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Pesticide Mixture Induced DNA Damage in Peripheral Blood Erythrocytes of Freshwater Fish, *Oreochromis niloticus*

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ABSTRACT

The present study was undertaken to examine the DNA damage induced by pesticide mixture (endosulfan+chlorpyrifos) in peripheral blood erythrocytes of freshwater fish, *Oreochromis niloticus* by using Comet assay. The 96-h LC_{50} value of pesticide mixture was estimated for 180-old fingerlings of *O. niloticus*in a static system and then four sub-lethal concentrations *viz.* 1/3rd of LC_{50}, 1/4th of LC_{50}, 1/5th of LC_{50} and 1/6th of LC_{50} were calculated and fish were exposed to these concentrations, separately in glass aquaria for 70 days at constant laboratory conditions. Peripheral blood erythrocytes were taken on 14, 28, 42, 56 and 70 day of exposure for the DNA damage assessment in-terms of percentage of damaged cells. In general, a dose dependent response was observed in fish erythrocytes with induction of maximum DNA damage at highest concentration (1/3rd of LC_{50}) of pesticide mixture. Statistically significant effects for both concentrations and time of exposure in terms of DNA damage were observed in treated fish as compared to control group. The results supported the use of Comet assay for evaluating the toxicity of pollutants which may be used as part of environmental monitoring programs.

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AF designed the experiments, collected and analyzed the data and wrote the manuscript. JM reviewed the final version of manuscript.

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Comet assay, Insecticides, Genotoxicity, Sub-lethal effects.

INTRODUCTION

Different classes of pesticides are now frequently used against a number of pests, to increase the production of crops even though these are highly toxic to other non-target species in the environment (Pandey *et al*., 2008; Naqvi *et al*., 2016). An increase in global food demand has resulted in a significant increase in the use of pesticides in agriculture. Residues of pesticides reach the aquatic environment, representing a risk for the non-target organisms threatening the ecological balance and biodiversity (Asita and Makhalemele, 2008). As a result non-target organisms are exposed to a mixture of pesticides which may induce genetic damage (Konen and Cavas, 2008), representing major problem at local, regional, national and global scale (Cerejeira *et al*., 2003). Organochlorine and organophosphate are commonly detected pesticides classes in freshwater. Endosulfan belongs to organochlorine class of pesticides which are hazardous for the survival of life. This may modulate antioxidant defense system and cause oxidative damage in aquatic organisms due to production of reactive oxygen species (Liu *et al*., 2006). Among all forms of organophosphate pesticides, chlorpyrifos is one of the most widely used pesticides in the world. It is neurotoxic and irreversible inhibitor of the cholinesterase. Chlorpyrifos is highly toxic to aquatic organisms, and is among the most detected pesticide in rivers, streams, ponds and reservoirs (Ensminger *et al*., 2011). Genotoxicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern. Fishes are being used as useful genetic model for the evaluation of pollution in aquatic environment (Nagrani *et al*., 2009). The DNA integrity has been proposed as a sensitive biomarker for monitoring environmental mutagens (Lourenço *et al*., 2013). Xenobiotics inducing strand breaks over extended time periods represent a constant threat to genomic integrity. Therefore, there exists need for rapid and sensitive assays that can be able to detect the foremost DNA lesions (Mohanty *et al*., 2013). Among different techniques used for the determination of genotoxic damage, the Comet assay (single cell gel electrophoresis) can detect DNA damage in single cell (Singh *et al*., 1988). This is very rapid and sensitive assay that can be successfully applied to the nucleated red blood cells of many fish species, exposed to different genotoxicants and environmental stressors (Mustafa *et al*., 2011). A number of studies have shown that Comet assay is the most effective assay for the detection of genotoxic effect of xenobiotics under the field (Polard *et al*., 2011) and laboratory conditions (Ali *et al*., 2008). Comet assay in alkaline conditions is a highly sensitive biomarker that can quantify and detect DNA damage such as single strand breaks, double strand breaks

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and alkali labile sites. These types of damages can be induced directly by the contaminants or indirectly through inactivation of repairing process (Tice *et al*., 2000). Therefore, the present study endeavor to investigate the DNA damage in terms of the percentage of damaged DNA in the freshwater fish, *Oreochromis niloticus* after in vivo chronic exposure to a binary pesticide mixture.

MATERIALS AND METHODS

Experimental fish and chemicals

The fingerlings of freshwater fish *O. niloticus* were purchased from local suppliers and transported to the Fisheries Research Farm, University of Agriculture, Faisalabad, Pakistan. Fish fingerlings were acclimatized under laboratory conditions in cemented tanks for two weeks and fed with pellet diet. The fecal matter and other waste materials were siphoned off daily to reduce ammonia contents in water. Endosulfan and chlorpyrifos of equal parts were dissolved, separately, in 95% analytical grade methanol (J.T Baker) as a carrier solvent to prepare the stock-I solutions (1g/100 ml) while binary mixture of pesticides were prepared by its further dilutions in deionized water (stock-II).

Determination of sub-lethal concentrations

The 96-h LC_{50} value of the pesticide mixture (endosulfan+chlorpyrifos) *i.e.,* 5.64 μgL^{-1} for the present study on *Oreochromis niloticus* was determined. Based on this value, four sub-lethal concentrations *viz*. 1/3rd of LC_{50} (1.88 μgL^{-1}), 1/4th of LC_{50} (1.41 μgL^{-1}), 1/5th of LC_{50} (1.13 μgL^{-1}) and 1/6th of LC_{50} (0.94 μgL^{-1}) were calculated and used for in vivo genotoxicity experiments.

Comet assay

Twelve fingerlings (180-day old with an average weight of 16.26±0.79 g) of *O. niloticus* were exposed to each of the four aforementioned test concentrations, separately, in glass aquaria having 70 L water capacity. Simultaneously, one group of fish was maintained in tap water also, which was considered as "control" (unstressed group). During 70 days of exposure period, the fish were fed daily small quantity of food. Water temperature (30 ºC), pH (7.75) and hardness (225 mgL^{-1}) were kept constant throughout the experiment. The exposure was continued for 70 days by using static water system and peripheral blood slides were prepared on day 14, 28, 42, 56 and 70 of exposure period and subjected to Comet assay. Aquaria water was renewed after every one week of interval. Experiment was conducted with three replications for each sub-lethal concentration. Blood samples were collected from caudal vein of fish, immediately transferred to eppendorf and treated with anticoagulants (heparin salt). Comet assay was performed as three steps procedure, followed by lysis, electrophoresis and staining (Singh *et al*., 1988). Two slides were prepared and one hundred and fifty cells per slide were randomly scored and analyzed by using an image analysis system attached to Epi-Fluorescence microscope (N-400M, American Scope; USA) at 400 X magnification and low lux (MD-800, American Scope; USA) camera. The DNA damage was quantified by visual classification of cells into the five categories "comets" corresponding to the tail length (measured through TriTekCometScore™) as undamaged (Type 0); low level damage (Type I); medium level damage (Type II); high level damage: (Type III) and complete damage (Type IV). The extent of DNA damage was examined as mean percentage of cells with medium, high and complete damaged DNA, which was calculated as the sum of cells with Types II+ III + IV. Statistical analyses were performed by using MSTATC computer software. Results were expressed as Means±SD. Means of data were compared for the statistical differences by using Duncan Range Multiple tests (Steel *et al*., 1996). A p-value less than 0.05 were considered statistically significant.

RESULTS

Acute toxicity test and sub-lethal concentrations

The 96-h LC_{50} value of endosulfan+chlorpyrifos mixture was calculated from the data obtained in acute toxicity bioassay by using Probit analyses method. The 96-h LC_{50} value for *O. niloticus* was 5.64 μgL^{-1}. Genotoxicity evaluation needs the live samples therefore; the sub-lethal concentrations were selected for Comet assay. Accordingly, four sub-lethal test concentrations were calculated as 1/3rd of LC_{50} (1.88 μgL^{-1}), 1/4th of LC_{50} (1.41 μgL^{-1}), 1/5th of LC_{50} (1.13 μgL^{-1}) and 1/6th of LC_{50} (0.94 μgL^{-1}) at which fish were exposed separately, for the detection of DNA damage.

DNA damage assessment

Proportions of undamaged nuclei (Type 0), damaged nuclei (Type I to IV) and percentage of damaged cells (Type II+III+IV) induced in the peripheral erythrocytes of *O. niloticus* under 70 day exposure of endosulfan+chlorpyrifos mixture are presented in Table I and Figure 1 (A-F).

Results showed that fish exposed to different concentrations of pesticide mixture exhibited significantly ($p<0.05$) higher DNA damage in their erythrocytes than the control specimens. Among all test concentrations, proportions of Type 0 cells were observed higher in control group on all sampling days. However, percentage of Type

I damaged cells were maximum on day 14 under 1/4th of LC_{50} while the maximum percentage of Type I damaged cells was observed on day 28, 42, 56 and 70 in fish exposed to 1/6th of LC_{50} as evident from their mean values of 30.00±2.00, 32.00±4.00, 34.00±2.00, 32.67±4.16 and 37.33±5.03%, respectively. However, Type I damaged nuclei exhibited non-significant differences between 1/5th and 1/6th of LC_{50} on 56th day of exposure. Percentage of Type II damaged cells ranged from 26.67 – 48.00% and exhibited concomitant increase in damage with exposure time from day 14 to 70 due to 1/3rd of LC_{50} exposure as compared to control group. Among different test concentrations, Type III damaged nuclei in peripheral blood erythrocytes of *O. niloticus* followed the sequence: 1/4thof $LC_{50} \geq 1/3^{rd}$of $LC_{50} > 1/5^{th}$ of $LC_{50} > 1/6^{th}$ of $LC_{50} >$ control on day 14; 1/3rd of $LC_{50} > 1/4^{th}$ of $LC_{50} > 1/5^{th}$ of $LC_{50} \geq 1/6^{th}$ of $LC_{50} >$ control on day 28; 1/3rd of $LC_{50} \geq 1/4^{th}$ of $LC_{50} > 1/6^{th}$ of $LC_{50} > 1/5^{th}$ of $LC_{50} >$ control on day 42; 1/3rd of $LC_{50} > 1/4^{th}$ of $LC_{50} > 1/6^{th}$ of $LC_{50} > 1/5^{th}$ of $LC_{50} >$ control on day 56 and 1/3rd of $LC_{50} > 1/4^{th}$ of $LC_{50} > 1/5^{th}$ of $LC_{50} > 1/6^{th}$ of $LC_{50} >$ control on day 70, respectively. However, on day 14 and 42, the proportion of Type III damaged cells showed non-significant differences between 1/3rd and 1/4th of LC_{50} exposures.

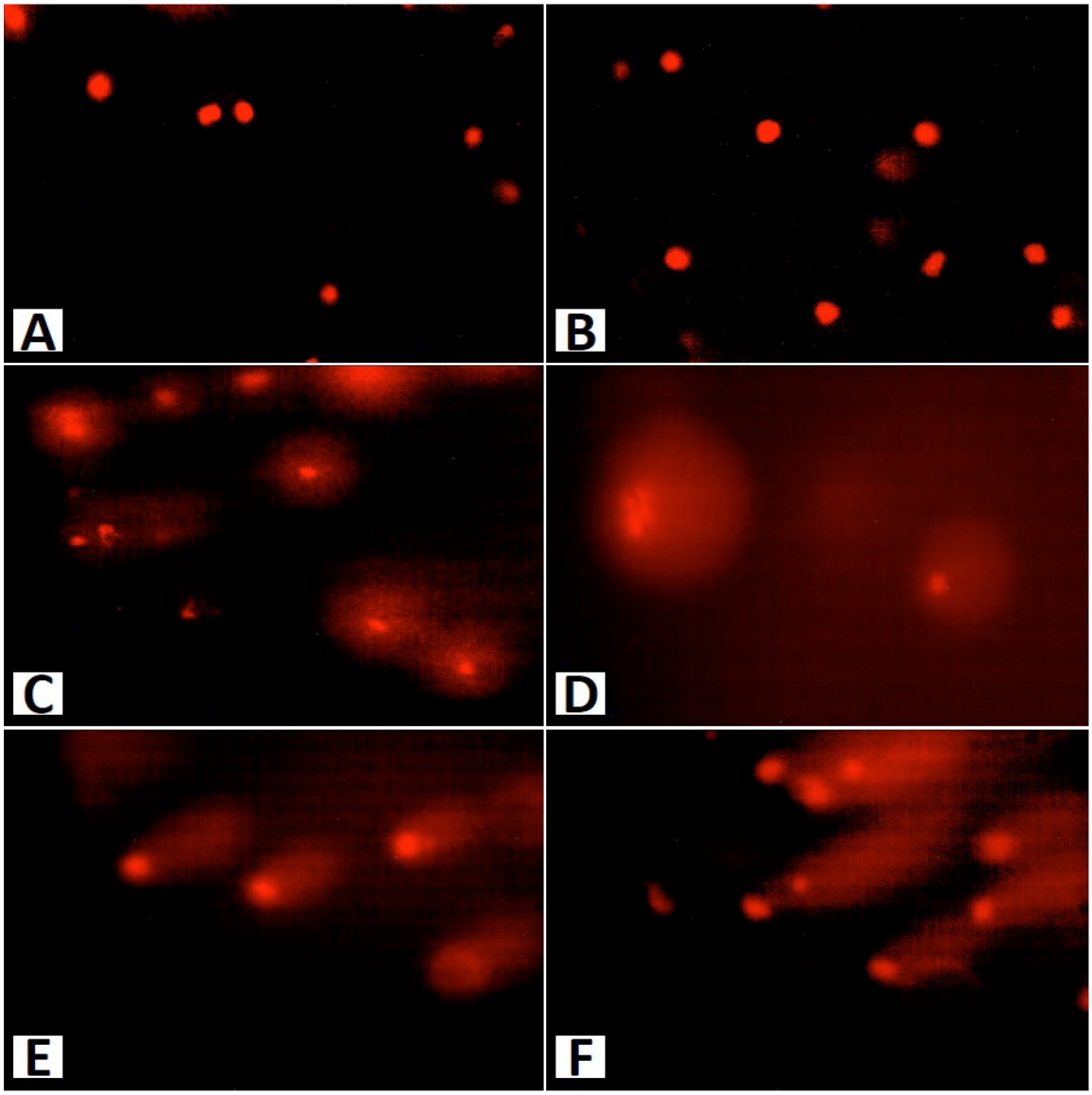

Fig. 1. Blood cells after Comet assay from control and treated group of *Oreochromis niloticus.* **A,** Control; **B,** Type 0 nuclei; **C,** Type I nuclei; **D,** Type II nuclei; **E,** Type III nuclei; **F,** Type IV nuclei.

Table I.- DNA damage in peripheral erythrocytes of *Oreochromis niloticus* exposed to endosulfan + chlorpyrifos mixture.

Exposure duration (days)	Test concentrations	Undamaged nuclei (%)	Comet class damaged nuclei (%)				Damaged cells (%) (II+III+IV)
		Type 0	Type I	Type II	Type III	Type IV	
14-day	Control	97.33±1.15a	2.67±1.15 e	0.00±0.00 e	0.00±0.00 e	0.00±0.00 d	0.00±0.00 e
	1/3rd of LC_{50}	22.67±2.31d	24.00±2.00 d	26.67±3.06 a	15.33±1.15 b	11.33±1.15 ab	53.33±1.15 a
	1/4th of LC_{50}	22.67±2.31d	30.00±2.00 abc	21.33±1.15 b	16.67±2.31 ab	9.33±1.15 b	47.33±1.15 b
	1/5th of LC_{50}	35.33±1.15c	26.00±2.01 cd	18.00±2.00 c	11.33±1.15 c	9.33±1.15 b	38.67±1.15 c
	1/6th of LC_{50}	50.67±1.15b	28.00±1.11 bcd	7.33±1.15 d	7.33±1.15 d	6.67±1.15 c	21.33±1.15 d
28-day	Control	96.00±3.46 a	2.67±1.15 e	0.67±1.15 e	0.67±1.15 d	0.00±0.00 e	1.33±2.31 e
	1/3rd of LC_{50}	17.33±2.31 e	19.33±1.15 d	30.67±1.15 a	19.33±1.15 a	13.33±1.15 b	63.33±1.15 a
	1/4th of LC_{50}	24.67±1.15 d	20.00±2.00 cd	22.00±2.00 bc	15.33±1.15 b	18.00±4.00 a	55.33±3.06 b
	1/5th of LC_{50}	32.00±2.00 c	27.33±3.06 b	19.33±1.15 c	12.00±2.00 c	9.33±1.15 c	40.67±2.31 c
	1/6th of LC_{50}	40.67±3.06 b	32.00±4.00 a	12.00±2.00 d	10.00±2.00 c	5.33±1.15 d	27.33±3.06 d
42-day	Control	97.33±1.15 a	2.00±0.00 e	0.67±1.15 e	0.00±0.00 d	0.00±0.00 c	0.67±1.15 e
	1/3rd of LC_{50}	14.67±3.06 d	16.67±3.06 d	38.00±2.00 a	20.00±2.00 a	10.67±1.15 a	68.67±1.15 a
	1/4th of LC_{50}	21.33±2.31 c	22.00±2.00 c	28.00±2.04 c	19.33±2.31 a	9.33±2.31 a	56.67±1.15 b
	1/5th of LC_{50}	22.00±4.00 c	30.00±2.00 b	30.00±3.46 bc	8.67±1.15 c	9.33±1.15 a	48.00±5.29 c
	1/6th of LC_{50}	29.33±2.31 b	34.00±2.00 ab	20.00±2.00 d	11.33±1.15 b	5.33±1.15 b	36.67±3.06 d
56-day	Control	96.00±2.00 a	3.33±1.15 d	0.67±1.15 e	0.00±0.00 e	0.00±0.00 e	0.67±1.15 e
	1/3rd of LC_{50}	8.67±1.15 e	20.00±2.00 c	40.67±3.06 a	22.67±1.15 a	8.00±2.00 bc	71.33±3.06 a
	1/4th of LC_{50}	14.67±1.15 d	23.33±3.06 bc	32.67±3.06 c	20.00±2.00 b	9.33±1.15 ab	62.00±2.00 b
	1/5th of LC_{50}	18.67±3.06 cd	30.00±2.00 a	35.33±1.15 bc	10.00±2.00 d	6.00±2.00 c	51.33±3.06 c
	1/6th of LC_{50}	25.33±4.16 b	32.67±4.16 a	22.67±1.15 d	16.00±2.00 c	3.33±1.15 d	42.00±2.00 d
70-day	Control	94.67±1.15 a	4.67±1.15 e	0.67±1.15 e	0.00±0.00 e	0.00±0.00 e	0.67±1.15 e
	1/3rd of LC_{50}	15.33±4.16 cd	16.00±4.00 d	48.00±2.00 a	18.00±2.00 a	2.67±1.15 cd	68.67±1.15 a
	1/4th of LC_{50}	10.67±1.15 e	25.33±1.15 c	39.33±1.15 b	15.33±1.15 b	9.33±1.15 ab	64.00±0.00 b
	1/5th of LC_{50}	12.00±2.00 de	32.67±3.06 b	34.67±1.15 c	12.00±2.00 c	8.67±1.15 b	55.33±1.15 c
	1/6th of LC_{50}	35.33±4.16 b	37.33±5.03 a	19.33±1.15 d	7.33±1.15 d	0.67±1.15 de	27.33±1.15 d

Means with similar letters in a single column for each variable are statistically non-significant at $p<0.05$. n, 100 cells per treatment; Type I-IV, extent of DNA damage.

Similarly, percentage of Type IV damaged nuclei were observed maximum and minimum on 14th day exposure to 1/3rd of LC_{50} and control treatment, respectively. However, on day 28, 42, 56 and 70 the same was maximum due to 1/4th of LC_{50} exposure with significant differences among them ($p<0.05$). Among four test concentrations, 1/3rd, 1/4th and 1/5th of LC_{50} showed non-significant difference for the induction of Type IV damaged cells in peripheral erythrocytes of *O. niloticus* on all sampling days, except on 28th day. The extent of DNA damage was examined as the mean percentage of cells with medium, high and complete damaged DNA, calculated as sum of Type II, III and IV. Statistically significant ($p<0.05$) DNA damage was observed during whole exposure period due to different test concentrations. Regarding different treatments (control, 1/3rd of LC_{50}, 1/4th of LC_{50}, 1/5th of LC_{50} and 1/6th of LC_{50}) the extent of DNA damage was observed significantly higher ($p<0.05$) due to 1/3rd of LC_{50} exposure followed by that of 1/4th of LC_{50}, 1/5th of LC_{50} and 1/6th of LC_{50} as compared to control group on all sampling days indicating dose dependent DNA damage. Statistically significant time based DNA damage was observed in treated fish as compared to control group under four different sub-lethal concentrations. Comparing the DNA damage during all sampling days, the peripheral blood erythrocytes of *O.niloticus* exhibited concomitant increase in damage from 14th to 56th day, followed by decline on day 70 due to 1/3rd of LC_{50} exposure (Fig. 2A). However, at 1/4th and 1/5th of test concentrations, damage was significantly increased with the passage of time from day 14th to 70 (Fig. 2B, C). Similarly, 1/6th of LC_{50} exposure exhibited gradual increase in DNA damage from day 14th to 56th, followed by sharp decline on day 70 (Fig. 2D).

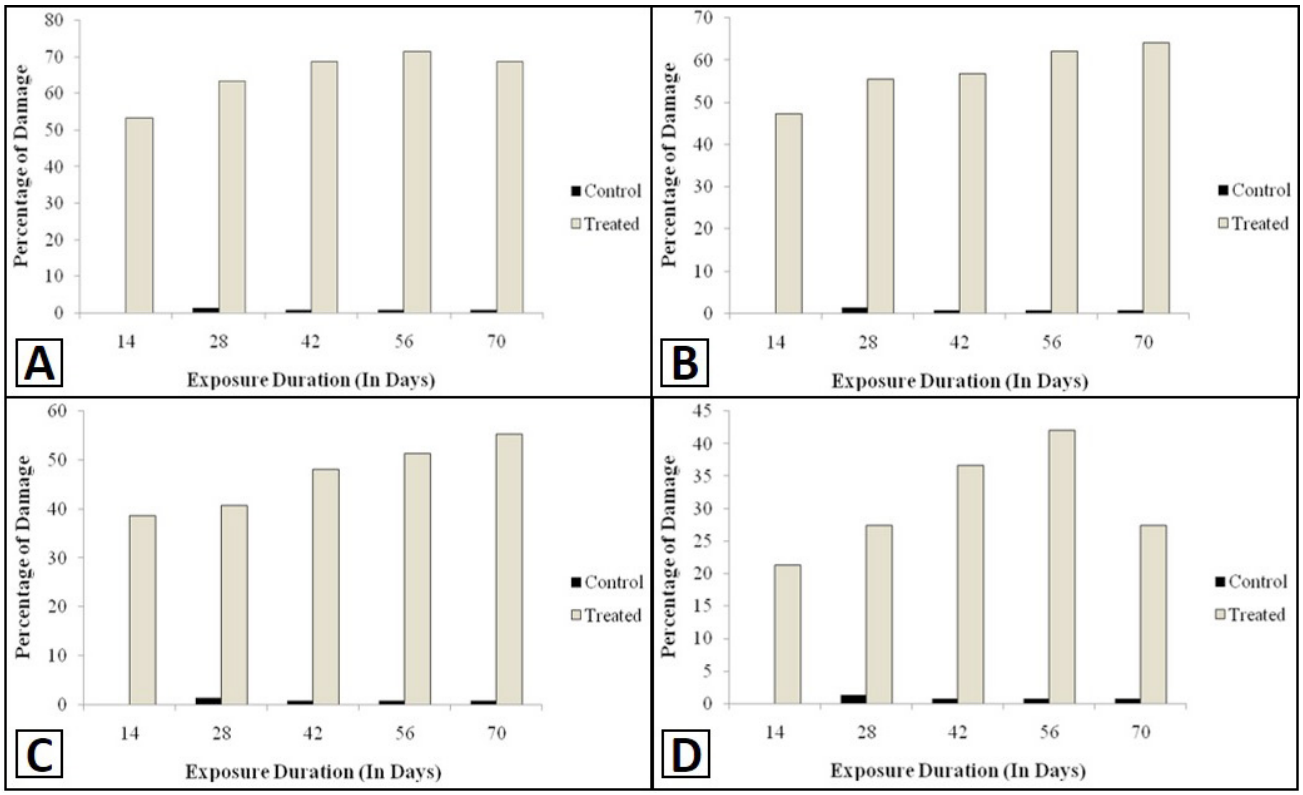

Fig. 2. DNA damage in peripheral blood erythrocytes of *Oreochromis niloticus* at **A,** 1.88; **B,** 1.41; **C,** 1.13 and **D,** 0.94µgL^{-1} of pesticide mixtur.

DISCUSSION

The present study elucidates the genotoxicity of pesticides mixture (endosulfan+chlorpyrifos) in freshwater fish, *O. niloticus* by using Comet assay. During the present experiments water temperature (ranged from 30.01 to 30.04ºC), pH (ranged from 7.75 to 7.77) and total hardness (ranged from 224 to 226 mgL^{-1}) were kept constant because toxicity of pesticides to the aquatic organisms has been reported to be affected by water temperature, pH, hardness, size, species and age of fish (Young, 2000). Effects of pesticides on organism were often evaluated by using single toxicant, however, under present environmental conditions pesticides are present in multiple combinations in aquatic ecosystems. The contamination of aquatic ecosystems by pesticides has gained increasing attention in recent decades (Byer *et al*., 2011). Biological monitoring using a target species could allow a sensitive approach to predict the potential risk of persistent pollutants in aquatic environment. Tested sub-lethal concentrations in the present study could be environmentally relevant concentrations, although repeated applications of the pesticides in most developing countries may be higher, suggesting the relevance of test concentrations. Pesticides in sub-lethal concentration present in water are too low to cause rapid death directly but may affect the functioning of organisms, disrupt normal behavior and reduce the fitness of natural population (Susan *et al*., 2010).

We applied the alkaline version of Comet assay to evaluate the DNA strand breaks in the peripheral blood erythrocytes of *O. niloticus* exposed in vivo to different sub-lethal concentrations of endosulfan+chlorpyrifos mixture for 70 days. Long-term genotoxicity studies can be an important approach for achieving the greater insight into the organism DNA repair ability and other protective mechanisms for excreting the xenobiotics. Present findings showed that exposure of fish to four sub-lethal concentrations of pesticide mixture induced significantly higher DNA damage in blood cells as compared to control group. Mixture of pesticides (endosulfan, chlorpyrifos and thiram) has been reported to cause DNA damage (Tope and Rogers, 2009). Similarly, Polard *et al*. (2011) observed significant increase in DNA damage under the exposure of five pesticides mixture (metolachlor + isoproturon + chlorotoluron + atrazine + deethylatrazine) as compared to control. Dose and time dependent response of *O.*

niloticus on DNA integrity was also observed by Rani and Kumaraguru (2013) due to exposure of endosulfan. Reactive oxygen species such as hydrogen peroxide, hydroxyl radical and superoxide anion have been shown to produce extensive damage such as DNA strand breaks, enzyme inactivation and apoptosis (Banudevi *et al*., 2006). It is possible that endosulfan and chlorpyrifos could cause alterations in DNA of *O. niloticus* resulting in comet induction. Altinok*et al*. (2012) observed significantly higher DNA damage in terms of tail length, tail intensity, tail moment and tail migration in *Oncorhynchus mykiss* exposed to various concentrations of carbosulfan for 60 days than that of control group.

Fish specimens exposed to sub-lethal concentrations viz. 1/4th of LC_{50}, 1/2nd of LC_{50} and 3/4th of LC_{50} of carbosulfan exhibited significantly higher DNA damage ($p<0.01$) in erythrocytes and gill cells in terms of percentage of tail DNA than the control group. DNA damage in both tissues was found to be dose and time dependent (Nwani *et al*., 2010). DNA damage observed in present study might have originated from DNA single strand breaks, double strand breaks, DNA-DNA/DNA-protein cross linking or inhibition of enzymes involved in DNA repair resulting from the interaction of pesticides or their metabolites with DNA (Guilherme *et al*., 2012). Dose and time related increase in DNA damage in the form of comet induction was observed during present investigation. Similarly, Nwani *et al*. (2013) observed highest DNA damage on day 14 at 1/10th, 1/8th and 1/5th of LC_{50} exposure of herbicide, followed by gradual non-linear decline on day 21, 28 and 35. Time dependent decrease in DNA damage may be due to repair of damaged DNA, loss of heavily damaged cells or both (Saleha *et al*., 2001). Similarly, Banu *et al*. (2001) observed a time dependent decrease in DNA breaks in erythrocytes of *Tilapia mossambica* exposed to different concentrations of monocrotophos pesticide by using comet assay. Exposure to different concentrations of chlorpyrifos induced higher DNA damage in fish as compared to controls, indicating genotoxic potential of this pesticide to aquatic organisms (Ali *et al*., 2008; Yong *et al*., 2011). Present results are also in accordance with findings of Pandey *et al*. (2011) who observed concentration dependent DNA damage in *Channa punctatus* under exposure of organophosphate pesticide. Chronic exposure to mutagenic pollutants, lead to accumulation of DNA strand breaks (Ternjej *et al*., 2010) because DNA repair capacity of fish cell is low as compared to other species.

CONCLUSION

Indiscriminate use of pesticides to improve agricultural practices may have impacts on non-target organism's especially aquatic lives which can ultimately pose a serious threat to the health of human communities. Biological monitoring approach help us to predict the potential risk of these contaminants and to devise the ''safe levels'' for such chemicals having genotoxic potential. Acute and chronic effects of individual pesticides have been widely studied for different fish species, but the information regarding the genotoxic potential of pesticides in the form of mixture is scant in literature. Our results on *O. niloticus* elucidate that this pesticide mixture (endosulfan + chlorpyrifos) possesses genotoxic threats. Overall, we focused on organochlorine and organophosphate exposures; however, the capacity of other pesticide classes to induce similar patterns of DNA damage should be investigated. Based on present results, we may suggest careful and sensible use of pesticides to guard against genetic hazards.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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