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Research Article
Genotoxic Effect of Endosulfan at Sublethal Concentrations in Mori (*Cirrhinus mrigala*) Fish Using Single Cell Gel Electrophoresis (Comet) Assay

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Abstract
The current study was designed to determine the genotoxic effect of endosulfan at sublethal concentrations (66, 50 and 33% of LC_{50}, 1.5 μg L^{-1}) in peripheral blood erythrocytes of an economically important indigenous Indian major carp Mori, *Cirrhinus mrigala*. A total of 180 fish were divided into four groups, each group received 45 individuals. Group 1st served as control (received no endosulfan), while group 2nd (0.5 μg L^{-1}), 3rd (0.75 μg L^{-1}) and 4th (1 μg L^{-1}) were exposed to endosulfan. For investigating the induced DNA damage, the blood samples were collected from the caudal veins of the fingerlings in all the groups after 7, 14, 21 and 28 days of endosulfan exposure. Endosulfan induced DNA damage in all the treated groups at all concentrations, in terms of percentage of damaged cell (% damage cell) and Genetic Damage Index (GDI) based on visual classification of the extent of damage (Class 0-4) and cumulative tail length (μm). A concentration and time dependent increase was observed in DNA damage in the exposed groups, the highest damage was observed in group 4th (1 μg L^{-1}) followed by group 3rd (0.75 μg L^{-1}). Similarly, the highest level of DNA damage was observed in peripheral blood erythrocytes sampled after 28 days, followed by 21 days after exposure. The current study displayed the severe genotoxic potential of endosulfan in *Cirrhinus mrigala*, even at sublethal concentrations. Therefore, the indiscriminate and injudicious use of endosulfan should be strictly monitored and banned or at least controlled by the responsible governmental authorities.

Key words: Endosulfan, *Cirrhinus mrigala*, sublethal, SCGE, genotoxicity, DNA damage

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

The enormous use of pesticides in modern world is as evident as bright day light. Pesticides are used to deter, control, mitigate and repel pests in both commercial and household agricultural activities, causing environmental pollution (Ullah, 2015). These are also used for controlling vectors of different diseases (Ullah *et al*., 2014). Chemical pollution poses a potential threat to humans as well as livestock specifically mammals, birds and fish. Among the various classes of pesticides, pyrethroids and organochlorine are widely employed (Ullah *et al*., 2015, 2016a). Organochlorine insecticides are majorly used for controlling pests of vegetables, fruits, tea and some non-food crops including cotton and tobacco.

Indiscriminate and injudicious use of pesticides has been a matter of concern for fisheries toxicologist since very long. Studies have shown that less than 0.1% of the applied pesticides reach the target while 99% lead to ecosystems, contaminating land, air and water (Yekeen and Fawole, 2011). Pesticides are one of the major contributors to water pollution. More than 200 types of pesticides are being used in thousand different products, containing different heavy metals including manganese, zinc, lead, copper, cadmium, nickel, iron and chromium (Latif *et al*., 2013). These heavy metals threaten the survival of different economically important aquatic organisms at lethal concentrations while adversely affect the biological systems of these organisms at sublethal concentrations (Ullah and Zorriehzahra, 2015).

Organochlorine insecticides are widely consumed on large scale due to their strong insecticidal properties and broader applications. However, higher persistence in the environment and being toxic to non-target organisms, some organochlorine insecticides are banned (Sharma *et al*., 2011). Endosulfan, an organochlorine insecticide is identified as one of the extensively used pesticides in Pakistan. Although, endosulfan is less persistent than other organochlorine insecticides, yet induces different form of toxicities in non-target animals (Lee *et al*., 2013). Despite being a higher agricultural yield due to endosulfan use, it has also been reported to be highly toxic to different non-target organisms including fish (Suneetha *et al*., 2010). It alters physiology, behaviour, metabolism, endocrine and defence systems of the fish and ultimately affects their survival (Ullah and Zorriehzahra, 2015; Ullah *et al*., 2016a).

The mutagenic and genotoxic effects of endosulfan have been reported in various fish species including *Mystus vittatus* (Sharma *et al*., 2007), *Clarias gariepinus* (Yekeen and Fawole, 2011), *Carassius carassius* (Dar *et al*., 2014, 2015) and *Labeo rohita* (Ullah *et al*., 2016b) but literature regarding sublethal genotoxic effects of endosulfan in mori (*Cirrhinus mrigala*) is still scanty. Therefore the current study was aimed to investigate endosulfan induced DNA damage in mori, an economically important indigenous Indian major carp.

MATERIALS AND METHODS

Test animal acclimatization: A total of 180 fingerlings of Indian major carp mori (*Cirrhinus mrigala*) (weight: 9.21±1.12 g; length: 10.45±1.32 cm) were acclimatized for fifteen days and were fed (35% basal protein diet) twice daily at the rate of 5% b.wt., prior starting the experiment. Feed remains and excretory wastes were siphoned off daily to avoid stress. Water quality parameters were checked daily.

Experimental design: After acclimatization, the fish were grouped (45 fingerlings in each group, 15 individuals per aquarium) and exposed to sublethal concentrations (0.5, 0.75 and 1 μg L^{-1}) of endosulfan in triplicates. Group 1st (control group) was not exposed to endosulfan while group 2nd, 3rd and 4th were exposed to 0.5, 0.75 and 1 μg L^{-1} of endosulfan, respectively. Blood was collected of caudal veins after 7, 14, 21 and 28 days for assessing DNA damage.

Comet assay: The DNA damage was assessed through comet assay by following Singh *et al.* (1988). The slides, gently neutralized (0.4 M tris buffer, pH 7.5) after electrophoresis were stained (Acridine orange stain, 3-4 mL of 0.2 mg mL^{-1} of distilled water) and analyzed through epifluorescent microscopy, 400X (Nikon AFX-1 Optihot).

Cells having comet like appearance were having damaged DNA while intact nuclei was having no DNA damage. The DNA damage was observed as DNA migration length in the tails of the comets (Grover *et al*., 2003). While comet scoring, cells having dispersed heads or no heads were excluded, considering them as apoptotic cell. The captured digital images were analyzed by following Collins (2004) through comets' visual inspection (Class 0-4, as given in Fig. 1).

The DNA damage was assessed in terms of Genetic Damage Index (GDI), percent damage cells and cumulative tail length of the comets, as this comet scoring method gives sufficient calculable and quantifiable resolution, rational for multipurpose (Liao *et al*., 2009).

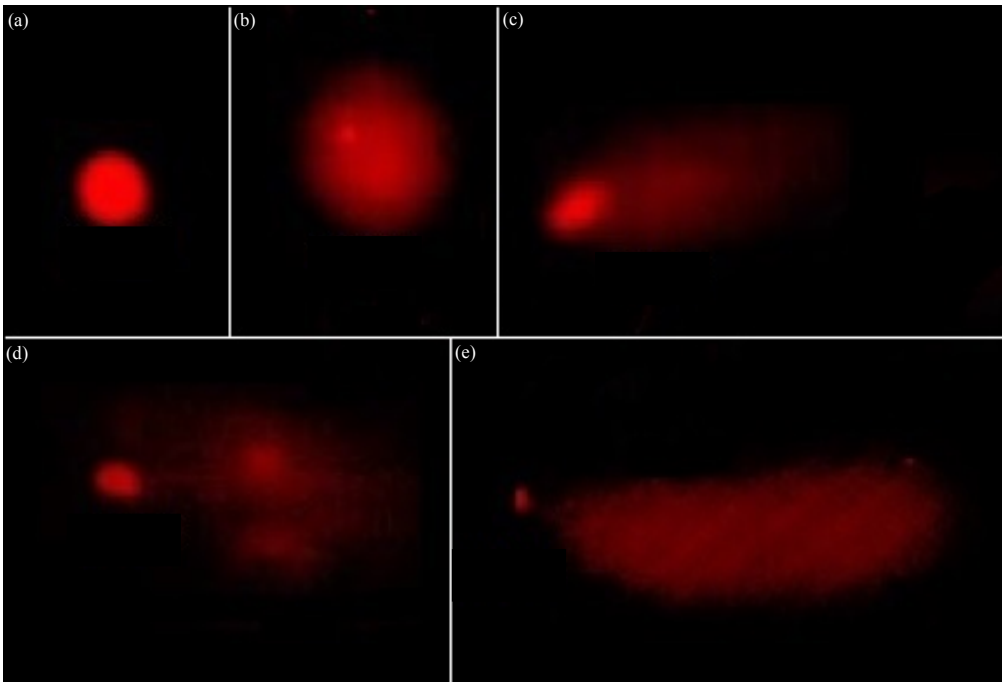

Fig. 1(a-e): DNA damage types/classes (comets), (a) Type 0, (b) Type 1, (c) Type 2, (d) Type 3 and (e) Type 4, induced in erythrocytes of *Cirrhinus mrigala*

Statistical analysis: Data expressed as Mean±SD was analysed through ANOVA followed by LSD test in Statistix Version X. Value of $p<0.05$ was considered as significant statistically.

RESULTS

The DNA damage was observed in all endosulfan treated groups. A time and concentration dependent increase was observed in DNA damage induced in peripheral blood erythrocytes of mori. Table 1-4 are showing DNA damage classes (damage type 0-4) induced after exposure to endosulfan at all three sublethal concentrations, while Table 5-8 are showing DNA damage, in terms of genetic damage index, percentage of damaged cell and cumulative tail length, observed after 7-28 days, respectively.

Water physico-chemical parameters: The experiment was carried out in ambient water, having physico-chemical parameters within permissible limits. During the experiment water temperature ranged between 22 and 25°C, pH <7.7, hardness <295 mg L^{-1}, ammonia <0.24 ppm while DO ranged between 6.9-7.5 mg L^{-1}.

Percentage of damaged cell (%): Percent damaged cell, in control group (not exposed to endosulfan) ranged from 3.50±0.1 to 4.60±1.0, in group 2nd (0.5 μg L^{-1}) 23.1±2.1 to 35.0±3.4, in group 3rd (0.75 μg L^{-1}) 34.0±1.2 to 43.7±1.3 while in group 4th (1 μg L^{-1}) percent damage cell ranged from 38.4±2.3 to 48.2±2.21 after 7-28 days of exposure to sublethal concentration of endosulfan.

Genetic Damaged Index (GDI): An increasing trend was observed in GDI with concentration, as the highest level of GDI was observed in Group 4th (1 μg L^{-1}) followed by Group 3rd (0.75 μg L^{-1}). A similar trend was observed with exposure time, as the highest GDI was observed after 28 days followed by 21 days of exposure. The GDI observed after 7 days was 0.1202±0.01, 0.9468±0.17, 1.3156±0.61 and 1.6350±0.71 in group 1st, 2nd, 3rd and 4th while 0.0848±0.00, 1.3262±0.06, 1.7088±0.02 and 1.8770±0.01 after 28 days of exposure, respectively.

Cumulative tail length of comets (μm): Cumulative tail length was highest in group 4th, followed by group 3rd after 28 days while least in group 1st after 7 days, followed by the same group after 14 days. Cumulative tail length ranged between 4.210±0.071 and 5.610±2.120 in group 1st, 113.87±5.91 and 152.51±12.6 in group 2nd, 155.56±9.87 and 198.42±10.6 in group 3rd and between 186.31±11.7 and 234.63±13.3 in group 4th.

Table 1: Endosulfan induced DNA damage in *Cirrhinus mrigala* after 7 days

	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
Groups	Type 0	Type 1	Type 2	Type 3	Type 4
Control	93.5±4.3^{a}	3.00±0.5^{c}	1.50±0.3^{c}	2.00±0.8^{d}	0.00±0.0^{d}
0.50 μg L^{-1}	64.2±5.3^{b}	12.7±1.8^{b}	9.10±1.5^{b}	11.5±0.6^{c}	2.50±0.3^{c}
0.75 μg L^{-1}	34.2±1.9^{c}	31.8±3.7^{a}	11.8±2.1^{a}	13.7±1.1^{b}	8.50±0.9^{b}
1.00 μg L^{-1}	31.6±2.3^{c}	30.0±4.1^{a}	10.0±1.7^{ab}	17.4±1.2^{a}	11.0±1.6^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test

Table 2: Endosulfan induced DNA damage in *Cirrhinus mrigala* after 14 days

	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
Groups	Type 0	Type 1	Type 2	Type 3	Type 4
Control	90.1±5.1^{a}	5.30±1.7^{d}	1.10±0.3^{c}	3.50±0.8^{c}	0.00±0.0^{d}
0.50 μg L^{-1}	52.3±7.2^{b}	20.4±3.4^{c}	13.3±1.9^{b}	9.81±1.2^{b}	4.19±0.7^{c}
0.75 μg L^{-1}	32.4±0.7^{c}	29.2±3.1^{a}	16.5±3.1^{a}	10.1±1.6^{b}	11.8±3.2^{b}
1.00 μg L^{-1}	30.2±1.1^{c}	27.5±3.1^{b}	13.5±2.1^{b}	15.4±2.1^{a}	13.4±2.4^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test

Table 3: Endosulfan induced DNA damage in *Cirrhinus mrigala* after 21 days

	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
Groups	Type 0	Type 1	Type 2	Type 3	Type 4
Control	88.2±7.3^{a}	8.10±0.7^{c}	1.40±0.7^{c}	2.30±0.4^{c}	0.00±0.0^{d}
0.50 μg L^{-1}	50.2±4.5^{b}	18.2±3.4^{b}	13.3±1.2^{b}	13.2±2.3^{b}	5.10±0.8^{c}
0.75 μg L^{-1}	30.2±1.3^{c}	28.9±2.4^{a}	17.2±0.9^{a}	14.1±1.1^{ab}	9.60±2.4^{b}
1.00 μg L^{-1}	28.2±1.1^{c}	26.2±1.2^{ab}	13.2±1.4^{b}	16.1±1.3^{a}	16.3±1.4a

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05)., ANOVA followed by LSD test

Table 4: Endosulfan induced DNA damage in *Cirrhinus mrigala* after 28 days

	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
Groups	Type 0	Type 1	Type 2	Type 3	Type 4
Control	88.2±6.6^{a}	8.07±0.9^{c}	1.40±0.4^{d}	2.30±0.6^{c}	0.00±0.0^{c}
0.50 μg L^{-1}	48.7±3.4^{b}	16.3±2.1^{b}	11.6±1.5^{c}	13.7±1.3^{b}	9.70±1.4^{b}
0.75 μg L^{-1}	29.8±1.7^{c}	26.5±0.9^{a}	18.7±2.4^{a}	14.8±2.1^{ab}	10.2±0.6^{b}
1.00 μg L^{-1}	26.2±1.5^{d}	25.6±1.2^{a}	14.5±1.7^{b}	15.4±1.1^{a}	18.3±3.3^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test

Table 5: Endosulfan induced geno-toxicity in *Cirrhinus mrigala* after 7 days

Groups	Damaged cells (%)*	**Genetic damage index (GDI)	Cumulative tail length (μm)
Control	3.50±0.1^{d}	0.1202±0.01^{d}	4.210±0.071^{d}
0.50 μg L^{-1}	23.1±2.1^{c}	0.9468±0.17^{c}	113.870±5.91^{c}
0.75 μg L^{-1}	34.0±1.2^{b}	1.3156±0.61^{b}	155.560±9.87^{b}
1.00 μg L^{-1}	38.4±2.3^{a}	1.6350±0.71^{a}	186.310±11.7^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test, Damaged cell (%)* = Type II+type III+type IV, **Genetic damage index = Type I+2(type II)+3(type III)+4(type IV)/type 0+type I+type II+type III+type IV

Table 6: Endosulfan induced genotoxic damage in *Cirrhinus mrigala* after 14 days

Groups	Damaged cells (%)	Genetic damage index (GDI)	Cumulative tail length (μm)
Control	4.60±1.0^{d}	0.1100±0.01^{d}	4.251±1.211^{d}
0.50 μg L^{-1}	27.3±2.3^{c}	1.0503±0.06^{b}	125.410±5.76^{c}
0.75 μg L^{-1}	38.4±1.7^{b}	1.5383±0.11^{a}	169.160±9.39^{b}
1.00 μg L^{-1}	42.3±2.1^{a}	1.7417±0.09^{a}	197.140±8.18^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test

Table 7: Endosulfan induced genotoxicity in *Cirrhinus mrigala* after 21 days

Groups	Damaged cells (%)	Genetic damage index (GDI)	Cumulative tail length (μm)
Control	3.70±0.9^{d}	0.1020±0.01^{d}	5.110±2.010^{d}
0.50 μg L^{-1}	31.6±3.1^{c}	1.2471±0.21^{b}	134.430±9.51^{c}
0.75 μg L^{-1}	40.9±1.2^{b}	1.6813±0.05^{a}	183.730±13.8^{b}
1.00 μg L^{-1}	45.6±2.3^{a}	1.8086±0.10^{a}	217.760±11.2^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test

Table 8: Endosulfan induced genotoxic damage in *Cirrhinus mrigala* after 28 days

Groups	Damaged cells (%)	Genetic damage index (GDI)	Cumulative tail length (μm)
Control	3.70±0.1^{d}	0.0848±0.00^{c}	5.610±2.120^{d}
0.50 μg L^{-1}	35.0±3.4^{c}	1.3262±0.06^{b}	152.510±12.6^{c}
0.75 μg L^{-1}	43.7±1.3^{b}	1.7088±0.02^{a}	198.420±10.6^{b}
1.00 μg L^{-1}	48.2±2.1^{a}	1.8770±0.01^{a}	234.630±13.3^{a}

Data are represented as Mean±SD (n = 6). Means followed by different letters within the column are significantly different (p<0.05), ANOVA followed by LSD test

DISCUSSION

Currently, over thousand chemicals are classified as pesticides. Some of these pesticides have been studied against different animal models and humans for their genotoxic potentials (Zeljezic and Garaj-Vrhovac, 2002; Bhalli *et al*., 2006, 2009). Animals including different species of mammals, birds, amphibians and fish have shown different extent of DNA damage after exposure to these pesticides. Different aquatic organism, specifically fish, have been employed at most of the occasions due to their prominent role in trophic web, economic worth and capability of accumulating toxic pollutants as well as responsiveness to different carcinogenic, genotoxic and mutagenic toxicants even at very low concentration (Osman *et al*., 2007; Banu *et al*., 2001; Ali *et al*., 2008; De Andrade *et al*., 2004; Jha, 2008).

Genotoxicological studies is a significant approach to achieve a greater intuition regarding the induced DNA damage, the capability of DNA repair of an organism as well as its protective mechanisms against different pollutants. Moreover, tissues specific response to specific mutagens can also be assessed. Such as, in case of fish, employing erythrocytes for evaluating DNA damage using single cell gel electrophoresis might be beneficial on account of being an easy tissues to collect and easy processing through SCGE, specifically in case of tiny fish species (Sumathi *et al*., 2001; Ullah *et al*., 2016c). Previous studies regarding investigations of DNA damage revealed SCGE as a significant, versatile and useful assay (Frenzilli *et al*., 2009; Galindo *et al*., 2010). The SCGE has been employed for measuring DNA damage in different organisms including, mollusks (Cotelle and Ferard, 1999; Canty *et al*., 2009), reptiles (Bronikowski, 2008), amphibians (Cotelle and Ferard, 1999; Yin *et al*., 2009), mammals (Park *et al*., 2007; Garaj-Vrhovac *et al*., 2009) and birds (Baos *et al*., 2006).

In the current study a significant ($p<0.05$) genotoxic effect of endosulfan was observed, from time of exposure as well as concentrations, which clearly indicated the genotoxic potential of endosulfan. The current findings were in agreement with the previous studies conducted on different fish species including *Ameiurus nebulosus* (Pandrangi *et al*., 1995), *Tilapia mossambica* (Banu *et al*., 2001), *Dreissena polymorpha* (Pavlica *et al*., 2001), *Channa punctatus* (Kushwaha *et al*., 2000; Pandey *et al*., 2006; Ali *et al*., 2008), *Mugil* sp. and *Netuma* sp., (De Andrade *et al*., 2004), *Carassius auratus* (Masuda *et al*., 2004), *Cyprinus carpio* (Buschini *et al*., 2004; Gustavino *et al*., 2005) and *Labeo rohita* (Ullah, 2015; Ullah *et al*., 2016b, c).

The DNA damage observed in the current study could possibly be initiated from DNA single or double strand breaks, formations of DNA adducts and DNA-Protein or DNA-DNA cross links, which might resulted due to the interaction of DNA and pesticide or its metabolites (Fairbairn *et al*., 1995; Mitchelmore and Chipman, 1998). However, further study is required in order to know the precise mechanism of endosulfan induced genotoxicity as well as to know regarding, which metabolite of endosulfan (alpha and beta) is responsible for DNA strand breaks or either both are responsible (Lu *et al*., 2000). Yet clastogenic activity, of either of the isomers or their metabolites may exist (Dzwonkowska and Hubner, 1986; Khan and Sinha, 1993). Genotoxicity in fish can be correlated with clastogenicity of endosulfan, as an elevation was observed in DNA migration in the current study as well as in previous studies after use of different environmental mutagens (Russo *et al*., 2004).

Endosulfan is highly toxic to different species of mammals and fish, which might be attributed to its estrogenic activity. It may possibly bio-accumulate in various edible aquatic organisms and can probably cause human fatalities (Naqvi and Newton, 1990; Belpaeme *et al*., 1998). However, the genotoxic potential of endosulfan is still to be explored on different fish species. The observed DNA damage in the current study might be linked to Reactive Oxygen Species (ROS) formation during biotransformation of endosulfan, as ROS is highly toxic to fish (Ullah *et al*., 2016a). The ROS directly break DNA via hydrogen peroxide or hydroxyl ions, subsequently result in oxidized bases of DNA (Akcha *et al*., 2003). Antioxidant defense system of fish neutralizes ROS but when ROS production exceeds antioxidant enzymes production, it leads to cellular lesions which result in DNA damage (Cadet *et al*., 2003; Cavalcante *et al*., 2008; Jha, 2008). Hence, DNA damage is much attributed by oxidative DNA damage due to higher production of ROS (Wilson *et al*., 1998; Pavlica *et al*., 2001).

CONCLUSION

Endosulfan was found as highly genotoxic to mori, even at lower sublethal concentration. The results of the current study revealed apprehension regarding the potential dangers of endosulfan to fish, as the similar concentration of endosulfan has been reported in river systems of some African countries. The current findings indicated SCGE/comet assay, as a reliable sensitive assay for investigating genotoxicity induced in fish after pesticides or other toxicants exposure.

Moreover, four weeks period of experimentation appears to be satisfactory for determining DNA damage induced by sublethal concentrations of endosulfan in fish using SCGE/comet assay.

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