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Investigation of the Genotoxicity of Malathion to Grass Carp *(Ctenopharyngodon idella)* Using Comet Assay

Fida Ullah Khan[1], Ahmad Ullah Khan[2], Faisal Ahmad Lodhi[1], Wasibullah Khan[2], Meraj Khalid[2]

[1]Department of Zoology, Quaid-i-Azam University Islamabad, Pakistan.
[2]Institute of Zoological Sciences, University of Peshawar, KP, Pakistan.

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Corresponding Author: Fida Ullah khan
Department of Zoology, Quaid-i-Azam University Islamabad, Pakistan.
Email: fidaullahkhan@bs.qau.edu.pk

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ABSTRACT

Malathion, an organophosphorus insecticide, used around the world, can be harmful to the environment because it doesn't break down easily, especially in aquatic ecosystems. As it accumulates in these environments, it can cause serious damage, disrupting biodiversity and harming water quality. This study assessed the genotoxic effects of malathion on the blood cells of grass carp (Ctenopharyngodon idella). Firstly, the LC50 value of malathion for 96 hours' exposure was identified to be 2.42 ppm. The fish were then subjected to three varying concentrations of malathion: 0.403 ppm/day, 0.605 ppm/day, and 1.21 ppm/day, over periods of 10, 20, and 30 days. The control group remained unexposed to malathion. The results indicated that DNA damage was significant across all concentrations after 10 days ($P < 0.05$). Moreover, after 20 and 30 days of trials, the extent of DNA damage exhibited a markedly significant increase ($P < 0.000$).The comet assay was used to evaluate the extent of DNA damage in the fish, taking into account exposure time and dosage. Our results show that higher concentrations and longer exposures aggravated DNA damage. Authorities must regulate and monitor the inappropriate use of malathion, as this study demonstrated its significant genotoxic effects on Ctenopharyngodon idella.

INTRODUCTION

Pesticides are either chemical or natural substances used to eliminate harmful and unwanted organisms such as insects, weeds, nematodes, and other pests that threaten crops, health, and food supplies in gardens, public areas, and agricultural lands (Rani *et al.,* 2021). Higher concentrations of pesticides have been reported in various urban environmental matrices in developing countries, especially in the tropical region of South Asia, which includes India, Bangladesh, Nepal, Pakistan, and Sri Lanka. These areas are considered significant sources of contaminants, both regionally and globally (Ali *et al.,* 2014). While effective at managing pests, these substances pose significant environmental risks, particularly when they enter water sources, leading to contamination of aquatic ecosystems.

Pesticides can have detrimental effects on fish populations, as well as other aquatic organisms, by disrupting their habitats and impairing their health (Kumar et al., 2021). Pesticides are considered potential mutagens because they contain compounds that may cause DNA fragmentation. According to the World Health Organization

(WHO), around one million people suffer severe injuries as a result of pesticide exposure. We observe a steady increase in the death rate from 0.4% to 1.9% (Eddleston, 2020; Jia *et al.,* 2020). Fish and other non-target creatures may be seriously endangered by pesticide residues in aquatic habitats (Ruiz-Suárez *et al.,* 2015). These days, there are serious worries about the genotoxic effects of pesticides on non-target species as well as their consequences on the ecosystem (Rasmussen et al., 2015).

Malathion, an aliphatic organophosphate first used in 1950, is one of the insecticides that belongs to the family of organophosphate compounds and can be among the oldest and most widely used (Ware and Whitacre, 2004). Because of its shorter environmental half-life and huge selectivity for the target pests, the insecticide Malathion (MAL) is a highly preferable option among this category of compounds (Prathibha *et al*., 2014). An assortment of feed and food crops, including wheat, mushrooms, broccoli, garlic, citrus, cabbage, squash, celery, rice, cotton, root crops, nuts, greens, berries, roughage, organic produce, and hay, all of which are treated with malathion (EPA, 2009). In the United States, the largest use of malathion has been linked to an effort to rid cotton-growing regions of the boll weevil (EPA, 2004).

Because of this, fish are frequently utilized in biomonitoring research worldwide. When assessing the genotoxic consequences of dangerous substances present in aquatic ecosystems, fish are a crucial vertebrate (Siraj *et al.,* 2018; Shah *et al.,* 2021; Qu *et al.,* 2017) examined how the glycogen, protein, and cholesterol levels in the heart muscle of freshwater gobiid fish, namely *Glossogobius giuris,* were affected by sub-lethal concentrations of malathion (0.05, 0.25, and 0.5 ppm) across exposure periods of 24, 48, 72, and 96 hours. They looked at the changes in protein, glycogen, and cholesterol and discovered that these parameters changed for different malathion exposure durations.

Comet essays are a useful tool for determining whether DNA structure has been damaged. According to Khisroon *et al.,* (2015), the Comet measure is a useful technique for differentiating between DNA cross-joins, soluble base labile locations, single-strand breaks, and two-fold strand breaks. To evaluate DND damage, the method may be applied to both in vitro and in vivo testing (Azqueta *et al.,* 2019). One of the most amazing methods for determining whether a person has DNA damage is single cell gel electrophoresis (Lu et al., 2006). Due to its responsiveness, the comet measure has emerged as one of the most often used techniques (Pitarque et al., 1999).

The purpose of this study was to use the comet test to examine the genotoxic effects of the malathion an organochlorine pesticide on *Ctenopharyngodon idella* while accounting for the challenges associated with previously discussed chemicals.

MATERIALS AND METHODS

Malathion, an organophosphate insecticide, was acquired from a local market for the study. The decision to purchase Malathion locally ensures accessibility and cost-effectiveness for the research while also providing a sample representative of the product available to consumers or professionals in the area.

Procurement of Fish

The fingerling grass carp (*Ctenopharyngodon idella*) were obtained from the Hatchery and Training Center of Carp in Sherabad, Peshawar, Khyber Pakhtunkhwa. After being safely transported to the laboratory, they were placed in glass tanks. The entire process of collection, transport, and placement in aquariums took approximately three hours. The fish were inspected to ensure they were free from bacterial, fungal, or ectoparasitic infections. To ensure the fish could adapt to the new conditions, they were given a one-week acclimation period. During this time, the fingerlings were monitored closely to ensure they adjusted well to their new surroundings, including the water quality, temperature, and overall tank conditions. The fish were kept in twelve 15-liter glass aquariums, each containing aerated dechlorinated tap water. The water conditions were maintained at a temperature range of 25 to 30°C, a pH between 7 and 8, and fully saturated dissolved oxygen levels. During the acclimation period, the fish were fed daily at a rate of 2% of their body weight.

Determination of Sublethal Concentrations

The acute toxicity bioassay for malathion was performed following standardized protocols to determine the 96-hour LC50 value (APHA,

AWWA, WPCF, 2005). Malathion was dissolved in water to prepare the test solutions, and a preliminary range-finding test was conducted before the main trial. In the primary experiment, ten samples were exposed to six different concentrations of malathion (1, 3, 5, 7, and 9 ppm) over 96 hours. The test was repeated, and the 96-hour LC50 value was calculated to be 2.42 ppm using probit analysis according to the method described by Finney (1971). Based on this LC50 value, three sublethal concentrations of malathion were selected to assess its genotoxic effects: sublethal I (SL-I; 1/2 of the 96-hour LC50 at 1.21 ppm), sublethal II (SL-II; 1/4 of the 96-hour LC50 at 0.605 ppm) and sublethal III (SL-III; 1/6 of the 96-hour LC50 at 0.403 ppm)

Laboratory trials and Sampling

Ten fish were placed in each of the twelve aquaria, for a total of 120 fish specimens. Three aquaria served as controls, while nine were assigned to the treatment groups. The aquaria were covered with nets to prevent the fish from jumping out, and each tank was equipped with air pumps to provide a continuous oxygen supply. Malathion was replenished under the daily (every 24 hours) water change.

To collect blood, three fish from each tank were humanely euthanized on days 10, 20, and 30. The blood was collected and transferred into labeled EDTA tubes, with five to eight drops of heparin added to prevent clotting. Any remaining fish tissue was disposed of following the guidelines established by the University of Peshawar's ethics committee and the advanced research board. Additionally, the health of fish behavior was monitored throughout the 30-day observation period.

Evaluation of DNA Damage

DNA damage was assessed using the comet assay, slightly modified from Khisroon et al. (2015). Normal melting agarose (NMA) was used to form the initial layer of the slides. One-third of the slides were melted in the oven, then placed in a Coplin jar to be covered with NMA. Afterward, the slides were removed and allowed to cool. For the second layer, 75 µL of 0.5% low-melting point agarose (LMPA) was carefully applied to the pre-coated agarose slide after mixing 10 µL of blood with 500 µL of phosphate-buffered saline (PBS).

After the slides were solidified with an ice pack, 85 microliters of low-melting-point agarose (LMPA) were added to fill the remaining gaps. To break down the cells, the slides were removed and left in a lysing solution at 4 °C overnight. The slides were then placed in a horizontal gel electrophoresis tank with freshly prepared cold electrophoresis buffer and left for 20 minutes. Afterward, electrophoresis was started and run at 25 V and 300 mA for 30 minutes

The slide was first treated with a neutralizing buffer and then dehydrated in 70% ethanol. Using a micropipette, acridine orange was applied to the slide at a concentration of 20 µg/mL for staining. The slides were examined under a Nikon Eclipse 801 epi-fluorescent microscope at ×400 magnification, using 450–490 nm excitation filters to capture the comet image. The images were recorded for further visual analysis. The total comet score (TCS), an indicator of DNA damage, was calculated for each fish sample using the method outlined by Collins (2004).

Data Analysis

For statistical analysis, IBM SPSS Statistics 26 software was used to process the data. The software calculated the mean and standard deviation for the measurements taken during the study. The mean provides an average value, while the standard deviation indicates how much individual data points deviate from the mean, helping to assess the variability of the data. The average differences in percentage tail DNA (a measure of DNA damage) were found to be below 0.05, suggesting that any observed differences in DNA damage between the groups were minimal and statistically insignificant. This threshold (0.05) is typically considered the level of significance for determining whether the results are likely due to chance or represent a real difference.

RESULTS

Physiochemical Properties of Test Water

The water tried showed a pH level somewhere in the range of 7.2 and 8.0, with temperatures going from 23.6°C to 25.0°C. All through the examination, it was found that the disintegrated oxygen content was inside the normal scope of 7.5-8.1 mg/L. Somewhere in the range of 253 and 310 µM/cm was the water's conductivity range. The reaches for complete alkalinity and all out hardness

as CaCO3 were 280-325 mg/L and 198-232 mg/L, separately.

DNA Damage

The DNA damage observed on the 10th day is shown in Table 1. Throughout the day, the treated groups exhibited significantly higher DNA damage compared to the control group ($aP < 0.030$, $bP \leq 0.038$, $cP \leq 0.020$). The corresponding values for aP, bP, and cP were 0.403, 0.605, and 1.21 ppm, respectively.

The DNA damage observed on day 20 is shown in Table 2. Throughout the day, the DNA damage in the treated groups was significantly higher than that in the control group ($aP < 0.001$, $bP \leq 0.000$, $cP \leq 0.003$)

Table 3 presents the DNA damage observed on the 30th day of the study. The data show that the treated groups exhibited significantly greater DNA damage compared to the control group. Statistical analysis revealed highly significant differences, with p-values of $aP < 0.001$, $bP \leq 0.000$, and $cP \leq 0.000$, indicating that the DNA damage in the treated groups was far more pronounced than in the control group. These p-values suggest strong evidence that the observed differences are unlikely to be due to chance.

Table 1

Comet classes and TCS in response to Malathion treatment in erythrocytes of Ctenopharyngodon idella after 10 days

Concentration	Class 0	Class 1	Class 2	Class 3	Class 4	TCS
Control	97.7 ± 0.6	2.0 ± 1.0	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	2.7±0.6
0.403 ppm	96.0 ± 1.0	2.7 ± 0.6	1.0 ± 0.0	0.3 ± 0.6	0.0 ± 0.0	5.7±2.1[a]
0.605 ppm	95.7 ± 0.6	2.3 ± 0.6	1.3 ± 0.6	0.7 ± 0.6	0.0 ± 0.0	7.0±1.0[b]
1.21 ppm	95.0 ± 1.0	2.0 ± 1.0	1.3 ± 0.6	1.7 ± 0.6	0.0 ± 0.0	9.7±2.3[c]

S.D=standard deviation. *TCS*=Total comet score. *Ppm*=parts per million. Significant difference relative to control group at [a]$P \leq 0.030$, [b]$P \leq 0.038$, and [c]$P \leq 0.020$.

Table 2

Comet classes and TCS in response to Malathion treatment in erythrocytes of C. idella after 20 days

Concentration	Class 0	Class 1	Class 2	Class 3	Class 4	TCS
Control	97.7 ± 0.6	2.0 ± 1.0	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	2.6±0.6
0.403 ppm	94.0 ± 1.0	2.3 ± 0.6	1.7 ± 0.6	1.0 ± 0.0	0.7 ± 0.6	11.3±1.5[a]
0.605 ppm	93.0 ± 1.0	2.3 ± 0.6	2.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	12.7±1.5[b]
1.21 ppm	93.0 ± 1.0	2.3 ± 0.6	1.7 ± 0.6	1.3 ± 0.6	1.3 ± 0.6	15.0±4.3[c]

S.D=standard deviation. *TCS*=Total comet score. *Ppm*=parts per million. Significant difference relative to control group at [a]$P \leq 0.001$, [b]$P \leq 0.000$, and [c]$P \leq 0.003$.

Table 3

Comet classes and TCS in response to Malathion treatment in erythrocytes of C. idella after 30 days

Concentration	Class 0	Class 1	Class 2	Class 3	Class 4	TCS
Control	97.7 ± 0.6	2.0 ± 1.0	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	2.7±0.6
0.403 ppm	92.0 ± 1.0	3.0 ± 0.0	2.0 ± 0.0	1.3 ± 0.6	1.3 ± 0.6	16.3±4.0[a]
0.605 ppm	90.7 ± 0.6	3.3 ± 0.7	2.0 ± 0.0	2.0 ± 0.0	1.7± 0.6	19.3±2.1[b]
1.21 ppm	89.0 ± 1.0	3.7 ± 0.6	2.7 ± 0.6	2.0 ± 0.0	2.3± 0.6	24.3±3.2[c]

S.D=standard deviation. *TCS*=Total comet score. *Ppm*=parts per million. Significant difference relative to control group at [a]$P \leq 0.001$, [b]$P \leq 0.000$, and [c]$P \leq 0.000$.

DISCUSSION

The genotoxic effects of various pesticides have been investigated in different animal models, including fish, amphibians, birds, and mammals (Siraj *et al.*, 2018; Shah *et al.*, 2021; Suliman *et al.*, 2020). These studies have revealed varying levels of DNA damage across species. Fish, in particular, are frequently used in such research due to their economic significance, essential role in food webs, and their sensitivity to even small amounts of toxic substances (Ali *et al.*, 2008; Jha *et al.*, 2008; Osman *et al.*, 2007).

The current study's objective was to evaluate the effects of the harmful chemical malathion on grass carp DNA using the comet test.The comet assay is the most commonly used method for

assessing DNA damage, due to its simplicity, reliability, sensitivity, and proven effectiveness in various fish species (Clara Ersson 2011).The comet assay has several advantages, including: a) requiring only a small number of cells; b) measuring variations in sensitivity or response between cells; c) detecting low levels of DNA damage with high sensitivity; and d) having the potential to evaluate damage in almost any eukaryotic cell

Since fish blood is a readily accessible and easily collectible tissue from the fish's body, it was selected for the experiment Furthermore, pollutants only encounter the RBCs when they circulate in the body of fish. As a result, this tissue is frequently used to measure DNA damage in fish employing the comet test technique (Tasneem and Yasmeen, 2018). Furthermore, because peripheral blood reflects an organism overall health, erythrocytes in blood of fish are the best option to find damage in the DNA. The goldfish *(Carassius auratus)*, gulfam *(Cyprinus carpio)*, thalapia (0. Niloticus), Rohu (*Labeo rohita)*, *Catla catla* are all used in previous studies to determine the suitability of erythrocytes (peripheral) studies genotoxic (Pandey *et al.*, 2018).

In ecotoxicological research, the comet assay is a widely used technique to detect damage to the DNA strands of fish and other aquatic animals (Ullah *et al.*, 2017). The most reliable metric for computing breakage of DNA strand is the quantity of DNA in the tail region (Nataraja *et al.*, 2018). As a result, it was used to demonstrate how malathion doses affected *C. idella*. After 10, 20, and 30 days, blood samples were collected and analyzed, showing a dose- and time-dependent increase in DNA tail length also described by Sharma and Chadha (2021). They compared control and treatment groups with BPA doses resulted in a significant change in both parameters. The most severe damage was seen after 30 hours of exposure to the highest dose of malathion (10µl/L), with tail length increasing and tail moment increasing in relation to the control group.

DNA damage exhibited a concentration-dependent relationship, with concentration 1 showing the highest level of damage, followed by concentrations 2 and 3. According to Gulsoy et al. (2015), although treatment with boric acid produced the most significant effect after 96 hours of exposure, zebra fish (*D. rerio*) exposed to borax showed notable DNA damage after 24 hours, followed by a decrease at 48 and 72 hours. Ateeq *et al.*, (2005) found that the erythrocytes of *Clarias batrachus* exhibited increased DNA damage in a concentration- and time-dependent manner following exposure to 2,4-dichlorophenoxyacetic acid.

CONCLUSION

The result of this study show that grass carp *(Ctenopharyngodon idella)* may be utilized as a biomarker to assess malathion genotoxicity using comet assay, which substantiated to be sensitive instrument for detecting a positive reaction in blood tissue in response to dosage and exposure duration. According to the results, at sub-lethal concentrations, malathion can cause DNA damage and a decrease in the total protein concentration in fish erythrocytes; these effects are exacerbated by both exposure duration and concentration Furthermore, the negative impact of malathion induces oxidative stress and genetic damage in *C. idella* erythrocyte and alteration in total protein contents. This might be due to inhibition of the DNA repair system as well as microtubular disruption activities.

RECOMMENDATIONS

- The quality of water resources should be examined on a regular basis, as well as the general environment. As a result, any anomalous changes in the quality of water will be obvious, and proper combat process would be conducted prior to an epidemic outbreak. Regular water quality checks should be taken by protection agencies such as (Pak-EPA) to preserve aquatic species from pollution.
- it is recommended that an appropriate effluent treatment system be adopted to ensure proper discharge into the environment.
- Numerous regulations control the quality assurance of aquatic reserves in Pakistan; however, the guidelines are seldom followed. Developed countries shows an excellent example, we should take cues from

them. They have more successful institutions monitoring the environment and stricter environment norms and regulations.

- Further work can be done on the impacts of malathion on muscle protein, endocrine system and behavior of fish.

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