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Dose dependent DNA damage in *Cyprinus carpio* under sub lethal concentration exposure to pesticides mixture

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Abstract

Water pollution due to various pesticides is become a global problem now a days because their contents reach in the aquatic ecosystem where it poses considerable toxicological risks to non-target organisms like fish. Therefore, this study was designed to determine the in-vivo induction of DNA damage in *Cyprinus carpio* (common carp) under sub lethal concentration exposure to binary pesticides mixture (endosulfan – chlorpyrifos) by using single cell gel electrophoresis / comet assay. At first phase, 96-hr LC_{50} was determined, based on this value four sub-lethal concentrations viz. $1/3^{rd}$, $1/4^{th}$, $1/5^{th}$ and $1/6^{th}$ were calculated. The *C. carpio* exposed, separately, to aforementioned concentrations for a period of 15 days along with two control groups i.e. negative and positive. The fish peripheral erythrocytes were sampled on day 15^{th} of exposure period from each sub lethal concentrations / treatments for the estimation of DNA damage in. The DNA damage was determined in terms of %age of damaged nuclei, genetic damage index (GDI) and cumulative tail lengths of comets (CTL). Results exhibited statistically significant ($p^{<0.05}$) effects at different sub lethal concentrations in fish groups along with positive control as compared to negative control. The DNA damage was found to be concentration dependent with highest damage observed at $1/3^{rd}$ LC_{50} exposure as compared to control groups in terms of %age of damaged nuclei, GDI and CTL of comets. The present experiment showing the potential genotoxicity of pesticides in common carp (which is non-target organism) and comet assay is useful tool to study the genotoxic effect of water-borne pollutants.

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Introduction

Pesticides has significantly enhanced agricultural yield in the modern world. However, the agricultural runoff events introduce pesticides residues into the aquatic ecosystem. These residues pose high risks to the aquatic organisms; accumulated in the food chain, ultimately threatening the human health (Burkepile *et al.,* 2000; Ondarza *et al.,* 2014). Toxicity of pesticides in the aquatic ecosystems fluctuates, depending on their chemical structures, length of exposure period, species, water quality species etc. (Dutta *et al.*, 1992). The higher solubility of pesticides, indiscriminate use, repeated applications, accidental spillage, spray drift and careless handling may result to huge build-up of pesticide residues in the aquatic animals, especially fish (Jordan *et al.,* 2013). Pesticides may bind to the materials in suspension can amassed in the sediments or absorbed by the aquatic organisms. Pesticides not only affect the physiology of fish but may also interact with their genetic material which can lead to mutation, genetic damage, teratogenesis and carcinogenesis (Ambreen and Javed, 2016; Corredor-Santamaria *et al.,* 2016). Among different commonly used pesticides, endosulfan is highly toxic and due to its toxic effect it is banned in more than seventy countries, but unfortunately it is still used in most of the developing countries (Ondarza *et al.,* 2014). Chlorpyrifos belongs to organophosphate class of pesticides, it is one of the mainly used insecticide in the feilds, potential to cause acute toxicity, for that reason it may elicit a number of other effects including mutagenic effects, immunological abnormalities, developmental disorders, hepatic dysfunction, teratoginicity, neuro-behavioral and neuro-chemical alterations (Dam *et al.*, 1999; Gomes *et al.*, 1999).

Significance of genotoxicity determination in fish lies in the fact that fish is an important source of protein in diet. Due to stability of pesticides, they can contaminate the aquatic ecosystem even at sub-lethal concentrations; tend to accumulate in the organs of aquatic animals also (Kumar et al., 2009). Fish used as a bio-indicators of pollution in water, play significant role in assessing the potential risk associated with pollution in the aquatic environment as they are directly exposed to chemicals, resulting from agricultural production/indirectly through the food chain (Lakra and Nagpure, 2009).

Therefore, it is very crucial to evaluate the brutality of genotoxic compounds which released into the aquatic ecosystems. The degree of DNA integrity has been proposed as a sensitive sign or effective biomarker for the monitoring of environmental carcinogens, mutagens, and teratogens (Cavas, 2008; Lourenco *et al.*, 2013). Among different techniques, comet assay is a fast, sensitive and reliable genotoxicity test (Altinok *et al.,* 2012). This assay can be practically applied to the nucleated red blood cells of fish exposed to different genotoxic compounds and pollutants (Mustafa *et al.*, 2011; Adeyemi *et al.*, 2015). Therefore, this study was planned to assess the DNA damage in *Cyprinus carpio* after sub-lethal concentrations exposure to binary pesticide mixture.

Materials and methods

Cyprinus carpio fingerlings having 180-day old were purchased from market and transported to Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Fingerlings having similar weight and lengths were acclimatized in rectangular tanks (cemented) for two weeks under laboratory conditions, prior to start of experiment. Pesticides viz. endosulfan and chlorpyrifos were dissolved separately, in 95% analytical grade methanol (J.T Baker) as a carrier solvent to prepare the stock-I solutions (1g/100ml) however, the binary mixture of pesticide was prepared by further mixing of stock solutions.

Determination of Sub-Lethal Concentrations

The 96-hr LC_{50} value of endosulfan- chlorpyrifos on *Cyprinus carpio* was determined during previous study of Ambreen and Javed (2015). Based on this 96-hr LC_{50} ($0.42\pm0.02\mu gL^{-1}$) value, four sub-lethal concentrations viz. $1/3^{rd}$, $1/4^{th}$, $1/5^{th}$ and $1/6^{th}$ of LC_{50} were calculated for this experiment.

Comet Assay/Single Cell Gel Electrophoresis (SCGE)

Healthy fingerlings of *Cyprinus carpio* were exposed, separately, to 1/3rd, 1/4th, 1/5th and 1/6th of LC_{50} concentrations in glass aquaria (70L water capacity) along with control groups i.e. negative and positive control. One group of *Cyprinus carpio* was kept in tap water, which was considered as "Negative control" (unstressed group) while 4% saline solution of cyclophosphamide (CP) was used as "Positive control". During 15 days of exposure period, *Cyprinus carpio* were fed daily small quantity of food. Water pH (7.75), temperature (30°C) and hardness (225mgL^{-1}) were kept constant throughout the duration of experiment. The exposure was continual for 15 days and peripheral blood slides were made on day 15th of exposure period and subjected to comet assay. This experiment was conducted with three replications for each sub-lethal concentration. Blood were sampled from caudal vein of fish, transferred to eppendorf and treated with anticoagulant salt. Comet assay was performed by following Singh *et al*. (1988) as three layer procedure, followed by lysis, unwinding, electrophoresis, neutralization and staining with ethidium bromide. Blood slides were examined at 400X magnification power by using the Epi-Fluorescence microscope (N-400M, American Scope; USA) equipped with mercury light and low lux digital camera. For each sub-lethal concentration/treatment, three slides and 50 cells/slide were scored randomly. Each image was classified according to the intensity of fluorescence in the comet tail and designated as following five categories (measured through Tri Tek Comet Score™):

Categories of Damage	Types
Undamaged:	Type-0
Low level damage:	Type-I
Medium level damage:	Type-II
High level damage:	Type-III
Complete damage:	Type-IV

Parameters for DNA Damage Estimation

%age of damaged nuclei

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

% age of damaged nuclei = Types-II+ III + IV

Genetic Damage Index (GDI)

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

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

Cumulative Tail Lengths (CTL)

TriTek CometScore ™ software was used to calculate the comet tail length of damaged cells (Jose *et al*., 2011) and cumulative tail length (µm) was obtained by adding the tail length of all examined cells (n = 50/replicate).

Statistical analyses of data

Statistical analyses were performed through MSTATC software and results were expressed as Means±SD. Data means were compared for statistical differences by using Duncan Multiple Range test (DMR) by following Steel *et al*, (1996) and a value of $p<0.05$ was accepted as statistically significant.

Results

Table 1 shows the variable proportions of Type-0 (undamaged nuclei), Type-I, II, III and IV nuclei (damaged), %age of damaged nuclei, GDI and CTL (µm) which was observed under sub-lethal concentrations of pesticide mixture (endosulfan-chlorpyrifos) in the peripheral blood erythrocytes of *Cyprinus carpio* (common carp) along with negative and positive control groups after 15 days of exposure period.

Undamaged and Damaged Nuclei (%)

At all sub-lethal concentrations viz. 1/3rd, 1/4th, 1/5th and 1/6th of LC_{50}, frequencies of undamaged nuclei (Type-0) were observed higher under negative control however, same was lower due to 1/3rd of LC_{50} exposure. The percentage of Type-I nuclei were significantly higher under 1/5th of LC_{50} while it was minimum as 3.33±1.15% in negative control group of fish. Regarding other damaged nuclei, frequency of Type-II damaged nuclei were observed maximum at

positive control while Type-III nuclei were observed higher under 1/4th of LC_{50} exposure. Proportions of Type-II nuclei under 1/4th and 1/5th of LC_{50} exposure did not vary significantly at $p<0.05$. The frequency of Type-IV damaged nuclei under sub-lethal concentrations and control groups in peripheral blood erythrocytes of *Cyprinus carpio* followed the sequence: 1/3rd > positive control > 1/4th > 1/5th > 1/6th > negative control.

Table 1. DNA damage in *Cyprinus carpio* under sub-lethal exposure to endosulfan and chlorpyrifos mixture.

		Undamaged Nuclei (%)	%age of Damaged Nuclei						
Exposure Duration	Treatments	Type-0	Type-I	Type-II	Type-III	Type-IV	%age of Damaged Nuclei (II+III+IV)	GDI	*CTL (µm)
15 Days	Negative Control	96.67±1.15 a	3.33±1.15 e	0.00±0.00 e	0.00±0.00 f	0.00±0.00 f	0.00±0.00 f	0.03±0.01 f	2.50±0.09 f
	Positive Control	24.67±1.15 c	18.00±2.00 d	22.00±2.00 a	14.00±2.00 d	21.33±2.31 b	57.33±2.31 b	1.89±0.06 b	115.26±0.08 e
	1/3rd of LC_{50}	14.67±4.16 d	20.67±1.15 cd	14.67±1.15 bc	20.00±2.00 bc	30.00±2.00 a	64.67±3.06 a	2.30±0.13 a	910.32±0.08 a
	1/4th of LC_{50}	28.00±2.00 c	22.00±2.00 c	12.00±2.00 c	21.33±2.31 ab	16.67±1.15 c	50.00±2.00 c	1.77±0.03 c	577.60±0.10 b
	1/5th of LC_{50}	24.67±2.31 c	32.00±2.00 a	12.67±2.31 c	18.00±2.00 c	12.67±2.31 d	43.33±4.16 d	1.62±0.11 d	466.28±0.07 c
	1/6th of LC_{50}	49.33±3.06 b	26.00±2.00 b	8.00±2.00 d	8.00±2.00 e	8.67±1.15 e	24.67±2.31 e	1.01±0.08 e	267.40±0.13 d

The means with similar letters in a single column for each variable are statistically non-significant at $p<0.05$.
*CTL = Cumulative Tail Length (µm).

%age of damaged nuclei

The %age of damaged nuclei under all treatments viz. 1/3rd, 1/4th, 1/5th, 1/6th of LC_{50}, negative control and positive control varied significantly at $p<0.05$.

The %age of damaged nuclei were observed higher at 1/3rd of LC_{50} exposure as compared to control groups, representing concentration/dose dependent DNA damage.

GDI

Tested *Cyprinus carpio* also respond differently towards damage induction measured in terms of genetic damage indices (GDI). Incidence of GDI was observed higher (2.30±0.13) at 1/3rd of LC_{50} mixture exposure while same was least in negative control group.

CTL (µm)

Statistically significant cumulative tail length (CTL) of comets were observed at various sub-lethal concentrations endosulafan-chlorpyrifos mixture, negative and positive control groups which ranged between 2.50±0.09 to 910.32±0.08µm observed at negative control and 1/3rd of LC_{50} exposure, respectively.

Discussion

During the present study, the alkaline version of comet assay was successfully applied to estimate the DNA damage in the peripheral erythrocytes of *Cyprinus carpio* exposed to sub-lethal concentrations of binary pesticide mixture i.e. endosulfan-chlorpyrifos and compared with negative and positive control. Pesticides can cause direct DNA damage due to action of their parental compounds or indirectly due to over-production of reactive oxygen species (Oliveira *et al.*, 2009). The central idea of the present study was to characterize the DNA damage induced by prolonged exposure to mixture. DNA damage was estimated by measuring the %age of damaged nuclei, GDI and CTL. Statistically significant increase in DNA damage was observed in the peripheral blood erythrocytes of fish under polluted water exposure (Klobucar *et al.*, 2010) while tertiary mixture of pesticides (chlorpyrifos+endosulfan+thiram) has been reported to cause significantly higher DNA damage (Tope and Rogers, 2009). Altinok *et al.* (2012) also observed higher ($p<0.05$) DNA damage in terms of comet tail length, tail intensity, tail moment and tail migration in the erythrocytes of *Oncorhynchus mykiss* which was exposed to different sub-lethal concentrations of carbosulfan as compared to positive control group.

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 (Guilherme *et al.*, 2012). Pesticides have ability to make variety of reactive oxygen species like H_2O_2, O^{2-} and OH^- and electrophilic free radicals that can interact with nucleophilic sites of DNA causing strand breakage (Banudevi *et al.*, 2006). Pesticides can form covalent bonds with DNA, resulting in the formation of DNA adducts (Hartwell *et al.*, 2000), alter antioxidant defense systems and may induce oxidative damage in the aquatic organisms (Monteiro *et al.*, 2006).

The present study showed that 1/3rd of LC_{50} exposure of pesticide mixture to the fish caused significantly ($p<0.05$) higher DNA damage while negative control treatment exerted significantly slightest damage to the nuclei. Dose dependent DNA damage in fish erythrocytes under exposure to carbosulfan by employing comet assay technique was also observed by other author. Exposure of fish to the sub-lethal concentrations like 1/4th, 1/2nd and 3/4th LC_{50} of carbosulfan gave significantly ($p<0.01$) higher DNA damage in fish erythrocytes in terms of %age of tail DNA than that of control group (Nwani *et al.,* 2010).

Genotoxic potential of different pesticides in the peripheral blood erythrocytes of fish by using micronuclei assay was evaluated by Naqvi *et al.* (2007). Genotoxicity of pesticides in fish was found to be in the order of cypermethrin > chlorpyrifos > malathion > lambda-cyhalothrin > buctril. Cavalcante *et al.* (2008) observe significantly ($p<0.05$) higher DNA damage in fish erythrocytes under exposure to 10mgL^{-1} of roundup than that of negative control.

Tested sub-lethal concentrations in the present study could be environmentally relevant concentrations, even though repeated applications of 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, but may affect the performance of organisms, disrupt their developmental stages, behavior, physiology and ultimately may reduce their survival rate (Susan *et al.*, 2010).

Present results are also in accordance with Ambreen and Javed (2016) who also reported simultaneous increase in DNA damage with increase in the concentrations of mixture in erythrocytes of *Cyprinus carpio* as compared to controls i.e. negative and positive control. The DNA damage induced due to mixture of pesticide recommended a severe health concern towards their possible danger for the survival of *Cyprinus carpio* (common carp) in their aquatic environment.

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