ORIGINAL RESEARCH

Induction of DNA damage in the peripheral blood of zebrafish (*Danio rerio*) by an agricultural organophosphate pesticide, monocrotophos

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Abstract The use of organophosphate pesticides (OPs) in agricultural practices in India has gained popularity due to their broad spectrum, low cost and high potency. Monocrotophos (an OP) which is a systemic and contact broad-spectrum cholinesterase inhibitor is being widely used in India. However, its use entails the damage to other non-target organisms such as fish. In the present study, zebrafish (*Danio rerio*) were exposed to various doses of monocrotophos (0.125, 0.625 and 1.25 μL/L) and genotoxicity studies were carried out employing the comet assay and micronucleus test in the peripheral blood of these fish after 24, 48 and 72 h of exposure. Significant DNA damage in the form of % tail DNA and micronuclei was observed in all the treated fish compared to the control. Both % tail DNA and micronuclei frequency were found to increase significantly as the concentration increased. DNA damage was also observed at all the time intervals for all treatments except in the 0.125 μL/L-treated group. A positive correlation was also observed between the comet assay and micronucleus test. The study thus suggests the use of zebrafish as an experimental model to study the genotoxic effects of agricultural pesticides using the comet assay and micronucleus test.

Keywords Comet assay · Micronucleus test · Genotoxicity

Introduction

Organophosphate pesticides (OPs) are used extensively in agricultural practices around the world due to their high potency and rapid mode of action on a broad spectrum of pests. They are preferred over organochlorines due to their rapid degradation and less environmental persistence. Monocrotophos (MCP), an OP, is used in many parts of the world, particularly in developing countries because of its low cost and is a broad-spectrum pesticide. It is a systemic and contact poison that inhibits cholinesterase and acts as a neurotoxin. Its use, however, entails several undesirable effects particularly in non-target organisms. It is an organic pollutant which is soluble in water and can, therefore, be easily taken up by aquatic fauna. It is labeled as a marine pollutant (FAO/UNEP 1997) and is included under category 1 (highly toxic) by USEPA (1985). It can easily enter the body through oral, dermal or inhalation routes. It is highly toxic to insects, birds and mammals and is moderately toxic to fishes. MCP, as with other pesticides finds its way to the aquatic environment by

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wastewater discharge and monsoon run-offs from agricultural land and thereafter inevitably enters the aquatic food chain.

A number of studies are available on the inhibitory effect of MCP on acetycholine esterase (AChE) activity in different fish species (de Croux et al. 2002; Rao 2004; Kavitha and Rao 2007).

The uptake of xenobiotics also has the capability to induce damage at the level of DNA. They may either affect the DNA directly by causing DNA strand breaks or indirectly by producing reactive oxygen species (ROS) which then damages the DNA by forming adducts or causing lesions (O'Brien 1988; Bagchi et al. 1995; Marnett 2000). DNA repair systems which try to heal the damaged DNA can do so only up to a certain threshold, beyond which DNA damage can persist, in the form of lesions or misrepaired DNA, leading to mutagenesis and carcinogenesis (Moustacchi 2000). Two reliable genotoxicity endpoints which are used for screening the genotoxicity of such xenobiotics are the micronucleus (MN) test and the single-cell gel electrophoresis (comet assay) (Anbumani and Mohankumar 2012; Praveen Kumar et al. 2014, 2015). The MN test is basically used to check the clastogenicity of a particular compound, whereas the comet assay is used to check the effect of the compound on the integrity of DNA (Hartmann et al. 2001; Bolognesi and Cirillo 2014). A number of studies are available on the genotoxicity of other organophosphate pesticides using these tests. Ali and Kumar (2008) studied the genotoxic effects in *Channa punctatus* exposed to acute treatments of chlorpyrifos and observed DNA damage in the form of micronuclei and percentage tail DNA from the peripheral blood. Similarly, Kumar et al. (2010) also observed the induction of micronuclei and tail DNA in different tissues of *C. punctatus* exposed to malathion. The genotoxicity of MCP has been reported in estuarine bivalves (Revankar and Shyama 2009). However, few studies are available on the genotoxicity of this pesticide in teleost fishes. *Tilapia mossambica* treated with different concentrations of MCP were reported to have significant DNA damage in their erythrocytes as observed by the comet assay (Banu et al. 2001). In another study, Ali and Kumar (2008) reported the DNA damage induced by MCP in the gills, kidneys and lymphocytes of *C. punctatus* using the comet assay. Besides fish, the genotoxicity of MCP has been reported in mice (Mahboob et al. 2002), in rats (Yaduvanshi et al. 2010) and in human peripheral blood lymphocytes (Das et al. 2007).

Although a number of fish models are used for toxicity screening of aquatic environment, the teleost cyprinid, zebrafish (*Danio rerio*) is the most popular choice because of its prominence as a toxicological model (Hill et al. 2005; Sipes et al. 2011). A few studies have been carried out on the different effects of MCP on zebrafish such as the estrogenic and feminizing effects of MCP (Zhang et al. 2013) and cortisol-mediated stress responses (Zhang et al. 2015). In a recent study, different concentrations of MCP were used to evaluate the developmental response of zebrafish embryos (Pamanji et al. 2015). Mortality was observed in all the test concentrations along with a large number of morphological abnormalities. However, there is no information on the DNA damaging effects of MCP in adult zebrafish, and therefore, the present study was carried out to assess the genotoxicity of different concentrations of this OP.

Materials and methods

Quality assurance and quality control

The appropriate quality assurance methods of sample preparation, handling and preservation were carried out in accordance with US EPA procedures. All chemicals used were of analytical grade from Himedia (Himedia, India).

Experimental fish

Zebrafish were handled and maintained as per the European Union directive on the protection of animals used for scientific purposes (EU 2010). Male zebrafish (*D. rerio*) (12-month old and size 4.0 ± 0.3 cm) were procured from a fish rearing and breeding farm from Pune, India and were transported in well-oxygenated polythene bags to the laboratory. They were maintained in filtered tap water in aquaria (25 L) with aerators

and temperature regulators at 28 °C, 12 h light/dark cycle, pH 7.0 ± 0.15 and DO 8.5 ± 1.0. They were fed with commercial feed pellets. Foam filters were used to collect the accumulated fecal matter and were cleaned every alternate day. Water was changed once a week. Thus, these fish were acclimatized in the laboratory conditions for 30 days.

Monocrotophos

Monocrotophos [$C_7H_{14}NO_5P$, dimethyl (E)-1-methyl-2-(methylcarbamoyl) vinyl phosphate, MCP], of commercial grade (37% water soluble) was procured from Sds Ramcides Cropscience Pvt Ltd, Chennai, India (Batch No. R/P/879).

Treatment schedule

The fish were distributed into groups; each containing ten fishes in the same aquaria mentioned above and was used for dose–response studies as well as time–response studies. Based on the LC50 value of MCP on *Tilapia mossambicus* as per Mustafa et al. (1982), three sub-lethal doses, i.e., 0.125, 0.625, 1.25 μL/L were selected and exposed in triplicates. Acute toxicity studies were also carried out at 24, 48 and 72 h of exposure without renewal of MCP.

Validation of the comet assay and micronucleus test

A separate group of fish (ten individuals) treated with cyclophosphamide (1, 3 and 5 mg/L in water), a well-known genotoxin was used as a positive control at different time intervals (24, 48 and 72 h) and the comet assay and micronucleus test were validated.

Cell viability

Prior to the comet assay and micronucleus test, the cell count and cell viability of the peripheral blood were checked to ensure that there were enough living cells to perform the assay employing trypan blue dye exclusion test. The samples showing more than 90% viability and a cell count of a minimum of 10^6 cells/mL were used for the tests.

Comet assay (alkaline single-cell gel electrophoresis)

The comet assay was performed according to the protocol of Ferraro et al. (2004) with slight modifications. All the steps were carried out in dim light and at 4 °C to prevent photo-oxidation of DNA. Peripheral blood was collected from the fish by caudal puncture and suspended in phosphate buffered saline (Ca + Mg + free, pH 7.2). 20 μL of this blood suspension was mixed with low-melting agarose (LMA) and overlaid on a base layer of normal-melting agarose on frosted microscopic slides. These slides were placed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 Mm Tris, 10% DMSO And 1% Triton-X pH 10, 4 °C) for 1 h. The slides were then transferred to an electrophoretic unit containing unwinding buffer (pH > 10) for 20 min to allow the DNA to unwind. Electrophoresis was then performed under alkaline conditions (pH 10) for 20 min at 300 mA, 25 V. After electrophoresis, the slides were placed in neutralization buffer (400 mM Tris base, pH 7.5) for 5 min. About 25 μL of ethidium bromide stain was applied in four-to-five small, equally sized droplets over the gel, covered with a coverslip and examined using a fluorescence microscope (Olympus BX53) with a green filter at 200× magnification. Two slides per fish were prepared and 100 "comets" were screened per slide. Images of the comets of non-overlapping cells were captured using an attached camera and analyzed with the help of computer software, CASP (Konca et al. 2003). The % tail DNA was recorded which is used as a reliable measure of DNA damage (Kumaravel and Jha 2006; Praveen Kumar et al. 2014).

Micronucleus test

The MN test was performed as per Baršiene et al. (2006). Briefly, a small drop of the blood collected by caudal puncture was immediately smeared on clean glass slides, allowed to air-dry, fixed in absolute methanol for 10 min and stained with 5% Giemsa for 30 min. Three slides per fish were prepared. The frequency of micronuclei (MNi) in erythrocytes was evaluated by scoring 5000 intact cells per fish at 1000× magnification. MNi were identified as structures with the following morphological features: (1) spherical or ovoid-shaped extra-nuclear bodies in the cytoplasm (2) a diameter of 1/3–1/20 of the main nucleus (3) non-refractory bodies (4) color texture resembling that of the nucleus, and (5) the bodies completely separated from the main nucleus (Fenech et al. 2003).

Statistical analysis

Statistical analyses of the data were carried out using IBM SPSS 23 statistical software package. Since the data are expressed as percentage values, arc sine transformation of the variables were computed and were tested to check the normality and homogeneity of variance using Shapiro–Wilk test and Welch's test, respectively, before being analyzed. A two-way ANOVA was applied to test the effect of dose as well as time on the DNA damage parameters and also the interactions between them followed by a post hoc Dunnet's test to compare the different groups with the control within the same treatment group or time interval group. Pearson's correlation with a scatter plot was also used to test the relationship between the frequency of MNi and % tail DNA. The data were considered to be statistically significant at $p < 0.05$.

Results

The DNA damage (% tail DNA) induced by different concentrations of CP (1, 3, and 5 mg/L) in the blood cells of zebrafish at various time intervals (24, 48 and 72 h) is shown in Fig. 1a ,b. All the doses of CP induced a significant increase in % tail DNA and % MNi compared to the control (Figs. 1, 2).

A significant increase of dose-dependent damage ($p < 0.05$) was observed in the form of % tail DNA in the erythrocytes of *D. rerio* compared to the control (Fig. 3). Tail DNA (%) was also found to increase significantly with advancing time.

A significant increase in the frequency of micronuclei (MNi) was also observed in the erythrocytes for the 0.62 and 1.25 µL/L treatment groups (Fig. 4). However, in the time-dependent studies, there was no significant increase of MNi formation after 24 and 48 h in the 0.125 µL/L treatment group although there was a significant increase after 72 h.

The results of the two-way ANOVA (Table 1) indicate the effect of treatments and time as well as their interactions (treatment × time) on the % tail DNA and MNi. The treatment variable contributed the most to the % tail DNA ($F = 1739.89$, $p < 0.001$) and to a lesser extent, MNi ($F = 697.98$, $p < 0.001$). On the other
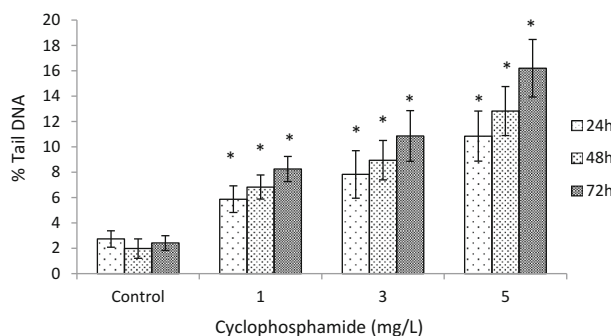

Fig. 1 Tail DNA (%) in erythrocytes of *D. rerio* exposed to different treatments of cyclophosphamide at different intervals of time. Statistical differences (*$p < 0.05$) are in relation to the control of the respective time interval
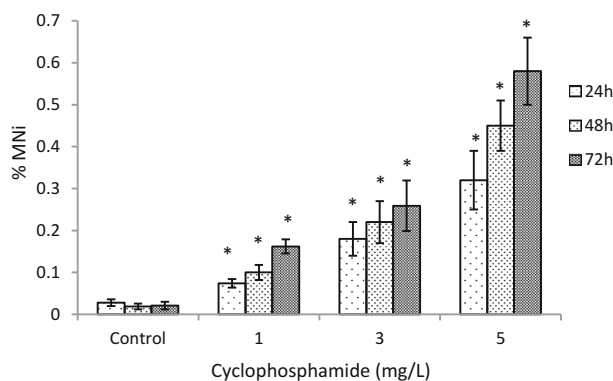
Fig. 2 MNi (%) in erythrocytes of *D. rerio* exposed to different treatments of cyclophosphamide at different intervals of time. Statistical differences (*$p < 0.05$) are in relation to the control of the respective time interval
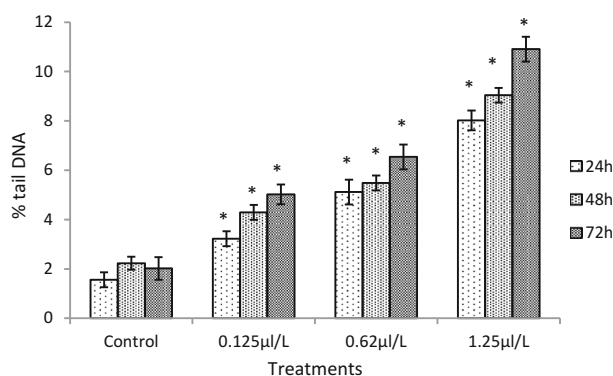

Fig. 3 Tail DNA (%) in erythrocytes of *D. rerio* exposed to different treatments of monocrotophos at different intervals of time. Statistical differences (*$p < 0.05$) are in relation to the control of the respective time interval
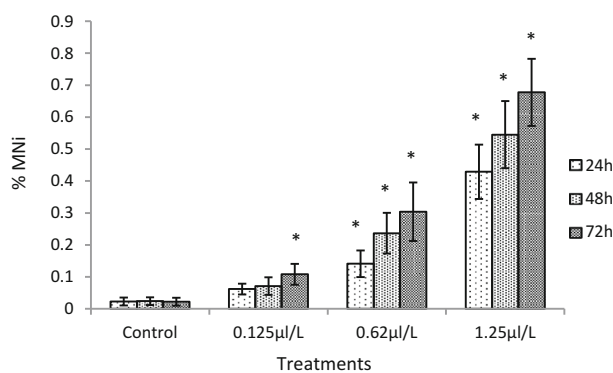

Fig. 4 MNi (%) in erythrocytes of *D. rerio* exposed to different treatments of monocrotophos at different intervals of time. Statistical differences (*$p < 0.05$) are in relation to the control of the respective time interval

hand, the time variable had the most effect on MNi ($F = 25.8$, $p < 0.001$) followed by the % tail DNA ($F = 10.96$, $p < 0.001$). The interaction of the two independent variables (treatment × time), however, influenced only the formation of MNi ($F = 4.56$, $p < 0.01$).

The data of the arc sine-transformed % tail DNA and % MNi were found to be highly correlated by Pearson's correlation analysis ($R = 0.92$) and the relationship is plotted in the form of a scatter plot in Fig. 5. A significant positive correlation is, therefore, found to exist between these two parameters of DNA damage ($R^2 = 0.86$).

Table 1 Two way ANOVA testing the influence of treatments and time as well as the interaction between them (treatment × time) on the DNA damage parameters in erythrocytes of *D. rerio* exposed to monocrotophos

Dependent variable	Independent variables					
	Factors				Interaction	
	Treatment		Time		Treatment × time	
	F value	*p*	*F* value	*p*	*F* value	*p*
% tail DNA	1739.89	< 0.001	10.96	< 0.001	1.36	ns
MNi	697.98	< 0.001	25.80	< 0.001	4.56	< 0.01

ns non-significant
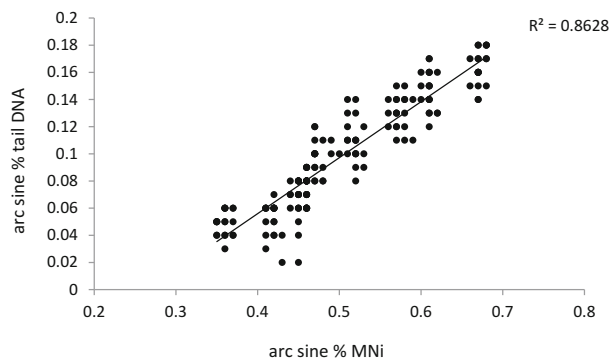
Fig. 5 Scatter plot showing the relationship between arc sine-transformed data of % tail DNA and MNi

Discussion

The present study revealed the genotoxicity of MCP in the peripheral blood cells of zebrafish as indicated by the comet assay and the micronucleus test. Further, genetic damage increased with increasing doses and time intervals. Our results are in agreement with that of Anbumani and Mohankumar (2015) in which they observed that freshwater fish (*Catla catla*) exposed to various doses of MCP exhibited a dose and time-dependent increase of DNA damage in the form of micronuclei. Our results also find similarity with the studies of Ali and Kumar (2008) wherein significantly high % tail DNA was induced in the erythrocytes of *C. punctatus* exposed to MCP. They reported that the DNA damage was highest on the fourth day, i.e., 96 h after exposure and the same was also evident in the present study in which DNA damage was highest on the third day, i.e., 72 h after exposure for all the doses. However, Banu et al. (2001) observed maximum DNA damage in the erythrocytes of *Tilapia mossambica* exposed to different doses of MCP at 24 h after exposure and a subsequent decrease of DNA damage at later time intervals. These differences in DNA recovery may be attributed to the rate of metabolite removal/detoxification or DNA repair capacity of different species. In the time-dependent study, MCP did not induce significant DNA damage at 0.125 μL/L at 24 and 48 h of exposure. This may probably be due to the initial low concentration of the toxicant which could not induce any immediate noticeable effects but did affect DNA at the 72 h time interval. This subsequent increase in DNA damage may have occurred due to gradual bioaccumulation of MCP over the span of 72 h. Further, the results of the two-way ANOVA suggest that both time and treatment play an important role in the formation of DNA strand breaks and micronuclei.

Our results also find similarities with studies on other related organophosphate pesticides by several workers. Ali et al. (2009) reported the induction of micronuclei and tail DNA by various treatments of chlorpyrifos, in the erythrocytes and lymphocytes of *C. punctatus*. They also reported that DNA damage was highest on the third day after exposure at all the concentrations of chlorpyriphos. Similar observations were reported by Mohanty et al. (2011) in which the erythrocytes of *Labeo rohita* fingerlings exposed to another OP, phorate exhibited significant DNA damage which declined after 72 h of exposure. Sub-lethal doses of

profenofos, an OP pesticide were found to induce DNA strand breaks in the gill cells of *C. punctatus* and was found to increase in a dose-dependent manner (Pandey et al. 2011). DNA damage was also observed in the gill cells of the European eel (*Anguilla anguilla*) exposed to the OP glyphosate (Guilherme et al. 2012). Ali et al. (2014) also reported the time-dependent and dose-dependent increase of genotoxicity of an OP insecticide, dimethoate in *C. punctatus,* which were found to be highly correlated with oxidative stress. Our results along with those of Pamanji et al. (2015) mentioned earlier, provide information on the toxic effects of MCP in both the adults and embryos of zebrafish suggesting that MCP can affect zebrafish at any time during its life cycle.

Organophosphate pesticides such as MCP and those cited above cause the alkylation of DNA bases either directly or indirectly by protein alkylation leading to DNA disintegration (Bhinder and Chaudhry 2013). The presence of a methyl ester group is more likely to cause alkylation than ethyl esters or other higher esters and thus MCP is more reactive than other organophosphate pesticides (Yaduvanshi et al. 2010). Alternatively, the phosphorous group in OPs may be a favorable substrate for nucleophilic attack which in turn may cause DNA phosphorylation and consequently, DNA damage (Rahman et al. 2002; Das et al. 2007). Another reason for damaged DNA could be oxidative stress due to the formation of reactive oxygen species (ROS). Oxidative stress may be caused due to the production of ROS above the level of detoxification and repair. The free radicals thus formed can affect DNA indirectly by lipid peroxidation which in turn causes single-stranded breaks (SSB) (Yaduvanshi et al. 2010; Nwani et al. 2011). Lipid peroxidation products such as 4-hydroxynonenal and malondialdehyde (MDA) which are the most genotoxic and mutagenic, respectively, form adducts with DNA thereby causing DNA damage (Luczaj and Skrzydlewska 2003). Thus, DNA damage prevailing in the erythrocytes of zebrafish may be due to the combined direct or indirect effects.

We also observed that the induction of tail DNA had a strong positive correlation with induction of micronuclei in zebrafish erythrocytes. The reason for correlating these two genotoxicity tests is that they reflect different forms of environmental stress. MCP, as mentioned earlier can induce SSBs which may be easily repaired and these transient DNA breaks can be easily detected by the comet assay. However, DNA damage which is difficult to repair tends to form extra-nuclear bodies or micronuclei which can be detected by the micronucleus test (Hartmann et al. 2001). These micronuclei are formed when a whole chromosome or a fragment of a chromosome do not get incorporated into either of the two daughter cells during cell division due to aneugenic agents that affect the spindle apparatus or clastogenic agents that damage and break the chromosome (Udroiu 2006). The comet assay and micronucleus test can, therefore, be applied on zebrafish erythrocytes and be used as an integrated part of a monitoring program of water bodies that might be contaminated with organophosphate pesticides.

Conclusions

The present study thus revealed the ability of MCP to induce DNA damage in zebrafish erythrocytes. The formation of MNi and tail DNA is highly correlated with each other and may occur in a dose-dependent and/or time-dependent manner. The MN test and comet assay can, therefore, be reliably used to assess the genotoxicity of MCP in the aquatic environment using zebrafish as a toxicological model. Despite several reports of induced toxicity mentioned earlier, MCP still continues to be used in agricultural practices and may affect aquatic fauna at the DNA level. Therefore, it is suggested to limit its use in agricultural practices to avoid the potential contamination and of nearby water bodies. Although there is a lack of data concerning the health implications of consuming fish contaminated with organophosphate pesticides, care and caution should be taken due to probable persistence of these residues.

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