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Research Article

Chlorpyrifos and Endosulfan Mixture Induced DNA Damage and Nuclear Anomalies in RBCs of *Labeo rohita* Assessed Through Comet and Micronucleus Assay

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HN conduct experiments and prepared draft. SA and KA supervised the study. TA assisted in writing of draft. KA and SQA reviewed the draft. MR assisted in data collection. FL assisted in data analysis. AAQ and MAH reviewed and edited the draft.

Keywords
Fish, Insecticides, RBCs, DNA damage

Abstract | Insecticides are widely used to control the insect damaging food and cash crops as well as the fruit plants. However, terrestrial and aquatic biodiversity is directly affected by overuse of insecticides. Present study was conducted to find out the genotoxic effect of insecticides on DNA damage and nuclear anomalies in RBCs of *Labeo rohita* after exposure to chlorpyrifos+endosulfan (CPF+END) by using Comet and Micronucleus Assay. Twenty fingerlings of *L. rohita* was kept in LC_{50} concentration of CPF+END, (1.95±0.02 μgL^{-1} for 96 h) for 5 days after acclimatization. The blood sample of 5 fish was collected after 24, 48, 72 and 96 h to see the DNA damage and nuclear anomalies. Results of the current study revealed that damaged nuclei (DN%) and genetic damage index (GDI) in RBCs of *L. rohita* exposed to insecticides mixture was increased throughout the experiment. Similarly, the frequency of micronuclei (MN) and other nuclear anomalies (NAs) were also increased as the duration of exposure passed. It is concluded that exposure of fish to insecticides may cause considerable genetic damage to the fish. The both Comet and MN Assay are good biomarkers to identify the insecticides pollution. The results of our study can be utilized in environmental risk evaluation and in bio-monitoring approaches.

Novelty Statement | This research sheds light on the genotoxicity and environmental risks associated with endosulfan and chlorpyrifos pesticides in freshwater ecosystems and offers new insights into the potential synergistic effects of these pesticides on aquatic organisms.

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Introduction

In the field of agriculture pesticides, insecticides and herbicides are abundantly used as chemicals to control

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pests, insects and unwanted weeds from crops (Hussain *et al.*, 2019). Terrestrial and aquatic biodiversity is directly affected by overuse of pesticides (Latif, 2020). Fish comes in direct contact with insecticides, as these chemicals are widely used on crops growing in the vicinity of fish farms (Stanley and Preetah, 2016). Pakistan ranks second in consuming pesticides among the countries included in the Indian Subcontinent (Muazzam *et al.*, 2019).

Class of pesticides called organophosphate is widely used against pests of cash crops like rice, cotton, vegetables and fruit trees i.e., bananas and apple (Gomez, 2009; Grube *et al.*, 2011). An organophosphate, chlorpyrifos also seems to affect fish by creating oxidative stress damaging hereditary material, affecting blood characteristics, creating histopathological alterations and inhibition of acetylcholine esterase (Banaee *et al.*, 2014). Insecticide organochlorine is also being widely used against many insects since long (Rehman *et al.*, 2016). Endosulfan, an organochlorine has the potential to kill wide range of insects (Corsini *et al.*, 2013). DNA is severely damaged by endosulfan by different ways including structural modifications, cellular transformation and gene amplification (Ullah, 2015). According to Ghaffar *et al.* (2015) endosulfan also reported to produce reactive oxygen species (ROS) as endosulfan is involved in redox reaction. Previous studies had shown that the effect of pesticides on water bodies consequences oxidative stress, cell death and DNA damage (Alak *et al.*, 2019; Bright, 2018; Ucar *et al.*, 2020).

Antioxidants badly affects organisms by producing oxidative stress and this disturbance can be seen when organism's body absorb such harmful pollutants which increase level of free radicals and ROS. Ultimately generated imbalance cause problem to organism health. Hematology of fish seems to be changed after exposure of damaging pollutants stresses (Xing *et al.*, 2011).

Damage of chemical mutagens, pollutants to organisms DNA can be estimated by a widely used method called Comet Assay. Basic principle of Comet Assay is based on movement of genetic material along charged poles (Nagarani *et al.*, 2012). Knowing genotoxic effects of pollutants to fish, Comet Assay is taken as one of the best and flexible technique. Significance of Comet Assay includes analyzing effects of physical and chemical mutagens, detecting low level of DNA damage, indicating DNA structural changes, apoptosis and determining inter strand cross linkages (Ullah *et al.*, 2016). According to Bolognesi and Hayashi (2011), pesticides affect cell division badly and creates micronuclei by affecting chromosomal damage. Micronucleus (MN) Assay is adopted to estimate genotoxic changes after exposure of aquatic organisms to chemical pollutants (Ventura-Campos de *et al.*, 2008). MN Assay is widely used to estimate aneugenic and clastogenic effects *in vitro* (Ali *et al.*, 2011) and *in vivo* (Norppa and Falck, 2003). The focus of this study was to find out the genotoxic effect of insecticide mixture on *Labeo rohita* by adopting two different techniques such as Comet Assay and Micronuclei Assay.

Materials and Methods

Acclimatization of experimental fish

Fingerlings of *L. rohita* (N 30) were purchased from the Fish Seed Hatchery Faisalabad. In order to become accustomed to new environment fishes were kept for two weeks in cemented tanks. LC_{50} (96-h) conc. of chlorpyrifos+endosulfan used was 1.95±0.02 μgL^{-1} as reported by Naz *et al.* (2019). Twenty fingerlings were kept in LC_{50} conc. of (CPF+END) mixture for 96 h. Five fish samples were maintained in same conditions without pesticides as a negative control while 5 fish were exposed to cyclophosphamide as a positive control. Five fish were removed from the experimental group after 24, 48, 72 and 96 h, for blood sampling.

Stock solutions

Stock solution of chlorpyrifos (CPF) and endosulfan (END) was prepared by dissolving 1g/100ml of technical grade CPF and END in analytical grade (95%) methanol separately. Required concentrations of solutions of CPF and END mixture (1:1) were prepared by diluting it in deionized water.

Comet assay

During sampling, blood was collected from the caudal vein of each fish. Eppendorf tube was filled with blood and anticoagulant was added to prevent blood clotting. Lysis, electrophoresis and staining was done as recommended in Comet Assay (Singh *et al.*, 1988). Epi-Fluorescence microscope were used for scoring randomly two slides that were prepared for each treatment. Five categories of damaged cells were designed as "comet" according to comets tail lengths (Type IV: complete damage, Type IIII: high level damage, Type II: medium level damage, Type I: low level damage and Type 0: undamaged). For measuring the comet tail length of damaged cells TriTek Comet Score TM software was used (Jose *et al.*, 2011).

$$\text{Damaged cells (\%)} = \text{Types II} + \text{III} + \text{IV}$$

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

Micronucleus assay

A slide was prepared by instantly smearing droplets of blood which were taken from the caudal vein of a fish. Methanol was added to fix smears and slides were left for drying in air for 10 minutes. Wright-Giemsa stain were used for 8 minutes for staining (Barsiene *et al.*, 2004). According to Fenech *et al.* (2003) binocular microscope

was used for scoring of micronuclei and the NAs on coded slides. To calculate the MN frequency following formulae was used:

$$MN\% = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

Statistical analysis

Data was expressed in mean (±SE) and was analyzed by non-parametric Mann-Whitney U-test obtained from DNA damage and nuclear abnormalities. The Microsoft Excel was used to draw the graphs.

Results and Discussion

The results of present experiment showed that different types of comet, DN and GDI in RBCs of insecticides mixture exposed *L. rohita* was increased throughout the experiment (Figure 1). Although GDI and damage nuclei were observed on the first day of experiment but as compared to day 2, 3 and 4, it seemed to be gradually increased and on day 4 GDI and DN was at its higher level. Similarly, MN and NAs frequency was also increased as the duration of exposure passed (Figure 2). The duration specific response was observed for both DNA damage and nuclear anomalies in *L. rohita*. Same trend was shown by Ambreen and Javed (2018), who reported that *Oreochromis niloticus* exposed to insecticides for 70 days showed GDI and it was totally dose and duration dependent. More DNA damage was reported on 70 days of experiment as compared to non-exposed group (Figure 1 and 2).

Results of the present study showed that as time of exposure of fish to insecticides increased, damage of DNA also increased significantly. This may be due to some biochemical effects of insecticides. The ROS produced as a result of organophosphate metabolic processes that damage the pyrimidine and purine bases which induce DNA strand breaks (Lu *et al.*, 2013). A direct relation of increased oxidative stress and DNA damage was reported by Ansari *et al.* (2011). DNA strand breaks, enzyme inactivation, and even carcinogenic effects on excessive accumulation of ROS (hydrogen peroxide, superoxide anion, and hydroxyl radical was reported by many researchers (Guney *et al.*, 2007). The hydroxyl radical, with a lifetime of a few nanoseconds, is the most important free radical of biological and toxicological importance, because of its potent oxidative potential and indiscriminate reactivity with cellular components, such as lipids of biological membranes, proteins of enzymes, and DNA (Jackson and Loeb, 2001). Pesticides composed different heavy metals such as manganese, copper, zinc, lead, cadmium, iron and nickel, etc. that causes DNA damage (Hayat *et al.*, 2007) by Fenton-like reactions (Ercal *et al.*, 2001). For repairing these DNA damages, living organism have ability to synthesize and control specific enzymatic systems (Fenech and Ferguson, 2001). Alkyl and phosphoryl groups are two electrophilic groups which are produced by the metabolism of organophosphate pesticide and are a good substrate for nucleophilic attack. Interaction with DNA may be resulted by phosphorylation process (Ali *et al.*, 2009).

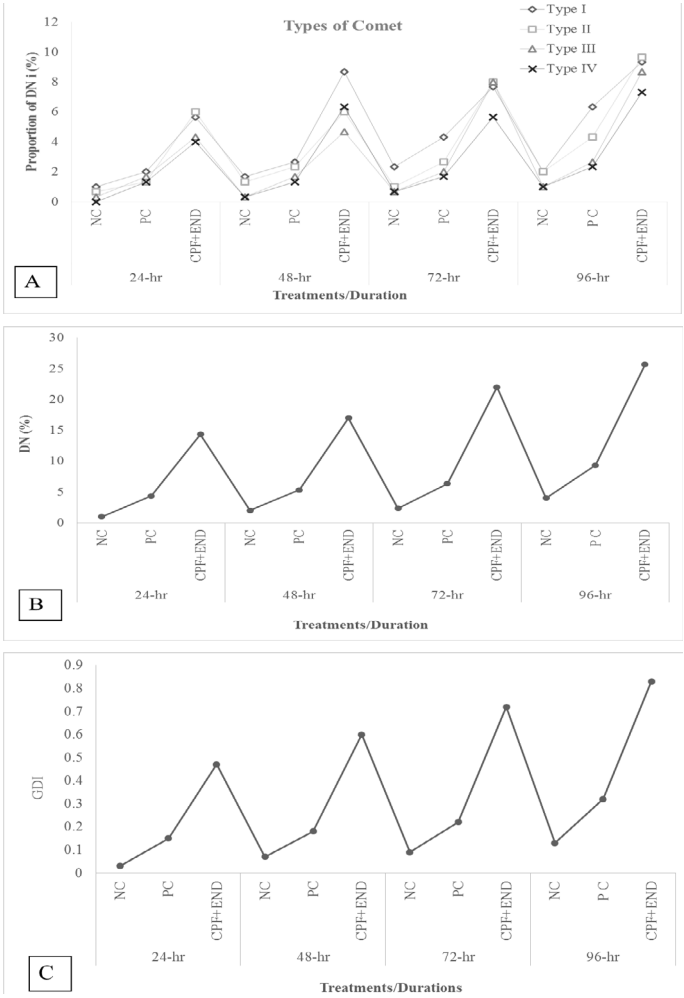

Figure 1: The different types of comet (A), DN (B) and GDI (C) In RBCs of *L. rohits*.

In the present study it was found that DN and GDI in RBCs as well as MN and NAs frequency of insecticides mixture exposed *L. rohita* was increased throughout the experiment (Figures 1 and 2). Ullah (2015) also reported different extents of damage to DNA of *L. rohita* subjected to endosulfan for 28 days at different concentrations. Wang *et al.* (2018) reported that highest concentration of organophosphate sumithion disturbed organisms ROS and damaged DNA as estimated by Comet Assay. Pawar *et al.* (2019) noted DNA damage (% tail DNA) by Comet Assay and found similar results to our study that damage is totally time and dose dependent. El-Bouhy *et al.* (2018) documented significant increase in tail length, tail DNA% and tail moment when Juvenile *Nile Tilapia* was exposed to chlorpyrifos. Hussain *et al.* (2018), stated that significant damage to DNA was observed with the help of Comet Assay in *L. rohita, C. mrigala* and in *C. catla*. According to Khisroon *et al.* (2021) when the concentration and exposure duration of endosulfan increased, DNA damage level was also increased. For MN and NAs, results similar to our study were reported by Shoaib and Ali (2021); Ali

et al. (2018). Islam *et al.* (2019) observed that level of MN of striped catfish, *Pangasianodon hypophthalmus* increased when exposed to different concentration of insecticide sumithion. It was reported that MN formation in *L. rohita* micronucleus was totally time dependent and their number increased after 96 hours of exposure to chlorpyrifos (Ismail *et al.*, 2018).

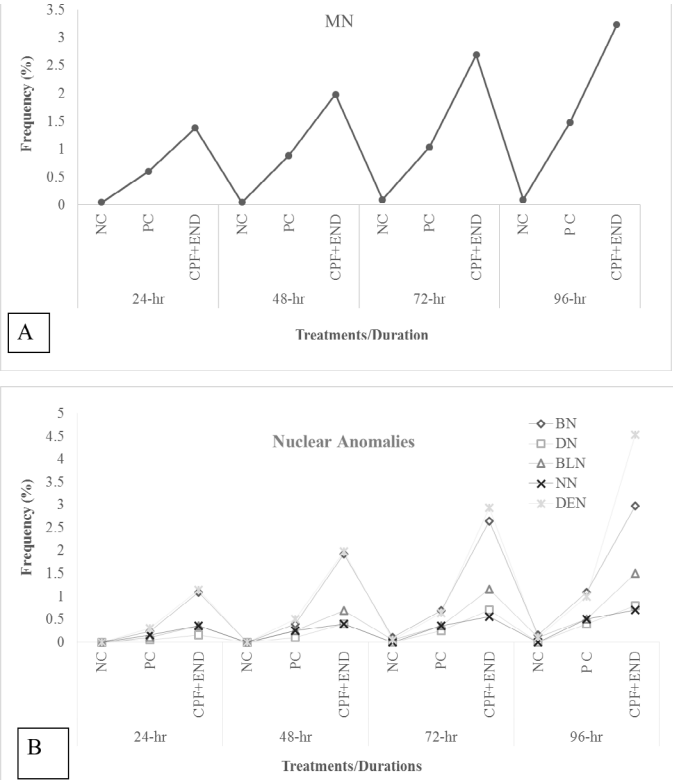

Figure 2: The MN (A) and NAs (B) frequency in RBCs of *L. rohita*.

As reported in the present study, in *L. rohita* exposed to insecticides, GDI increased with the passage of time. Same trend was observed by Naz *et al.* (2019), who reported significant GDI and damaged nuclei in *C. catla* exposed to chlorpyrifos and endosulfan. Low level exposure of chlorpyrifos to common carp significantly caused DNA damage forms nuclear abnormalities (Mitkovska and Chassovnikarova, 2020). After exposure of monocrotrophos and organophosphate to zebra fish Micronuleus test and comet assay indicated formation of micronulei and DNA damage (Dcosta *et al.*, 2018). Similarly, many Authors (Naz *et al.*, 2021; Hemalatha *et al.*, 2020; Davico *et al.*, 2020) also documented that the Micronucleus Test shows formation of MN and NAs in fish erythrocytes after exposing to insecticides. Naz *et al.* (2021) also documented that In *C. mrigala* damaged DNA, nuclear anomalies, micronuclei are totally time dependent when exposed to three different mixtures of B+C, C+B and B+E.

Conclusions and Recommendations

The current research concluded that CPF+END mixture is very toxic to fish even at very low concentration. It has ability to cause considerable genetic damage to fish. The both Comet and MN Assay are good biomarkers to identify the insecticides pollution. The results of our study can be utilized in environmental risk evaluation and in bio-monitoring approaches.

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Consent for publication

All authors are fine with the current version of the manuscript and give their consent for publication.

Ethical approval

Not applicable.

Conflict of interest

The authors have declared no conflict of interest.

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