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DNA Damage in Erythrocytes of *Cyprinus Carpio* under Sublethal Concentrations Exposure of Pesticides Mixture

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ABSTRACT
This experiment was conducted in order to assess the genotoxicity of commonly employed pesticides viz. chlorpyrifos, endosulfan and bifenthrin in the form of tertiary mixture on fish, *Cyprinus carpio* by using comet assay. At first, 96-hr LC_{50} was determined and then sublethal concentrations viz. $1/3^{rd}$, $1/4^{th}$, $1/5^{th}$ and $1/6^{th}$ were calculated. *Cyprinus carpio* exposed, separately, to aforementioned concentrations for a period of 30 days along with negative and positive control. Peripheral blood erythrocytes were sampled on day 15 and 30^{th} of exposure for the estimation of DNA damage. Damage was determined in terms of percentage of damaged nuclei and genetic damage index. Statistically significant ($P<0.05$) effects were observed at various concentrations and time of exposure in stressed and control fish. DNA damage was found to be dose and time dependent with highest damage was observed at $1/3^{rd}$ LC_{50} exposure as compared to control groups. Time dependent response was also observed in fish erythrocytes with induction of maximum DNA damage on day 30^{th} of exposure. The current study of DNA damage caused by pesticides mixture may become a key indicator in assessing the general health of freshwater fish.

Keyword: *Pesticides, DNA, Erythrocytes, Comet assay, Cyprinus carpio.*

INTRODUCTION
Different water sources are used in aquaculture and it is not uncommon for some of these sources to become polluted with different types of xenobiotics [1]. Deterioration of water quality significantly leads towards decrease in aquatic biodiversity at both individual or population levels, as well as decline in the quality of aquatic products [2]. Exposure of aquatic organisms to genotoxic compounds could pose health hazards to humans through the food chain, an ecological risk linked with induction of transmittable mutations leading to loss of biodiversity [3]. Fish are relatively sensitive to changes in their surrounding environment; therefore health status of fish is a suitable reflection for the monitoring of environmental pollutants [4,5]. Many studies that evaluate the toxicity of pesticides to aquatic organisms have focused only on individual toxicants. However, aquatic environments are collectively exposed to a mixture of toxic substances [6,7].

Pakistan economy largely depends on agriculture; therefore, pesticides are likely to represent an important source of xenobiotics in freshwater ecosystems because agricultural development has led a parallel growth in the use of these chemicals for crop protection. Pesticides comprise an extensive range of synthetic organic compounds [8], that are marketed as herbicides, insecticides and fungicides [9]. The aquatic environment is actually the last receptacle for pesticide residues [10]. Endosulfan (class organochlorine) is a very controversial chemical because of its highly acute toxicity and bio-accumulative behavior. Manufacturing and use of endosulfan is globally banned by the Stockholm Convention in the year 2011, but unfortunately it is still extensively used in more than seventy countries, including Pakistan, India (major producer and consumer), China and Cuba [11]. Chlorpyrifos (class organophosphate) is one of the most widely used insecticide with potential of acute toxicity and it elicit a number of other effects including immunological abnormalities, developmental disorders, hepatic dysfunction, teratoginicity, genotoxicity, neurobehavioral and neurochemical changes [12,13]. Bifenthrin (class pyrethroid) is less toxic in mammals as they are in insects and fish because mammals have ability to break the ester bond of bifenthrin. Bifenthrin stay longer in the fish body because fish have a slow metabolic rate therefore, very small concentration of bifenthrin may adversely affect the fish health [14]. Among the different techniques used for the determination of genotoxic damage, the comet assay can detect DNA damage (single and double strand breaks) at single cell level [15], can be successfully applied to the nucleated red blood cells of many fish species, exposed to different genotoxic compounds and environmental stressors [16,17].

The objectives of present investigation were to assess the long term DNA damage in peripheral erythrocytes of *Cyprinus carpio* under various sublethal concentrations exposure of tertiary pesticides mixture (chlorpyrifos+endosulfan+bifenthrin).

MATERIALS AND METHODS
Experimental Organism and Solution
Fingerlings (180-day old) of *Cyprinus carpio* were purchased from local market and transported to Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Prior to experiment, fingerlings of similar weight and lengths were acclimatized in cemented tanks for two weeks under laboratory conditions. Pesticides viz. chlorpyrifos, endosulfan and bifenthrin were dissolved, separately, in 95% analytical grade methanol (J.T Baker) as a carrier solvent to prepare the stock-I solutions (1 g/100 ml) while tertiary mixture of pesticides were prepared by further mixing of these stock solutions.

Determination of Sublethal Concentrations
The 96-hr LC_{50} value of pesticides mixture (chlorpyrifos+endosulfan+bifenthrin) i.e. 0.217 µg L^{-1} on *Cyprinus carpio* was determined during previous study of Ambreen and Javed [18]. Based on this 96-hr LC_{50} value, four sublethal concentrations viz. $1/3^{rd}$, $1/4^{th}$, $1/5^{th}$ and $1/6^{th}$ of LC_{50} were calculated for these experiments.

Comet Assay

Cyprinus carpio (n=6) were exposed, separately to 1/3rd, 1/4th, 1/5th and 1/6th of LC_{50} in glass aquaria having 70 L water capacity along with negative and positive control. One group of fish was kept in tap water, which was considered as "Negative control" (unstressed group) while cyclophosphamide used as "Positive control". During the whole experimental period, *Cyprinus carpio* were fed with the feed on daily basis. Water temperature (30ºC), pH (7.75) and hardness (225 mg L^{-1}) were kept constant throughout the experimental duration. The exposure was continued for 30 days and peripheral blood slides were prepared on day 15th and 30th of exposure and subjected to comet assay. These experiments were conducted with three replications for each sublethal concentration. Peripheral blood erythrocytes were collected from caudal vein of fish and immediately transferred to eppendorf and treated with anticoagulant. Comet assay was performed as three layer procedure, followed by lysis and electrophoresis [15]. After electrophoresis, DNA was stained with ethidium bromide and slides were examined at 400X magnification by using the Epi-Fluorescence microscope (N-400M, American Scope; USA) equipped with light source of mercury and low lux digital (MD-800, American Scope; USA) camera. For each treatment, three slides and 50 cells per slide were randomly scored. Each image was classified according to the intensity of fluorescence in the comet tail and designated as following five categories (measured through TriTek CometScore™):

Undamaged: Type 0
Low level damage: Type I
Medium level damage: Type II
High level damage: Type III
Complete damage: Type IV

The extent of DNA damage was calculated as the mean percentage of cells with medium, high and complete damaged DNA by using following formula:

%age of DNA Damage = Types II+ III + IV

From the arbitrary values assigned to the different categories (from Type=0 to Type IV=4) a genetic damage index (GDI) was calculated for each subject by using following formula:

$$GDI = \frac{(\text{Type I}) + 2(\text{Type II}) + 3(\text{Type III}) + 4(\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

Statistical Analyses

The data were statistically analyzed through MSTATC computer software and results were expressed as Means±SD. Computed means were compared for the statistical differences by using Duncan Multiple Range test [19] and a value of P<0.05 was accepted as statistically significant.

Table 1: Time and dose dependent DNA damage in peripheral erythrocytes of *Cyprinus carpio* exposed to tertiary pesticide mixture

		Undamaged Nuclei (%)	Damaged Nuclei (%)					
Exposure Duration	**Treatments**	**Type 0**	**Type I**	**Type II**	**Type III**	**Type IV**	***%age of Damaged Nuclei (II+III+IV)**	****GDI**
15 Days	**Negative Control**	96.67±2.31a	2.67±1.15e	0.67±1.15e	0.00±0.00d	0.00±0.00f	0.67±1.15f	0.04±0.03e
	Positive Control	28.67±1.15c	14.67±1.15bc	20.67±1.15b	12.67±1.15c	23.33±3.06b	56.67±2.31cd	1.87±0.10c
	1/3rd of LC_{50}	15.33±1.15e	12.00±2.00d	20.67±1.15b	20.67±2.31b	31.33±3.06a	72.67±2.31a	2.41±0.08a
	1/4th of LC_{50}	22.00±2.00d	14.00±2.00cd	32.00±2.00a	14.67±1.15c	17.33±3.06c	64.00±3.46b	1.91±0.12bc
	1/5th of LC_{50}	22.00±2.00d	24.00±2.00a	11.33±1.15c	30.00±2.00a	12.67±2.31d	54.00±4.00d	1.87±0.12c
	1/6th of LC_{50}	46.67±3.06b	24.00±2.00a	8.00±2.00d	14.00±2.00c	7.33±1.15e	29.33±1.15e	1.11±0.06d
30 Days	**Negative Control**	97.33±1.15a	2.00±0.00f	0.67±1.15f	0.00±0.00d	0.00±0.00d	0.67±1.15e	0.03±0.02e
	Positive Control	36.67±3.06b	12.00±2.00d	17.33±3.06d	13.33±3.06c	20.67±2.31c	51.33±3.06d	1.69±0.13d
	1/3rd of LC_{50}	5.33±1.15e	8.67±1.15e	22.67±1.15ab	28.00±2.00a	35.33±3.06a	86.00±0.00a	2.79±0.05a
	1/4th of LC_{50}	8.67±1.15d	20.00±2.00c	21.33±1.15bc	21.33±3.06b	28.67±2.31b	71.33±2.31b	2.41±0.08b
	1/5th of LC_{50}	8.67±1.15d	20.67±1.15bc	19.33±1.15cd	22.67±1.15b	28.67±4.16b	70.67±2.31b	2.42±0.11b
	1/6th of LC_{50}	20.00±2.00c	24.00±2.00a	12.00±2.00e	22.67±1.15b	21.33±2.31c	56.00±2.00c	2.01±0.09c

The means with similar letters in a single column for each variable are statistically non-significant at P<0.05

*%age of DNA Damage = Type II + Type III + Type IV, **GDI (Genetic Damage Index)

RESULTS

Pesticides Mixture Induced DNA Damage in Fish

DNA become damaged when it mutates or changed from its original conformation. DNA mutagens can increase the strand breakage due to time and concentrations of exposure. The alkaline version of comet assay was performed to see the genotoxic effects of pesticides mixture (chlorpyrifos+endosulfan+bifenthrin) on *Cyprinus carpio*. The percentage of damaged nuclei and genetic damage index (GDI) were calculated from the values assigned to different categories (Type 0 to Type IV) of peripheral erythrocytes of fish, exposed to various concentrations (1/3rd, 1/4th, 1/5th, 1/6th of LC_{50}) of mixture and compared with negative and positive controls at different time intervals. Table 1 shows the undamaged and damaged nuclei, %age of damaged nuclei and GDI examined in the peripheral blood erythrocytes of *Cyprinus carpio* exposed to

different concentrations of mixture.

Day 15th: Significantly variable frequency of Type 0, I, II, III and IV were observed under all the test concentrations, negative and positive control. Percentage of damaged nuclei in peripheral blood erythrocytes of *Cyprinus carpio* followed the sequence: 1/3rd > 1/4th > positive control > 1/5th > 1/6th > negative control on day 15th of exposure. GDI increased significantly with concomitant increase in the exposure concentration of mixture from 1/6th to 1/3rd of LC_{50}. Significantly maximum mean GDI values were observed at 1/3rd of LC_{50} exposure however, it was significantly minimum due to negative control treatment (Table 1).

Day 30th: Statistically significant DNA damage was observed in terms of %age of damaged nuclei during whole exposure duration. During different treatments (1/3rd, 1/4th, 1/5th, 1/6th of LC_{50}, positive and negative control) the %age of damaged nuclei were observed higher at 1/3rd of LC_{50} exposure, followed by that of 1/4th of LC_{50}, 1/5th of LC_{50}, 1/6th of LC_{50}, positive control and negative control. However, non-significant difference was observed between 1/4th and 1/5th of LC_{50} exposures. Genetic damage index due to different concentrations of pesticides mixture, negative and positive control, ranged between 0.03±0.02 to 2.79±0.05 with statistically significant difference at $P<0.05$ (Table 1).

Significantly higher damage (in terms of %age of damaged nuclei and GDI) was caused by 1/3rd of LC_{50} while negative control gave significantly least damage to the fish nuclei. Significantly maximum mean damage values in terms of %age of damaged nuclei and GDI were observed on day 30th while these were significantly minimum on day 15th of exposure as evident from their mean values.

DISCUSSION

Pesticides may cause direct DNA damage due to action of parental compound or their metabolites or indirectly due to over-production of reactive oxygen species [20]. During present study, time and concentration dependent DNA damage was observed under sublethal exposures of chlorpyrifos, endosulfan and bifenthrin mixture. Dose and time dependent genotoxicity in fish associated with pesticide exposure using the Comet Assay/Single Cell Gel Electrophoresis in fish erythrocytes is well documented [21,22,23,24]. However, previously discussed genotoxicity assessment was based on evaluation of acute exposure. Therefore, such approach fails to provide appropriate information regarding the long-term effects of pesticide burden on the genome. The main idea of the present study was to characterize DNA damage induced by prolonged exposure to mixture.

During present investigation, the alkaline version of comet assay was applied to evaluate the DNA damage (strand breakages) in the peripheral blood erythrocytes of *Cyprinus carpio* exposed to sublethal concentrations of pesticides mixture and compared with negative and positive controls for the duration of 30 days. DNA damage was estimated by using the %age of damaged nuclei and genetic damage index. Statistically significant increase in DNA damage was observed in the erythrocytes of *Cyprinus carpio* due to exposure of polluted water [25]. Mixture of pesticides (chlorpyrifos, endosulfan and thiram) has been reported to cause significantly higher DNA damage [26]. Altinok *et al*. [27] also observed significantly higher DNA damage in terms of comet tail length, tail intensity, tail moment and tail migration in *Oncorhynchus mykiss* blood erythrocytes exposed to various concentrations of carbosulfan for 60 days than positive control.

Genotoxicity of pesticides regarding their ability to make variety of highly reactive oxygen species (ROS) such as H_2O_2, O^{2-} and OH^- and electrophilic free radical metabolites that interact with nucleophilic sites of DNA, cause strand breakage [28,29]. Pesticides can form strong covalent bonds with DNA resulting in the formation of DNA adducts, modulate antioxidant defensive systems and cause oxidative damage in aquatic organisms [30]. The DNA damage detected in the present study could have originated from DNA single strand breaks, DNA double strand breaks, DNA-DNA/DNA-protein cross linking or inhibition of the enzymes involved in DNA repair resulting from the interaction of pesticides or their metabolites with DNA [31]. Chronic exposure of mutagenic pollutants leads to accelerated DNA strand breaks [32] because DNA repairing capacity of fish is low as compared to other animals [33].

Genotoxicity not only reduces the fitness of fish populations but also causes risk to the humans through the food chain [34]. The present study reveals that 1/3rd of LC_{50} exposure of pesticides mixture to the fish caused significantly higher DNA damage while negative control exerted significantly least damage to the nuclei. The frequency of damaged nuclei and GDI increased concomitantly with the duration of exposure i.e. from day 15th to day 30th. Exposure of fish to sublethal concentrations viz. 1/4th, 1/2nd and 3/4th of LC_{50} of carbosulfan gave significantly higher DNA damage ($P<0.01$) in erythrocytes and gill cells (in terms of percentage of tail DNA) as compared to the control group. DNA damage in both the tissues was found to be dose and time dependent [35]. Similarly, [36] reported significantly higher DNA damage in fish, *Prochilodus lineatus* exposed to 10 mg L^{-1} of roundup as compared to negative control. Ilyas [37] observed dose-dependent response of Major Carps towards waterborne exposure of individual pesticides viz. endosulfan, bifenthrin and chlorpyrifos to induce DNA damage in their peripheral blood erythrocytes.

CONCLUSION

Statistically significant ($P<0.05$) genotoxic effects in peripheral erythrocytes of *Cyprinus carpio* were observed at different concentrations and duration of pesticides mixture (chlorpyrifos+endosulfan+bifenthrin) exposure. Among all the exposure concentrations, 1/3rd of LC_{50} caused significantly highest DNA damage to the nuclei. A concomitant increase in percentage of damaged nuclei and genetic damage index in peripheral erythrocytes of fish was found with a rise in pesticides mixture exposure duration i.e. from day 15th to 30th.

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