Pinter et al., 1989). Studies on the genotoxicity of aldicarb, aldicarbonsulfone, aldicarb oxide, carbofuran and propoxur, reported that all these pesticides were ‘suspect genotoxic’ after S9-activation in mutatox test (Canna-Michaelidou & Nicolaou, 1996). Genotoxicity of carbofuran, carbosulfan and methyl isothiocyanate, a component of the pesticide carbaryl, has also been reported (Chauhan et al., 2000; Rencuzogullari and Topaktas, 2000; Kassie et al., 2001). Gupta et al., 2010 reported cisplatin-induced DNA damage in Drosophila larval midgut tissues, as evidenced by a significant increase in the Comet parameters such as tail length (mm), TM(arbitrary units) and tail DNA(%) in the exposed organisms.

We used D. melanogaster as a model in this study. During the last decade, the use of model organisms, especially lower eukaryotes like Caenorhabditis elegans and Drosophila generated much interest after the unraveling of the genome sequences of the organisms (Gupta et al., 2010). Previous studies have shown that flies showed dose-response to cytotoxic, genotoxic assessment (Hirsch et al., 2003; Siddique et al., 2005b). Thus, these laboratory based experimental evidences using D. melanogaster are useful in generating information that could be of value for their efficient extrapolation to higher mammals.

5.4 CYTOTOXICITY ASSESSMENT IN D-Mel-2 (S2) DUE TO PESTICIDE EXPOSURE:

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. However, sometimes DNA damage is also observed even at non-
cytotoxic doses, suggesting that pesticides are able to induce DNA damage independent of cytotoxicity. Chemically induced cell injury, cytotoxicity, may be initiated by the formation of a stable complex with an enzyme, receptor site, or via the formation of highly chemically reactive species (electrophiles, free radicals, carbenes, nitrenes), or by provoking physicochemical changes (e.g., pH, redox, ionic composition) (Bridges et al., 1983). Studies have shown the toxicological effect of chlorpyrifos and neem extract (Biosal B) against 3rd instars larvae of *D. melanogaster* (Anjum et al., 2010). Obeidat, (2008) also studied the toxicity of *Bacillus thuringiensis* isolates in *Drosophila*.

Cell lines provide a useful tool to investigate the molecular mode of action of insecticides. The present study attempted to use *Drosophila* cell line to determine the cytotoxic effect of paraquat and malathion which are the major environmental toxin used in the present era. In the present study these two environmental toxin inhibit cellular proliferation. Paraquat has cytotoxic effects *in vitro* at the applied doses (IC50 values ≤ 200 µM and 100µM) as measured by MTT method after 24h and 48h. After three different hours of exposure, paraquat induced dose dependent cytotoxic effects in D-Mel-2 (S2) cell with IC50 values ≤ 55.5±4.1 after 24h and IC50 values ≤ 46.4±4.9 after 48h respectively. Total cells viability of D-Mel-2 (S2) dropped to 29.3%, 18.05 and 11.4% level due to the acute toxicity of 1mM/ml of paraquat after 24h, 48h and 72h respectively. Whereas in malathion exposure total cell viability dropped to 44.05%, 20.7% and 13.4% level due to the acute toxicity of 1mM/ml of malathion after 24h, 48h and 72h respectively. This result indicates that paraquat exhibits more lethal effects on the viability of the cells. Total cytotoxicity of paraquat was assessed by LDH method where cytotoxicity of cell increased to 61.24%, 93.84% and 93.08% level due to acute toxicity of paraquat after 24h, 48h and 72h. Whereas in malathion
exposure cytotoxicity of cells increased to 61.55%, 92.85% and 96.85% level after 24h, 48h and 72h respectively. In terms of sensitivity D-Mel-2 (S2) cells indicated low level of cytotoxicity (approx 22.51%) upon treatment with 100µM paraquat at 24h, whereas in malathion low level of cytotoxicity (approx 18.89%) recorded after 24h of treatment. These results indicate that malathion has more potential at cytotoxic level in comparison to paraquat. The difference in the result of paraquat and malathion cell viability and cytotoxic tests may be due to their difference in mechanism of cytotoxicity, although further investigations are warranted for its confirmation. In a related study treatment by tebufenozide, another diaclyhydrazine, leads to the same effects in two other lepidoptera cell lines, IAL-PID2 from *Plodia interpunctella* (Auzoux-Bordenave *et al.*, 2005) and Se4 from *Spodoptera exigua* (Decomber *et al.*, 2005). Methoprene and another analog of JH, fenoxycarb, significantly inhibit cell proliferation of the IAL-PID2 cell line (Oberlander *et al.*, 2000). Auzoux- Bordenave *et al.*, (2005) reported that tebufenozide arrested the cell cycle in G2/M in the *Spodoptera exigua* cell line. Several studies have examined the effects of hormones on cell lines (Gauhar *et al.*, 2009; Wills *et al.*, 2010). In addition, Mosallanejad *et al.*, (2010) reported microarray data on *Drosophila* cell line S2 resistant to methoxyfenozide. According to Zhang *et al.*, (2011 ii) there are differences in the sensitivity to camptothecin and hydroxycamptothecin at the cellular level, indicating selective cell-type-dependent cytotoxicity due to the different origin and cell cycle parameters.

Our present investigation suggests that sub-lethal exposure of these pesticides to *D. melanogaster* exhibit key aspects of neurodegeneration at different parameters which are mentioned above such as, locomotor deficit at various doses of malathion and paraquat, damage to DNA content in brain tissues in response to dose dependent
treatment of paraquat and malathion. *in vitro* studies also suggests cellular degradation in D-Mel-2 (S2) cells due to different doses of paraquat and malathion at different time exposure.

*Drosophila* is an attractive organism due to the conserved gene function between fly and humans, the short life span and the availability of vast range of scientific manipulations. In recent era, various studies are conducted using *Drosophila* in genetic, biochemical and behavioral research. Here we tried to present the cytotoxic, genotoxic and neurotoxic effects of two important environmental toxins. This study will lay the groundwork for understanding the effects of these harmful pesticides and for future testing of potential harmful toxins in whole organism system. For these potential reasons and many more reasons, the use of *D. melanogaster* in research will continue to grow and provide the scientific community with valuable knowledge for at least the next 100 years.