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Research Article

Malathion Induced DNA Damage in Freshwater Fish, *Labeo rohita* (Hamilton, 1822) Using Alkaline Single Cell Gel Electrophoresis

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Abstract

The current study was aimed to investigate the genotoxic effect of an organophosphate pesticide malathion in the gills of a freshwater teleost rohu, *Labeo rohita* using alkaline Single Cell Gel Electrophoresis (SCGE)/comet assay. The 96 h LC_{50} of malathion was estimated for rohu in a semi-static system and was found to be 5 μg L^{-1}. Specimens of rohu were exposed to LC_{50} of malathion. Gill tissues were sampled after 24, 48, 72 and 96 h of exposure. DNA damage was evaluated by studying different indices, including tail length (μm), percentage of DNA in tail, tail moment and olive tail moment using TriTek CometScore™. A linear relation was observed between exposure time and DNA damage in the gill cells. The current study revealed malathion as a potent inducer of DNA damage and comet assay as a reliable and sensitive assay for investigating and detecting DNA damage *in vivo*, induced in fish by genotoxic pesticides. In order to conserve the vanishing populations of rohu in natural aquatic bodies across the country, indiscriminate use of genotoxic pesticides such as malathion should be minimized.

Key words: Malathion, *Labeo rohita*, LC_{50}, DNA damage, comet assay

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pesticides are used for preventing, repelling, destroying, mitigating and controlling agricultural and household pests (Ullah, 2015). These are also used for preventing infectious diseases. Although these chemicals are beneficial but pose a serious threat to humans as well as to the environment. Due to greater toxicity and higher persistence, organochlorine pesticides are banned worldwide or their use has been restricted, which established the second line pesticides, including pyrethroids and organophosphates as the most common and widely used pesticides (Ullah and Zorriehzahra, 2015). These pesticides might be beneficial, but their indiscriminate and inappropriate application can kill natural enemies of pests and can increase resistance of the pests. Some of these pesticides are highly persistent, thus contaminate the ground, soil and surface water. The toxic effects of these pesticides manifest in various ways, such as bioaccumulation and biomagnification, acute immune response, allergic reaction, chronic toxicity, teratogenic, carcinogenic and mutagenic effects (Ecobichon, 1996). These pesticides have led to serious environmental concerns, as some of these might not show instant effects *in vivo* but might lead to long term health hazards to humans. The ill environmental impacts of pesticides are often far greater as compared to the projected ones by those who use these pesticides. Approximately more than 98% employed insecticides and 95% herbicides lead to edible, non-target species, consequently reaching food and food chains, and eventually human beings (Damalas and Eleftherohorinos, 2011).

Malathion is one of the most widely employed organophosphate pesticides in agriculture, public health and for hygiene purpose in different countries around the globe (Ojha and Srivastava, 2014). Previous studies revealed organophosphates, including malathion, methyl parathion, chlorpyrifos and monchrotophos etc., as anticholinesterase agent and brought about different alterations in the level of neurotransmitters and neurobehavioral processes in different animals after exposure (Kamel and Hoppin, 2004; Satoh and Gupta, 2011). These pesticides also produce oxidative stress and extensive production of free radicals of oxygen, which is involved in pesticides induced toxicities in animals (Lukaszewicz-Hussain, 2010), *in vitro* experiments (Gultekin *et al*., 2001), pesticide manufacturing workers (Ranjbar *et al*., 2002) and pesticide sprayers (Lopez *et al*., 2007). Oxidative stress induced DNA damage has proposed mechanistic link of pesticide exposure to different health outcomes in previously conducted epidemiological studies (Olinski *et al*., 2002; Muniz *et al*., 2008). The diseases linked with the release of Reactive Oxygen Species (ROS), e.g., hydroxyl radical, superoxide anion radical, singlet oxygen, hydrogen peroxide, etc.) include atopic dermatitis, hepatitis, different autoimmune diseases (systemic lupus erythematosus and rheumatoid arthritis, etc.), male infertility and defective sperm function, aging, Alzheimer's disease, Huntington's disease and Parkinson's disease (Shen and Ong, 2000; Olinski *et al*., 2002; Cooke *et al*., 2003; Tvrda *et al*., 2011).

Reactive oxygen species causes DNA oxidation, known as the most common kind of DNA damage. Hydrogen peroxides and superoxide anions induce base oxidation of intracellular DNA and strand breaks whereas, hydroxyl radicals induce DNA damage by direct reaction with DNA molecules (Friedberg *et al*., 1995). Despite being different tools for assessing DNA damage comet assay or Single Cell Gel Electrophoresis (SCGE) is the most commonly used measure for investigating induced damage in DNA. The SCGE is a well-established assay for the assessment and estimationof DNA damage both *in vitro* and *in vivo*, at the individual cell level due to its capability of detecting very low level oxidative damage induced in DNA (Singh *et al*., 1988).

Malathion is a potent source of ROS and is also an alkylating agent that can cause DNA damage (Wild, 1975; Braun *et al*., 1982). The technical grade of malathion induced changes in chromosome such as chromosomal aberration and micronuclei in germ and somatic cells in mice (Flessel *et al*., 1993). The DNA might be damaged due to the metabolites of malathion. Commercially available malathion contains isomalathion and malaoxan, which can induce *in vivo* disturbances in DNA such as DNA breakage at the site of tumour suppressor genes or oncogenes. Thus play a distinct role in inducing malignancies in exposed individuals. Keeping in view malathion can be considered as a potent carcinogen/mutagen (Blasiak *et al*., 1999).

Malathion is widely used organophosphate pesticide. However, there is no report regarding its genotoxic potentials against commercially important indigenous fishes of Pakistan. Keeping in view the current scenario, the current study was designed to find out the LC_{50} of malathion and assess its genotoxic potential against a freshwater fish rohu, *Labeo rohita*, economically an important teleost.

MATERIALS AND METHODS

Experimental animal acclimatization: A total of 180 uniform sized and healthy specimens (8.17±0.794 cm and 6.55±1.01 g) of *L. rohita* were collected. The fish were acclimated for 10 days in aquaria (60×30×30 cm) and were fed with 35% protein to satiation at the rate of 5% body weight. The aquaria were siphoned off on a daily basis for avoiding stress to the fish and different water quality parameters were investigated regularly. Dead fish were removed as quickly as possible in order to maintain water quality.

Test chemical: The commercial formulation of malathion was purchased from local market and used during the study. A stock solution was prepared in acetone. During the experiment, required amount of dilutions was used.

Determination of Lethal Concentration (LC_{50}): The LC_{50} of malathion against rohu was found through probit analysis. The fish were exposed to the different concentrations (2, 3, 4, 5, 6, 7 and 8 μg L^{-1}) of malathion, using semi-static method. Fish mortality was noted from 24 through 96 h of exposure. Dead fish were removed to avoid water deterioration.

Alkaline Single Cell Gel Electrophoresis (SCGE)/comet assay: The alkaline Single Cell Gel Electrophoresis (SCGE)/comet assay was carried out as three layer procedure by following Singh *et al*. (1988) with minor modifications (Klaude *et al*., 1996) as performed by Pandey *et al*. (2011). The 15 μL cell suspension was mixed with Low Melting-Point Agarose (LMPA): 85 μL, 0.5% and was layered on a frosted slide, already coated with a layer of 1% 200 μL normal agarose. Then again, it was layered with LMPA (100 μL) after solidification of the gel. The slides were kept submerged in lysing solution overnight at 4°C. The slides were positioned side by side in horizontal unit of gel electrophoresis, dipped in fresh cold alkaline electrophoresis buffer, left at 4°C for 20 min in the same solution in order to unwind DNA and convert alkali-labile sites to single strand breaks. The same alkaline buffer was used for carrying out alkaline electrophoresis using 300 mA, 15 V (0.8 v/cm) for 20 min at 4°C. Then the slides were gently neutralized using 0.4 M tris buffer (pH 7.5). The slides were stained with acridine orange and were inspected under an epifluorescent microscope (400x, Nikon AFX-1 Optiphot). Digital images were captured for succeeding scoring with TriTek CometScore™, Freeware v1.5. A total of 100 randomly selected cells were scored for each specimen (50 cells from each of the two replicated slides). The parameters including Tail Length (TL), Percent DNA in tail (TDNA%), Tail Moment (TM) and Olive Tail Moment (OTM) were selected for quantifying the level of DNA damage.

Statistical analysis: Data obtained from the experiment were expressed as Mean±SE. The data were analysed by using one way analysis of variances (ANOVA) followed by HSDTukey test using Statistix Version 8.1. The value of $p<0.05$ was considered statistically significant.

RESULTS

Physico-chemical parameters of water: The temperature of the water was varying from 24.3-25.8°C. The dissolved oxygen ranged from 6.1-7.4 mg L^{-1}, pH 6.9-7.5, conductivity 240-290 μM cm^{-1}, total hardness 162-178 mg L^{-1} and Ammonia was lying under 0.25 ppm.

Toxicity evaluation of malathion: Acute toxicity bioassay of different concentrations, log concentrations, Percent mortality and values for probit kill is given in Table 1. A concentration dependent increase was observed in mortalities. Plotting percent mortality and probit kill against log concentration resulted in formation of curves shown in Fig. 1 and 2, respectively. Both the analyses revealed 50% mortality at log concentration 0.653, equal to 5 μg L^{-1} (Table 1).

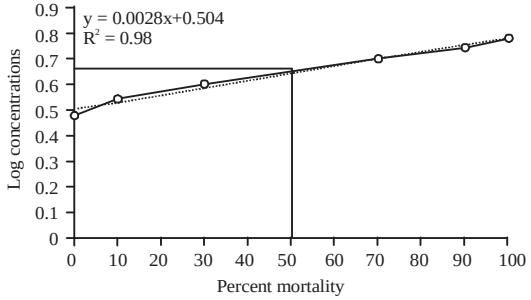

Fig. 1: Toxicity evaluation of malathion against rohu, *Labeo rohita*

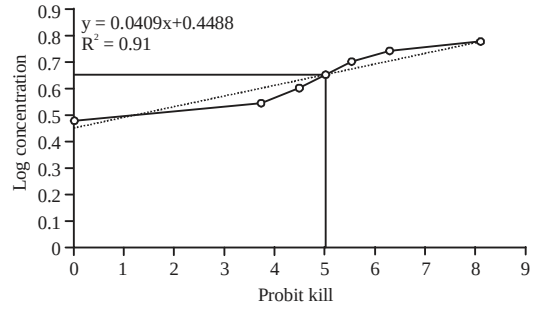

Fig. 2: Toxicity evaluation of malathion against rohu, *Labeo rohita*

Table 1: Determination of LC_{50} value of malathion for 96 h against *Lebeo rohita*

Concentrations CYP (μg L^{-1})	Log concentrations	No. of fish exposed	No. of alive fish	No. of dead fish	Percent mortality (%)	Probit mortality
2.0	0.477	10	10	0	0	0.00
3.0	0.544	10	9	1	10	3.72
4.0	0.602	10	7	3	30	4.48
5.0	0.653	10	5	5	50	5.00
6.0	0.699	10	3	7	70	5.52
7.0	0.740	10	1	9	90	6.28
8.0	0.778	10	0	10	100	8.09

DNA damage: Malathion induced DNA damage in gill tissues of *Labeo rohita* was quantified in terms of TL, TDNA%, TM and OTM. A significant ($p<0.05$) increasing trend was observed in TL of the comets, TDNA% and TM with exposure period in the treated group as shown in Table 2. The exposed fish specimens of treated group also exhibited significantly higher olive tail moment ($p<0.05$) in their gills as compared to fish specimens of control group (Fig. 3). The strand breaks in DNA of the fish in the treated group after malathion exposure is shown in Fig. 4.

With respect to duration of exposure, the lowest level of damage in DNA was observed at 24 h while, the highest after 96 h of exposure in the treated group in term of TL, TDNA%, TM and OTM.

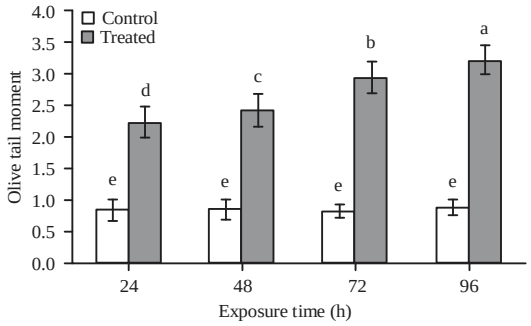

Fig. 3: Olive tail moment of comets in gills tissues of rohu at specific intervals after exposure to LC_{50} of malathion, data are presented as Mean±SE, (n = 6), Means with different superscripted letters are significantly different ($p<0.05$)

DISCUSSION

Previous studies revealed SCGE (Frenzilli *et al*., 2009; Galindo *et al*., 2010) as a handy assay for investigating the gentoxicological potential of different chemicals such as in birds (Baos *et al*., 2006), mammals (Park *et al*., 2007; Garaj-Vrhovac *et al*., 2009), amphibians (Cotelle and Ferard, 1999; Yin *et al*., 2009), reptiles (Bronikowski, 2008) and mollusks (Cotelle and Ferard, 1999; Canty *et al*., 2009). Aquatic organisms, including fish can serve as excellent sentries for assessing the carcinogenic, mutagenic and genotoxic potential of different toxicants (Banu *et al*., 2001; Ali *et al*., 2008). Different fish species from both marine and freshwater ecosystems have been employed for environmental biomonitoring such as *Channa punctatus* (Kushwaha *et al*., 2000; Pandey *et al*., 2006; Ali *et al*., 2008), *Cyprinus carpio* (Buschini *et al*., 2004; Gustavino *et al*., 2005), *Ameiurus nebulosus* (Pandrangi *et al*., 1995), *Dreissena polymorpha* (Pavlica *et al*., 2001), *Mugil* sp. and *Netuma* sp. (De Andrade *et al*., 2004).

Fish are used as a model organism in ecotoxicological studies on account of its economic value, crucial role in trophic web, sensitiveness even to lower concentration of toxicants as well as the capability of bioaccumulation of toxic substances (De Andrade *et al*., 2004; Jha, 2008). The fish is being used as an important bioindicator of pollution and earlier detection of problems in aquatic ecosystem (Cavas and Ergene-Gozukara, 2005; Steckert *et al*., 2009). Many tissues of fish, such as blood peripheral erythrocyte, gills, liver and brain

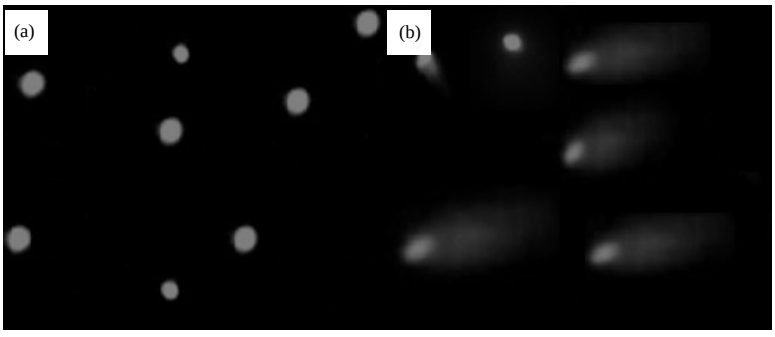

Fig. 4(a-b): Fluorescent photomicrograph (40×) of cells in gills of rohu, after 96 h of exposure to LC_{50} of malathion using comet assay, Stain: Acridine orange, (a) Intact DNA in control fish and (b) Comets with tail formed in treated fish

Table 2: Tail length, Tail DNA (%), tail moment observed in the gills of *Lebeo rohita* after exposure to malathion at different time intervals

	Tail length (μm)		Tail DNA (%)		Tail moment	
Time (h)	Control	Treated	Control	Treated	Control	Treated
24	5.73±1.11^{e}	11.21±1.32^{d}	8.13±1.01^{e}	18.25±4.11^{d}	0.86±0.12^{d}	1.81±0.21^{c}
48	5.34±0.87^{e}	17.72±2.13^{c}	8.55±1.11^{e}	28.34±4.92^{c}	0.83±0.14^{d}	1.95±0.24^{bc}
72	5.38±1.12^{e}	21.54±2.67^{b}	8.41±1.57^{e}	33.13±5.34^{b}	0.81±0.11^{d}	2.03±0.32^{b}
96	5.62±0.94^{e}	27.11±2.91^{a}	8.67±0.97^{e}	35.11±4.12^{a}	0.85±0.15^{d}	2.18±0.29^{a}

Data are represented as Mean±SE (n = 6), means followed by different letters are significantly different at $p<0.05$. ANOVA followed by HSD Tukey test

can be used for conducting toxicological studies such as evaluating DNA damage, however, non-invasive method is highly preferred (Lee and Steinert, 2003; Zhou *et al*., 2006).

Pesticides are extensively used in modern era of the industrial revolution (Ullah, 2015; Ullah and Zorriehzahra, 2015). Therefore, researchers are actively involved in conducting experiments on exposed populations in order to investigate the risks associated with occupational exposure. As cancer is having a mutational base, therefore, monitoring of exposed animals to different mutagenic and genotoxic pesticides in polluted environments is of great importance in assuring protection to human health (Pandey *et al*., 2011). Several reports are available, demonstrating the genotoxic effects of different pesticides in various living organisms (Bhalli *et al*., 2006, 2009). Pesticides bioaccumulate in the tissues of living organisms, hence, the estimation of these chemicals in tissues might be more valuable for assessing natural or wild populations (Kumar and Chapman, 2001; Rao *et al*., 2003).

In the present study acute concentration of malathion causing 50% mortality after 96 h was found to be 5 μg L^{-1}. This value was a bit higher than 4.5 μg L^{-1} (Patil and David, 2013) and much lower than 15 mg L^{-1} (Thenmozhi *et al*., 2011) for the same fish species. Changes in LC_{50} values of the same toxicant for the same fish species or different toxicants for various fish species might be due to the formulation, specification of the pesticides as well as the stereochemistry of their active molecules (Ullah, 2015). Toxicity of the pesticides mainly depends on stereochemistry of their molecules as isomers in formulations of pesticides vary in their precise toxicity (Ullah *et al*., 2014). Single isomer based pesticides are more toxic as compared to those having various combinations of isomers in their formulation (Bradbury and Coats, 1989).

Pesticide toxicity is also correlated with the carriers of contaminants and active and inert ingredients (Ullah *et al*., 2014). Moreover, the toxicity of pesticides against fish also depends on certain factors, including temperature, age of the fish, the size of the fish and health condition of the fish (Farah *et al*., 2004). The temperature during the study was ranging from 24.3 and 25.8°C. No association between temperature and the value of LC_{50} was observed while, an inverse relation between temperature and LC_{50} was observed by Kumaragura and Beamish (Kumaraguru and Beamish, 1981). Studies have shown that pesticides are less toxic in summer as compare to winter. Tenfold difference was observed at 10, 15 and 20°C in the value of LC_{50} for 96 h (Singh *et al*., 2010), while an inverse association between pesticide toxicity and body weight do exist (WHO., 1992).

The results of the current study revealed a significant DNA damage in the gills of rohu. An increase was observed in DNA damage with the time period of exposure. The higher DNA damage in gills might be due to the fact that the gills are constantly, directly and continuously exposed tissue to the toxicant (Pandey *et al*., 2006). Gill has been shown as the suitable tissues for conducting genotoxicological studies in goldfish, rainbow trout and shellfish (Liepelt *et al*., 1995; Sasaki *et al*., 1997; Masuda *et al*., 2004; Pandey *et al*., 2011). Some previous studies on various species of fish showed higher sensitivity of gills to DNA damage than other cells, including lymphocyte, erythrocytes, liver and kidney (Wilson *et al*., 1998; Kilemade *et al*., 2004; Ali *et al*., 2008, 2009).

The basic principle of electrophoresis is that damaged DNA migrate faster toward anode as compared to intact DNA, thus result in tail formation. The fragments of DNA freely move during electrophoresis, while tranquilloops of DNA drag out of the head/nucleus. Thus the tail length determines the extent of DNA migrated out of the nuclear head. As smaller fragments of DNA migrate farthermost, hence the tail length of the comets is primarily showed by the size of the fragments of DNA, produced during the alkaline unwinding step of SCGE (Kumaravel and Jha, 2006). Percent tail DNA shows the percentage of DNA moved out of the head and is also considered as a suitable parameter (Kilemade *et al*., 2004). Olive tail moment shows the distance from the head centre to tail centre and is used for the assessment of degree of DNA damage (Singh *et al*., 1988).

The biotransformation of xenobiotic leads to an increase in the production of Reactive Oxygen Species (ROS) highly toxic to fish. It can directly break DNA through OH^- and H_2O_2 resulting in oxidized bases of DNA (Akcha *et al*., 2003). Fish have an antioxidant defence system against ROS but when higher production of ROS surpasses the defence systems of fish, cellular lesions and DNA damage occur (Cadet *et al*., 2003; Cavalcante *et al*., 2008; Jha, 2008). Oxidative DNA damage due to production of ROS is attributing for variable and higher DNA damage in the cells of the gills (Wilson *et al*., 1998; Pavlica *et al*., 2001).

Our results are in congruence with the previous studies conducted on mutagenic and genotoxic potentials of different organophosphates pesticides in different organisms (Fahmy and Abdalla, 1998; Das *et al*., 2006; Rao *et al*., 2006; Ganguly *et al*., 2010; Li *et al*., 2010). The result of the present study demonstrated malathion as genotoxicity inducer at acute concentration. The findings, display a serious concern regarding the potential threats to aquatic organisms from malathion. Thus, it should be carefully and judiciously employed in domestic, industrial and agricultural practices. The current study has a broad perspective regarding aquatic toxicology as gills of the fish species are constantly exposed to different type of toxicants.

CONCLUSION

Our results clearly concluded that malathion possesses mutagenic and genotoxic potential as well as showed SCGE as a potent assay for bio-monitoring genotoxicity in aquatic environments. This study provides a biomarker for malathion induced mutagenicity and genotoxicity, which might be useful for evaluating the toxicants impacts in acute concentrations on freshwater fish species. This system might also work as a sensitive and useful tool for investigating the exposure of different fish species to genotoxins and assessing the mutagenic hazards in surface water.

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