Diagnosis of endosulfan induced DNA damage in rohu (*Labeo rohita,* Hamilton) using comet assay

Ullah S.[1,2*]; Hasan Z.[2]; Zorriehzahra M.J.[3]; Ahmad S.[4]

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Abstract

Use of different pesticides in the agriculture sector, in order to boost crop yield within a short time period and low labor, has been tremendously increased since the last decade. Pesticide use has elevated crop yield but has produced a number of pronounced problems regarding environmental and health safety. The continuously deteriorating toxicological effects of these pesticides are not only hazardous to humans and land animals but also to economically important aquatic organisms such as fish. One of these extensively used pesticides is an organochlorine insecticide, endosulfan. Experiments conducted in the past have shown the deleterious effects of endosulfan on different aspects of various fish species but its genetic toxicity has not been well studied. The present study was conducted to diagnose the DNA damage induced by endosulfan in peripheral blood erythrocytes of an economically important teleost fish rohu, *Labeo rohita* (Hamilton, 1822) using comet assay. The fish were exposed to three different sub lethal concentrations (1, 1.5 and 2 µg L^{-1}) of endosulfan for 7, 14, 21 and 28 days. Rohu showed different extents of DNA damage at different concentrations and time, in terms of genetic damage index (GDI), percentage of damaged cells (% damaged cell) and cumulative tail length (µm) of the comets. Increase in DNA damage was observed to be concentration and time-dependent. The current study revealed the severe genotoxic effects of endosulfan in rohu, *Labeo rohita*. Therefore its discriminate use should be avoided as it can contribute to the decline of rohu in natural habitats. Also it should be considered as a hazardous threat for human consumption.

Keywords: Endosulfan, DNA damage, Erythrocyte, Comet assay, Rohu

1-Fisheries and Aquaculture Lab., Department of Animal Sciences, Quaid-i-Azam University Islamabad 45320, Pakistan

2-Fisheries Lab., Department of Zoology, University of Peshawar, Peshawar, Khyber Pakhtunkhwa, Pakistan

3-Department of Aquatic Animal Health and Disease, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran

4-Department of Zoology, University of Malakand, District Lower Dir, Khyber Pakhtunkhwa, Pakistan

*Corresponding author's Email: sunyuop@gmail.com

Introduction

Geno-toxicological studies deal with the chemicals that damage DNA and genetic material of the cell as well as its consequent impacts on the health of organisms (Ullah and Zorriehzahra, 2015). Pesticides have been widely used in the agriculture sector in order to elevate yield with low labor and time but have deleterious effects on ecosystem. On account of their accumulation and hazardous impacts on environment, animals and humans, pesticides have gained much attention these days (Ullah, 2015). Studies concerning LC_{50} of various pesticides have been carried out on a number of fish species (Das and Mukherjee, 2000; Tripathi and Verma, 2004; Indirabai *et al.*, 2010; Marigoudar *et al.*, 2012; Ullah *et al.*, 2014; Ghaffar *et al.*, 2015) as well as other animals including rats, ciliates, toads and crabs etc. (El-Demerdash, 2007; Madkour, 2012; Zaki, 2012; Deshai *et al.*, 2012; Ismail *et al.*, 2014; Amamra *et al.*, 2015) but comparatively less studies have been undertaken on its genotoxic effects.

A number of pesticides are being used these days, posing a serious threat to the biosphere. Among these pesticides, endosulfan is an agent, equally toxic to mammals, birds, insects, fish and other aquatic life (Das and Mukherjee, 2000). Endosulfan, an organochlorine pesticide, can damage all these organisms by biomagnifying in them and affecting their homeostasis (Indirabai *et al.*, 2010) and metabolic activities (Adhikari *et al.*, 2006). It can cause DNA damage in cells via different mode of action (Bhattacharya and Bhattacharya, 2007) such as cellular transformation, gene amplification, breaking DNA protein cross links and DNA strand rupturing (Ullah, 2015). It also leads to the production of reactive oxygen species (ROS) because of its capability to contribute to redox cycling (Ghaffar *et al.*, 2015).

Comet assay is considered as one the most versatile and best approaches for studying geno-toxicological effects of toxicants on fish (Nagarani *et al.*, 2012). It is used to estimate DNA damage for evaluating genetic risks linked with xenobiotic exposure (Ullah, 2015). Xenobiotics monitoring in local species can help in estimating environmental quality and human health (Kousar and Javed, 2015). Comet assay is reliable, fast, capable of detecting low level of DNA damage, requires short time and is a responsive technique for single or double strand breakage in DNA, cell death, inter strand cross linkages, incomplete excision repair sites, and alkali labile sites that are induced by chemical or physical agents in individual eukaryotic cells (Kim *et al.*, 2002; Ali *et al.*, 2015; Ullah et al., 2016a). It has been used for evaluating different toxicant induced genetic toxicity in peripheral erythrocytes in a number of fish species (Ali *et al.*, 2015; Kousar and Javed, 2015; Ullah, 2015).

Evaluating localization and concentration of different toxicants is

very much important because these not only affect human health but also animals, important for humans (Nicareta, 2004). According to Khan *et al.* (2012), only 1% industrial effluents get treated before being disposed off into streams, lakes and rivers which clearly indicates that Pakistan is faced with acute freshwater pollution. Direct discharge of domestic waste, industrial effluents, agricultural runoffs and untreated water from various factories is adversely affecting freshwater fauna, fish being the most vulnerable and important (Jabeen and Javed, 2012). On account of aquatic pollution, indigenous fish species are on the brink of extinction in the rivers in Pakistan (Rauf *et al.*, 2009).

The current scenario is demanding toxicological studies for identifying adverse effects, tolerance levels of fish and permissible ranges of different pesticides in natural water bodies for aquatic organisms. This will not only help in maintaining a healthy environment but will also result in devising proper strategic management for fish fauna conservation in the natural habitats. Keeping in view the current condition of extensive pesticide use and decline of natural fisheries potential in Pakistan, the current study was conducted to assess the genetic toxicity induced by endosulfan in rohu, *Labeo rohita* (Hamilton, 1822), an economically important freshwater teleost.

Materials and methods

Test animal acclimatization

A total of 180 fingerlings of rohu, *Labeo rohita* (6.3±0.87 g weight and 7.9±0.65 cm length) were collected and acclimatized to lab conditions for fifteen days before starting the experiment. During acclimatization the fish were fed to satiation (35% basal protein diet) twice daily (5% body weight). Excretory wastes and feed remains were siphoned off every day in order to avoid stress to the fingerlings. Water was changed on a daily basis. During this period temperature (26.5°C), pH (7.5), hardness (300 mg L^{-1}), ammonia (<0.25 ppm) and DO (7−7.4 mgL^{-1}) were checked on a daily basis and efforts were made to keep them within optimum ranges.

Experimental design

To govern DNA damage in peripheral blood erythrocytes of rohu, the fingerlings were exposed to three sub lethal concentrations of endosulfan (1, 1.5 and 2 µg L^{-1}) after 15 days of acclimation. Group 1 served as the control group (having distilled water only without endosulfan) while Group 2, 3 and 4, designated as experimental groups received 1, 1.5 and 2 μgL^{-1} endosulfan respectively. After 7, 14, 21, and 28 days, blood was collected from caudal vein of the fish. The experiment was carried out in triplicates.

Comet assay

The collected blood samples were processed for the assay by following

Singh *et al.* (1988). After electrophoretic analysis, the slides were gently neutralized in 0.4 M Tris buffer (pH = 7.5) and were stained using Acridine Orange stain (300-400 µL of 20µg/ml of distilled water) and analyzed using epifluorescent microscopy (400X, Nikon AFX-1 Optiphot). The captured digital images were analyzed.

Cells having no DNA damage had intact nuclei without tails while cells having DNA damage had a comet like appearance in shape. DNA migration length in comets' tail was projected as DNA damage (Grover *et al.*, 2003). Cells without heads or dispersed heads were not included for analysis and were considered as apoptotic cells. DNA damage was evaluated in terms of percent of damage cells and genetic damage index (GDI) by following Collins (2004) through visual inspection of the comets, a) comet class 0 (no damage, hence no tail), b) comet class 1 (tail up to 1.5 times the diameter of the comet nucleus), c) comet class 2 (tail 1.5–2.0 times the diameter of the comet nucleus), d) comet class 3 (tail 2.0–2.5 times the diameter of the comet nucleus) and e) comet class 4 (maximally damaged with total DNA in its tail) (Fig. 1). This method of comet scoring gives enough quantifiable and calculable resolution which is reasonable for many purposes (Liao *et al.*, 2009).

Statistical analysis

Data obtained from experiment were expressed as mean±SE. The results were analysed through one way analysis of variances (ANOVA) followed by least significant difference (LSD) test using Statistix Version 8.1. $p<0.05$ was considered as statistically significant.

Results

All the three concentrations of endosulfan induced DNA damage. The severity of damage in the DNA of blood peripheral erythrocytes of rohu was time course and concentration dependent. Tables 1, 3, 5 and 7 show comet classes (Type 0 damage to type 4 damage) after 7, 14, 21 and 28 days of exposure of rohu to three sub lethal concentrations of endosulfan. Tables 2, 4, 6, and 8 show DNA damage in term of genetic damage index GDI, percentage of damaged cell (% damaged cell), and cumulative tail length (µm) after exposure of rohu to endosulfan for 7, 14, 21, and 28 days, respectively.

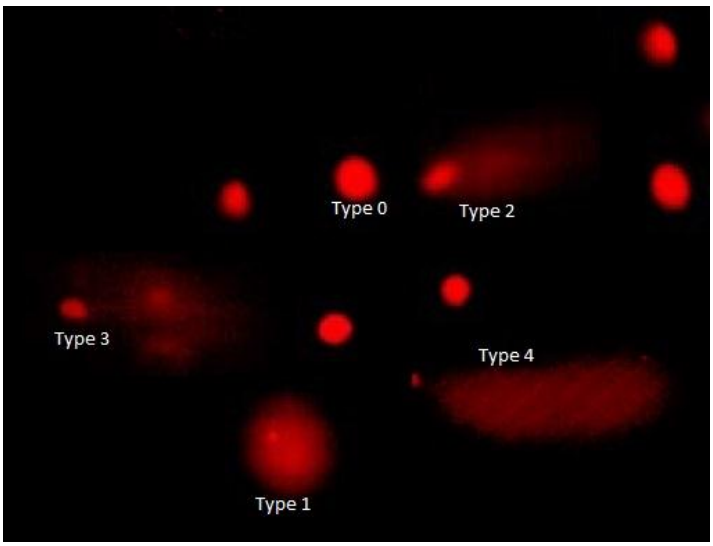

Figure 1: Comets showing DNA damage induced by endosulfan in erythrocytes of rohu. DNA damage types/ classes are also were showed.

Table 1: DNA damage induced by endosulfan (µg L^{-1}) in rohu after 7 days of exposure.

Groups	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
	Type 0	**Type 1**	**Type 2**	**Type 3**	**Type 4**
Control	92.66±0.01^{a}	4.00±0.58^{d}	2.00±0.58^{c}	1.340±0.01^{d}	0.00±0.00^{d}
2 µg L^{-1}	29.30±0.12^{d}	25.7±0.12^{a}	14.50±0.12^{a}	13.20±0.06^{c}	17.3±0.58^{a}
1.5 µg L^{-1}	47.33±0.012^{c}	12.18±0.01^{b}	11.09±0.01^{b}	20.40±0.12^{a}	9.00±1.16^{b}
1 µg L^{-1}	61.33±0.64^{b}	7.31±0.02^{c}	10.13±0.01^{b}	17.81±0.01^{b}	3.42±0.02^{c}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p< 0.05). (ANOVA followed by LSD test).

Table 2: Geno-toxicity caused by endosulfan in peripheral erythrocyte after 7 days.

Groups	%age of damaged cells*	Genetic damage index (GDI)**	Cumulative tail length (µm)
Control	3.34±0.01^{d}	0.12±0.01^{d}	3.01±0.01^{d}
2 µg L^{-1}	45.00±1.16^{a}	1.64±0.01^{a}	191.43±11.78^{a}
1.5 µg L^{-1}	40.49±0.02^{b}	1.32±0.01^{b}	163.56±9.87^{b}
1 µg L^{-1}	31.36±0.02^{c}	0.95±0.01^{c}	109.87±7.89^{c}

Data are represented as Mean±SE (n=3). Means followed by different letters ithin the column are significantly different (p< 0.05). (ANOVA followed by LSD test).

* %age of Damaged Cell = Type II + Type III + Type IV

** GDI = Type I + 2(Type II) + 3(Type III) + 4(Type IV)/Type 0 + Type I + Type II + Type III+ Type IV

Table 3: DNA damage induced by endosulfan (µg L^{-1}) in rohu after 14 days of exposure.

Groups	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
	Type 0	**Type 1**	**Type 2**	**Type 3**	**Type 4**
Control	94.00±1.55^{a}	3.20±0.12^{d}	1.20±0.12^{c}	1.00±0.01^{c}	0.60±0.12^{d}
2 µg L^{-1}	23.33±0.06^{d}	29.27±0.57^{a}	16.50±0.06^{a}	11.7±3.24^{b}	19.2±0.06^{a}
1.5 µg L^{-1}	35.33±0.01^{c}	21.70±0.2^{b}	12.07±0.1^{b}	15.6±0.12^{a}	15.3±0.12^{b}
1 µg L^{-1}	54.66±0.02^{b}	11.43±0.017^{c}	14.32±0.01^{ab}	13.4±0.24^{ab}	6.19±0.01^{c}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p<0.05). (ANOVA followed by LSD test).

Table 4: Genotoxic damage in peripheral erythrocyte of rohu after 14 days.

Groups	%age of damaged cells	Genetic damage index (GDI)	Cumulative tail length (µm)
Control	2.80±0.23^{d}	0.11±0.01^{c}	3.11±0.05^{d}
2 µg L^{-1}	47.40±0.12^{a}	1.74±0.01^{a}	203.71±15.45^{a}
1.5 µg L^{-1}	42.97±0.01^{b}	1.54±0.01^{a}	179.21±11.23^{b}
1 µg L^{-1}	33.91±0.01^{c}	1.05±0.01^{b}	117.54±8.89^{c}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p<0.05). (ANOVA followed by LSD test).

Table 5: DNA damage induced by endosulfan (µg L^{-1}) in rohu after 21 days of exposure.

Groups	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
	Type 0	Type 1	Type 2	Type 3	Type 4
Control	91.3±0.12^{a}	7.20±0.12^{d}	1.50±0.11^{c}	0.00±0.00^{c}	0.00±0.00^{c}
2 µg L^{-1}	20.00±1.15^{d}	27.52±0.02^{a}	21.44±0.01^{a}	13.70±0.12^{b}	17.34±0.02^{a}
1.5 µg L^{-1}	31.33±0.02^{c}	21.21±0.02^{b}	11.21±0.01^{b}	20.5±0.15^{a}	15.75±0.02^{ab}
1 µg L^{-1}	52.00±1.16^{b}	9.43±0.01^{c}	12.45±0.01^{b}	14.1±0.06^{b}	12.02±0.01^{b}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p<0.05). (ANOVA followed by LSD test).

Table 6: Genotoxic damage caused by endosulfan in peripheral erythrocyte of rohu after 21 days.

Groups	%age of damaged cells	Genetic damage index (GDI)	Cumulative tail length (µm)
Control	1.50±0.2^{d}	0.102±0.01^{c}	4.01±0.12^{d}
2 µg L^{-1}	52.48±0.05^{a}	1.81±0.01^{a}	223.39±14.05^{a}
1.5 µg L^{-1}	47.46±0.04^{b}	1.68±0.01^{a}	192.17±14.32^{b}
1 µg L^{-1}	38.57±0.04^{c}	1.25±0.02^{b}	128.56±7.65^{c}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p<0.05). (ANOVA followed by LSD test).

Table 7: DNA damage induced by endosulfan (µg L^{-1}) in rohu after 28 days of exposure.

	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)			
Groups	Type 0	Type 1	Type 2	Type 3	Type 4
Control	92.66±0.13^{a}	6.2±0.12^{c}	1.14±0.02^{c}	0.00±0.00^{d}	0.00±0.00^{c}
2 µg L^{-1}	17.33±0.02^{d}	26.42±0.01^{a}	26.11±0.01^{a}	11.5±0.06^{c}	18.64±0.01^{a}
1.5 µg L^{-1}	28.67±0.01^{c}	24.2±0.2^{a}	9.07±0.01^{b}	23.7±0.12^{a}	14.36±0.02^{b}
1 µg L^{-1}	48.00±0.58^{b}	13.18±0.01^{b}	10.32±0.01^{b}	15.2±0.12^{b}	13.3±0.17^{b}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p<0.05). (ANOVA followed by LSD test).

Table 8: Genotoxic damage caused in peripheral erythrocyte of rohu after 28 days.

Groups	%age of damaged cells	Genetic damage index (GDI)	Cumulative tail length (µm)
Control	1.14±0.01^{d}	0.08±0.001^{d}	3.41±0.01^{d}
2 µg L^{-1}	56.25±0.03^{a}	1.88±0.01^{a}	247.53±17.09^{a}
1.5 µg L^{-1}	47.13±0.01^{b}	1.71±0.02^{b}	207.15±15.53^{b}
1 µg L^{-1}	38.82±0.01^{c}	1.33±0.01^{c}	141.12±8.71^{c}

Data are represented as Mean±SE (n = 3). Means followed by different letters within the column are significantly different (p<0.05). (ANOVA followed by LSD test).

Percentage of damaged cell (%)

An overall damage from 45.00±1.16 to 56.25±0.03 was observed in Group 2 (2 μgL^{-1}), from 40.49±0.02 to 47.13±0.01 in Group 3 (1.5 μgL^{-1}) and from 31.36±0.02 to 38.82±0.01 was observed in Group 4 (1 μgL^{-1}) after exposure to sub lethal concentrations of endosulfan from day 7 to day 28, respectively. Maximum damage was observed for Group 2 followed by Group 3. For Group 1 (Control) values of % damaged cell ranged between 1.14±0.01 and 3.34±0.01 during the study period.

Genetic damaged index (GDI)

Genetic damaged index was highest for Group 2 followed by Group 3. An increasing trend was observed in GDI values with exposure time and endosulfan concentration. GDI after 7 days was observed to be 0.1202±0.01, 1.635±0.01, 1.3156±0.01 and 0.9468±0.01 for Groups 1, 2, 3, and 4, respectively. GDI after 28 days was 0.0848±0.001 in Group 1. GDI value increased to 1.877±0.01, 1.7088±0.02 and 1.3262±0.01 in Groups 2, 3 and 4 respectively on day 28 of exposure.

Cumulative tail length of comets (µm)

In terms of cumulative tail length, different values were observed for Group 1 to 4. Cumulative tail length followed the order of Group 2 > Group 3 > Group 4 > Group 1. Values for cumulative tail length were 3.01±0.01, 191.43±11.78, 163.56±9.87 and 109.87±7.89 after 7 days while 3.41±0.01, 247.53±17.09, 207.152±15.53 and 141.121±8.71 after 28 days in Groups 1, 2, 3, and 4, respectively.

Discussion

Growth of agricultural, industrial and commercial chemicals has resulted in increase of genetic disorders, mortalities diseases of exposed organisms in natural habitats (Lenártová *et al.*, 1997). Around the world, thousands of different pollutants are discharged off to the aquatic bodies. Insecticides are one of the major groups of pesticides, posing serious threats to functionality and integrity of cellular DNA in aquatic organisms, fish being the most important economically (Ullah and Zorriehzahra, 2015). Therefore it is the need of the day to study the adverse impacts of these chemicals on fish at DNA level. During the present study an increasing trend was observed in DNA damage level and severity with increase in endosulfan concentration and exposure time.

The genetic toxicity observed in the present study is in accordance with other previous studies conducted on DNA damage of different fish species for different toxicants. Arsenic induced DNA damage in blood cells of *Oreochromis mossabicus* and showed concentration dependent increase in damage (Ahmed *et al.*, 2011). Kousar and Javed (2015) observed DNA damage in blood erythrocyte of *Labeo rohita*, *Cirrhina mrigala*, *Catla catla* and *Ctenopharyngodon idella* after exposure to Arsenic, copper and zinc. They also observed the same pattern of DNA damage as being time and concentration dependent. Farombi *et al.* (2007) also reported time dependent genotoxic effects of arsenic, copper,

zinc, cadmium and lead in *Clarias gariepinus*. Findings of the current study were also in agreement with a previous study conducted on genotoxic effects of lead, iron, zinc, nickel, chromium and copper inducing DNA damage in *Hyphssobrycon luetkenii* (Scalon *et al.*, 2010). Arsenic induced DNA damage in *Carassius auratus* and *Channa punctatus* in a time dependent manner (Kumar *et al.*, 2013). Dinitrobisphenol A induced DNA damage in *Carrasius auratus* (Toyoizumi *et al.*, 2008).

Different pesticides such as endosulfan, cypermethrin, malathion, delmithrin, paraquat and λ–cyhalothrin etc. have been reported as causative agents of increment in oxidative stress enzymes' activities (El-Demerdash, 2007; Fetoui *et al.*, 2010; Madkour, 2012; Ullah *et al*., 2016b) such as superoxide dismutase (SOD), glutathione content (GSH), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSR), acetylcholine esterase (AChE) and induction of lipid peroxidation (LPO) activity (Lenártová *et al.*, 1997; Ullah, 2015) in different fish species such as *Clarias batrachus* (Tripathi and Verma, 2004), *Ctenopharyngodon idella, Labeo rohita* (Indirabai *et al*., 2010; Marigoudar *et al.*, 2012), *Cyprinus carpio* (David *et al.*, 2008) and *Tor putitora* (Ullah *et al.*, 2014) etc. These enzymes counteract the effects of reactive oxygen species (ROS) produced because of the pollutants such as pesticides, heavy metals, various drugs and other industrial effluents (Lenártová *et al.*, 1997; Van der Oost *et al.*, 2003; Ullah, 2015). All biomolecules including proteins, lipids and DNA etc. are severely damaged by these highly reactive compounds (Ali *et al.*, 2015; Ullah *et al*., 2016a, 2016b, 2016c). DNA is one of the more vulnerable biochemical molecules to these pollutants and is responsible for genotoxicity or genetic damage (Ullah *et al.*, 2016c).

During the present study significantly higher DNA damage in terms of cumulative tail length, genetic damage index and percent damaged cells was observed in Group 2, exposed to 2 μgL^{-1} endosulfan followed by Group 3, exposed to 1.5 μgL^{-1}. Induction of DNA damage in Group 4, exposed to 1 μgL^{-1}, clearly demonstrates strong genotoxic effects of endosulfan in *Labeo rohita*, even at low concentrations. The current study also showed concentration dependent DNA damage induced by endosulfan in the peripheral blood erythrocytes of rohu. Time course increase in DNA damage, evaluated through the studied parameters (GDI, % damaged cell and cumulative tail length), demonstrated time dependent increment in genotoxic effects of endosulfan to *Labeo rohita*.

Fish have the capability of metabolizing and accumulating contaminants within their body, hence they are being used as a vertebrate model for estimating potential risks (Diekmann *et al.*, 2004). Using fish blood cells for SCGE (single cell gel electrophoresis) has attained much attention because of the nucleated erythrocytes of fish, which render it

more suitable for obtaining nucleoids for SCGE (Costa *et al.*, 2011). Peripheral blood demonstrates the health status of any organism; hence peripheral blood erythrocytes of fish are more appropriate for DNA damage analysis and evaluating environmental contaminants in fish (Ateeq *et al.*, 2005; Ergene *et al.*, 2007).

The present investigation revealed that *Labeo rohita* is considerably sensitive to endosulfan by exhibiting significantly higher GDI, percent damaged cell and cumulative tail length of the comets. During the present study, the length of cumulative tail even at the lowest sub lethal concentration demonstrated higher susceptibility of rohu to endosulfan. The results also showed inability of rohu to interact against endosulfan, thus suggesting a serious concern regarding potential danger to carps survival in natural water bodies.

Generally, the results of the present study showed deleterious genotoxic damage induced by endosulfan at all three concentrations in peripheral blood erythrocytes of Rohu. Moreover it is concluded that comet assay can be used for detecting toxicants, chemicals or pollutants in natural environments as well, such as river, lakes and other aquatic habitats.

The toxicities of pesticides are often underestimated by the users but indiscriminate use of these pesticides can lead to some serious concerns regarding environment. Some of these might not reveal the ill impacts *in vivo* but can pose different serious threats on human health. Therefore pesticides should be employed with proper care and precautionary measures.

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