Original Article

Sub-Lethal Effects Of Chlorpyrifos On Geno-Neurotoxic Profiles In Freshwater Fish Cyprinus Carpio(L.)

Kareema Ambareen[1]*, Mididoddi Venkateshwarlu[2]

[1]*Department of Applied Zoology, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shimoga – 577451 Karnataka, India. ORCID iD: 0000-0003-1636-8320
[2]Professor, Department of Applied Zoology, Kuvempu University, Jnana Sahyadri, Shankaraghatta / Vice Chancellor, Tumkur University, B.H Road, Tumkur-572103, Karnataka, India., ORCID iD: 0000-0001-7122-3349

***Corresponding Author:** Kareema Ambareen

*Department of Applied Zoology, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shimoga – 577451 Karnataka, India. ORCID iD: 0000-0003-1636-8320
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Abstract

Pesticide toxicity is a global concern and chlorpyrifos is one of the most widely used organophosphate agrochemicals,it is frequently detected in surface ground waters of India. Chlorpyrifos(CPF) LC50 – 96 h was calculated as 0.318 mg/l using probit analysis based on LC50 value two sublethal concentrations of 1/5th of LC50 (0.0636 mg/l) and 1/10th of LC50 (0.0318 mg/l) were determined. The influence of CPF on the acetylcholinesterase (AChE) activity and Ach content and the Genotoxicity of *Cyprinus carpio* was evaluated. Results confer that significant inhibition in the AchE activity in brain tissue resulting in a build-up of Ach at the neuronal synapse resulting in a decrease of cholinergic transmission finally impairs the neurophysiological activity and ultimately result in the death of fish although studies on genotoxic effects of CPF were carried out to examine the prevalence of nuclear abnormalities in freshwater fish *Cyprinus carpio* using Comet and MN assay in blood erythrocytes and gill cells as percentile tail DNA damage as comets and micronuclei and other nuclear anomalies were scored in erythrocytes of *Cyprinus carpio* it was found that significant effect was observed for both concentrations and periods of exposure in the treated fishes. The highest DNA damage and increased micronuclei frequency and inhibitory AchE activity in *Cyprinus carpio* were found to be high on the highest sublethal 1/5th of LC50 followed by Sublethal 1/10th of LC50 concentration. Overall findings imply that AchE and genotoxic biomarkers may be useful diagnostic tools for chlorpyrifos poisoning in biomonitoring programs.

Keywords: Chlorpyrifos; *Cyprinus carpio;* AchE, Ach: Comet assay; Micronuclei assay

Significance statement:

The study of neurotoxicity and genotoxic impact of Chlorpyrifos on fish *Cyprinus carpio* helps in evaluating neurotoxic deformities and genomic instability and this study proved to be an effective tool for assessing the interaction between susceptibility of aquatic animals with the exposure of genotoxic contaminants and DNA damage. The findings of this study could help to assess the toxicant impacts at the sub-lethal concentration on freshwater species this could help to predict the possible mechanisms of toxicity in humans because of their diagnostic significance. It would be very advantageous in assessing the associated environmental risk of these pesticides and also the hazardous impact, thus establishing subsequent management strategies for safeguarding aquatic organisms and their associated fauna. it would be very advantageous in assessing the associated environmental risk of these pesticides and also the hazardous impact, thus establishing subsequent management strategies for safeguarding aquatic organisms and their associated fauna.

1. INTRODUCTION

Pesticides are a significant group of environmental pollutants used in intensive agriculture methods to protect crops from diseases and pests and to increase overall crop yields with minimal effort and labor [1]. Water pollution from agricultural and industrial wastes is an unavoidable and global problem [2]. Pesticides used in agricultural areas to manage pests are very toxic to non-target organisms such as fish and harm their health by impairing metabolism, which often leads to death [3]. Pesticides are commonly used in modern agriculture to aid in food production. However, some producers must have the potential to cause substantial health and/or environmental harm. Consequently, producers should take care to follow label requirements and use a low-risk product wherever possible [4].

Chlorpyrifos (O, O diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate) is one of the earliest evolved biggest selling organophosphate insecticides. In India, CPF was introduced in 1965[5] and widely used for controlling various pests

belonging to the orders such as Diptera, Coleoptera, and Homoptera in soil or on vegetation[6]. Likewise, it is also used in the control of domestic and agricultural pests under the trade name Dursban and Coroban, etc. However, due to its widespread use, it may cause adverse effects on the non-target organism, fish[7], and is categorized as a harmful pesticide[8]. It is one of the major insecticides found in fishery items. According to the report by[9], in 2015 about 200,000 tonnes of CPF were used, and it is anticipated that this insecticide will endure being in high demand universally, leading to widespread distribution. Earlier reports claimed that there were several hundred parts per billion of chlorpyrifos in the water associated with fish death instances, its half-life is 26.5 days[10] in water it penetrates the aquatic environment by the proximity of agricultural lands to water bodies or directly through reckless application in such environments affects the aquatic species. CPF residues were found in groundwaters in the range of 0.11-0.25μg L−1and surface waters in the range of 0.26-0.44μg L−1[11]. Hence allowable limit of CPF is surpassing in the environment. Its low persistence in the aquatic environment makes it the most potent agent to exert negative impacts on nontarget organisms, especially fish[12]. For these reasons, it is an excellent applicant for toxic studies on the health of aquatic organisms[13].

Chlorpyrifos acts as a neurotoxin and major inhibitor of acetylcholine esterase (AChE) which degrades the neurotransmitter acetylcholine (Ach) hence accumulation of Ach in the synaptic cleft results in unregulated neuronal transmission leading to asphyxiation loss of respiratory control, and finally death of an organism[14]. In animals, CPF is converted into 3,5,6-trichloro-2-pyridinol (TCP) and chlorpyrifos-oxon, both of which are significantly more harmful to the nervous system compared to chlorpyrifos[15]. As a result, AChE activity is frequently used as a biomarker of OP pesticide exposure in biomonitoring studies[16]. According to reports by [17], the CPF acts through several different routes to cause genotoxicity, hepatic dysfunction, neurobehavioral abnormalities, and neurochemical alterations. The prevalence of CPF in aquatic environments is harmful to fish and other aquatic organisms that are not the target of the chemical's toxicity[18]. For these reasons, it is an excellent candidate for toxic studies on the health of aquatic organisms[13].

One of the major concerns in the field of environmental studies is the presence of neurotoxic genotoxic contaminants in the aquatic environment, which has made it obligatory to develop sensitive ways to evaluate the hazardous effects of these compounds on aquatic organisms. The present study investigates the neurotoxic effects of chlorpyrifos using ACh and AChE levels in brain tissue, CPF is a potent neurotoxin inhibitor of acetylcholinesterase enzyme activity in the brain [19] it terminates the neurotransmitter Acetylcholine (Ach) by hydrolysis resulting in the release of choline and acetate [20]. This leads to loss of nerve impulse transmission at the synaptic cleft of cholinergic neurons thus ending the neurotransmission and causing neurotoxicity [21] and genotoxic effects using Micronucleus (MN) assay in erythrocytes and single cell gel electrophoresis (SCGE) or Comet assay in gill cells of *Cyprinus carpio* exposed *in vivo*. Both are sensitive, quick, reliable, and widely used recommended procedures for detecting the genotoxic and mutagenic effects of chemicals and xenobiotics in organisms in both field and laboratory settings. The advantage of assessing the MN and SCGE assays is that they may both be used to simultaneously measure DNA damage and are also useful in monitoring aquatic contamination.

Although chlorpyrifos's genotoxic property is previously assessed in green algae (Pseudokirchneriella subcapitata and Nannochloris oculata)[22], three-spined stickleback (Gasterosteus aculeatus)[23]. But research on the neurotoxicity and genotoxic properties of chlorpyrifos in aquatic organisms especially fish *Cyprinus carpio* is not yet done, there is a need for research on the toxicity level of chlorpyrifos, especially in terms of its long-term exposure in freshwater fish *Cyprinus carpio*, commercially available and an ideal model indicator for toxicological investigations. Hence an attempt has been made to assess the neurotoxicity and genotoxic effects of sublethal concentrations of chlorpyrifos in *Cyprinus carpio.*

2. MATERIALS AND METHODS

2.1. Experimental fish specimen and chemical

The freshwater fish *Cyprinus carpio* (L.) (Family: Cyprinidae, Order: Cypriniformes) is edible and commercially valuable. Live fish of size 5-6 cm and weight 3.5-4.5g weight were procured from State Fisheries Department, Bhadra Reservoir Project, Karnataka State, India. To avoid dermal infections, fish specimens were bathed twice in 0.05 percent potassium permanganate (KMnO4) for 2 minutes. The specimens were then acclimatized for two weeks in semi-static systems under laboratory conditions.

During the acclimatization, period fish were supplemented with commercial fish pellets and rice bran twice a day. To decrease the ammonia content in the water, feces and other waste materials were drained off daily. The physicochemical characteristic of test water such as Temperature 25±1ºC, pH 7.2±0.2 at 25ºC, Dissolved Oxygen 6.7±0.8 mg/L, Carbon-dioxide 6.2±0.3 mg/L, Total Hardness 23.2±3.4 mg as CaCO3/L, Phosphate 0.37±0.002 μg/L, Salinity 0.01 ppm, Specific Gravity 1.001 and the conductivity of the water is less than 10 μS/cm. were examined following the standard method [24].

For the present study, the technical grade of Chlorpyrifos (50%EC) with the trade name 'Premain strong' (manufactured by ADAMA India Pvt. Ltd. Hyderabad) was purchased from the local market. This grade of chlorpyrifos was found to be predominantly used in agricultural settings.

2.2.In Vivo exposure experiment

Acute toxicity was determined using the semi-static bioassay method[25], and the 96 h LC_{50} value of chlorpyrifos to *C.carpio* was determined to be 0.318 mg/l (L.) based on the LC50 – 96 h value, two sublethal test concentrations of

chlorpyrifos, 1/5th (0.0636 mg/l)- SL-I and 1/10th (0.0318 mg/l)- SL-II of LC_{50} were chosen as the nominal sublethal concentrations and were employed in the current study to examine the neurotoxic effects of chlorpyrifos using ACh and AChE assay in the brain and muscle tissues and genotoxicity of chlorpyrifos using Comet assay in gill cells and Micronucleus (MN) assay in erythrocytes of. of *C.carpio*. The fish specimens were exposed to these two test concentrations in a semi-static system with the change of test water on every alternate day to maintain the concentration constant. The exposure was continued for up to 21 days and tissue sampling was done during exposure periods of 7, 14, and 21 days.

For the estimation of ACHE activity and genotoxic assessment Sublethal concentration, I ($1/5^{th}$ LC50, 0.0636 mg/l)and Sublethal concentration II ($1/10^{th}$ LC50,0.0318 mg/l) were selected, as the nominal concentration for the analysis of AchE and Ach content in the brain and muscle tissue of *Cyprinus carpio* at the end of the exposure periods 7^{th},14^{th}, and 21^{st}day, sampling was done at the end of each exposure days.

2.3. Estimation of Acetylcholine (ACh) content

ACh content of the brain and muscle was calculated using Hestrin's technique, as reported by[26]. After separating and weighing the brain and muscle tissue, it was teased and transferred to tubes that had already been placed in a boiling water bath for 10 min to inactivate the enzyme acetylcholinesterase and release bound ACh. After cooling the tubes, the contents were homogenized in 2.0 ml of distilled water. A total of 2.0 mL of alkaline hydroxylamine hydrochloride and 1.0 mL of dilute HCl with H_2O at a ratio of 1:1 were added. The contents were centrifuged, and the supernatant was treated with 1.0 ml of ferric chloride. The optical density of the sample was measured in a spectrophotometer at 540 nm in comparison to a blank.

2.4. Estimation of acetylcholinesterase (AChE) activity

[27]the procedure was used to calculate acetylcholinesterase activity in the brain and muscle. In cold 0.25 M sucrose solution, 3% of the homogenate of brain and muscle tissue was produced and homogenated. The enzyme test was performed using supernatant. A total of 3.0 ml of reaction mixture included 12 µm of acetylcholine chloride, 100 µm of sodium phosphate buffer (pH 7.4), and 1.0 ml of homogenate. After 30 minutes of incubation at 37 $^{\circ}$C, the reaction was halted by adding 2.0 ml of alkaline hydroxylamine hydrochloride solution, followed by 1.0 ml of HCl (1:1 HCl: H_2O). The mixture was properly blended and filtered. 1.0 ml of 0.37 M ferric chloride solution was added to the clear filtrate, and the color was measured at 540 nm in a spectrophotometer with a blank. The protein content in each homogenate was estimated at [28] to determine the specific activity of the enzyme.

2.5. Alkaline single-cell gel electrophoresis (SCGE) or comet assay

The alkaline single-cell gel electrophoresis (SCGE)/comet test was performed as a three-layer approach with slight modifications [29,30]. The gill tissue and blood erythrocytes were processed for SCGE. Blood was collected By puncturing the fish's caudal vein with a heparinized syringe, Gill tissues were homogenized in an ice-cold homogenization buffer (1X HBSS, 20 mM EDTA, 10% dimethyl sulfoxide (DMSO), pH 7.0-7.5), and then centrifuged at 3000 rpm for 5 minutes at 4 °C. The cell pellet was then suspended in cooled phosphate-buffered saline (PBS). Using the trypan blue exclusion test technique, the viability of erythrocytes and gill cells was assessed[31]. Tissue samples having cell viability of more than 84% were processed for the comet assay.

The cells were randomly scored and analyzed with an image analysis system (Komet - 5.5 software) attached to a fluorescent microscope (Nikon E600) equipped with appropriate filters. As determined by the software, the parameter chosen for quantification of DNA damage was % Tail DNA (% tail DNA = 100 - % head DNA).

2.6. Micronucleus Assay

The MN test was carried out adopting the methodology of [32] using heparinized syringe peripheral blood samples collected by the caudal vein puncture technique. After collecting the blood sample, smeared on clean microscopic slide air dried, and then fixed for 15 min in 100% methanol. For 10 min, each slide was stained with a 5% Giemsa solution. The stained cells were examined and scored under a microscope.

To determine the frequency of cells with one, two, or more micronuclei, at least 1000 binucleated cells per duplicate cell culture are scored. Furthermore, the cells are categorized as mononucleates, binucleates, or multinucleate to calculate the proliferation index as a toxicity indicator. The major criteria for scoring the micronucleus (MN) were based on those of [33], which included the lack of connections with the main nucleus, identical color, and size of 1/10 to 1/30 of the main nucleus. According to [34], the nuclear abnormalities detected were divided into five categories: a) micronuclei, b) binucleated nucleus, c) lobed nucleus, d) notched nucleus, and e) other nuclear abnormalities. For each individual/slide of *C.carpio*, the frequencies of micronuclei and other nuclear damages were determined and expressed as a percentage of 1,000 cells (%). The various forms of nuclear abnormalities were calculated as

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

2.7. Statistical Analysis

To examine the mean differences in % tail DNA, Ach, and AChE levels between concentrations within the tissue, and between durations within concentrations were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple range tests, and statistical significance was assessed at 5% ($P < 0.05$) levels using GraphPad version (8.0) software.

3. RESULTS

3.1. Estimation of Acetylcholinesterase Activity

The analysis of Acetylcholinesterase alterations in fish has emerged as an important method for monitoring environmental exposure to pollutants both in laboratory and field research. The calculated values for *Cyprinus carpio* at a sub-lethal concentration of I and II of Chlorpyrifos after the 7th, 14th, and 21st days of exposure and percent change over control along with standard deviations are given. In the present study reduced AchE activity with increased Ach content was observed in the brain followed by muscle tissues of *Cyprinus carpio* for all the exposure time.

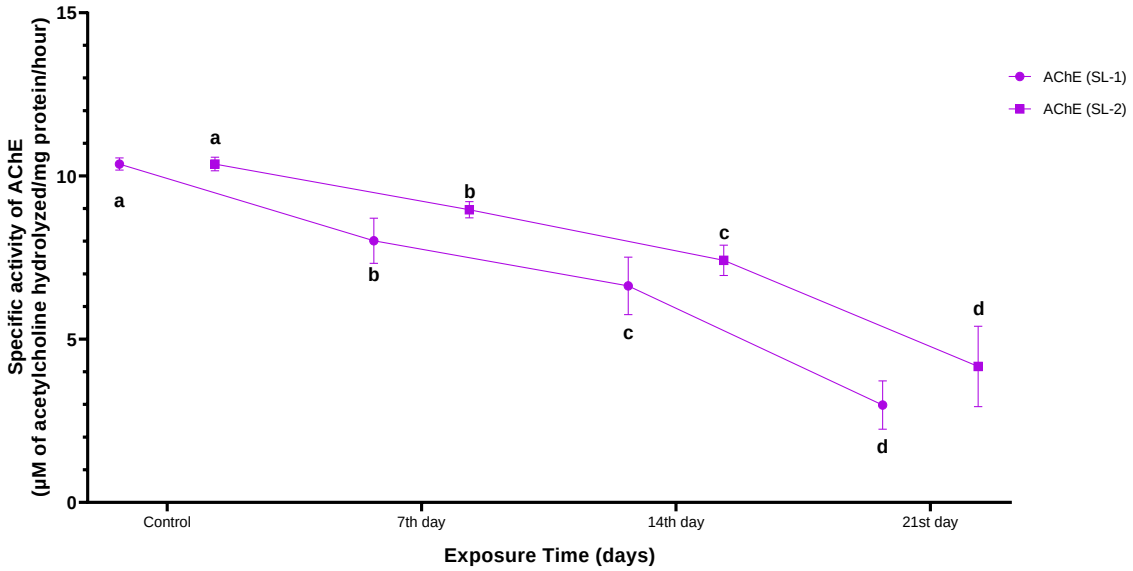

Fig. 1. Changes in the AChE content in brain tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of acetylcholine hydrolyzed/mg protein/hour. Different alphabets within each tissue between exposure times denote significance at a 5% level ($P<0.05$) and the same alphabets denote nonsignificance. Since $P<0.05$ there is a significant decrease in the AChE level of brain tissue of *C.carpio* with the different days of exposure in both the exposure concentrations(Fig.1).

3.2. Acetylcholine(Ach) level in Brain

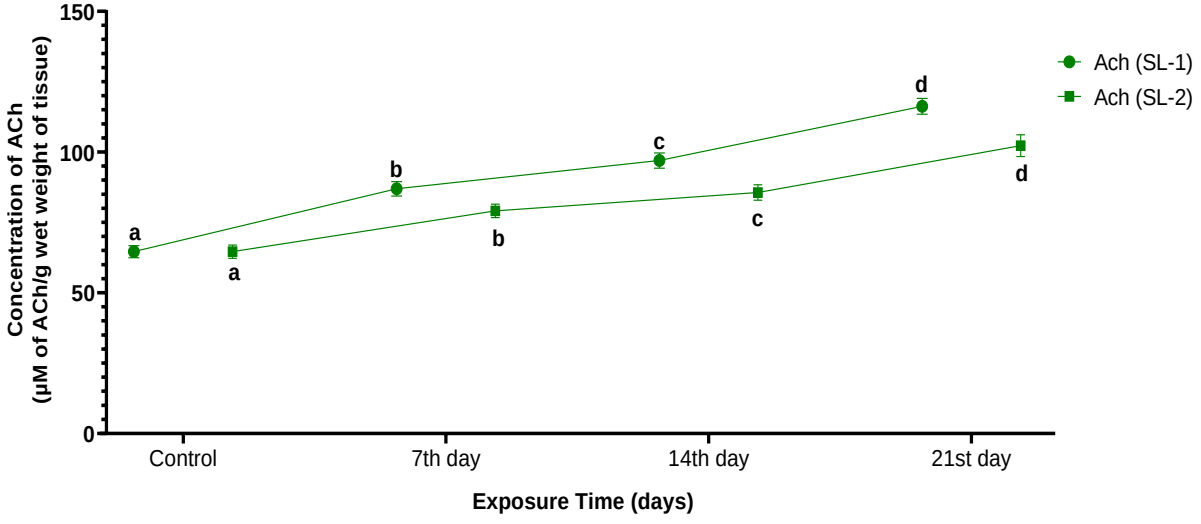

Fig. 2. Changes in the ACh content in brain tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of ACh/g wet weight of tissue. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant increase in the ACh level of brain tissue of *C.carpio* with the different days of exposure in both the exposure concentrations(Fig.2).

3.3. Muscle AChE Activity

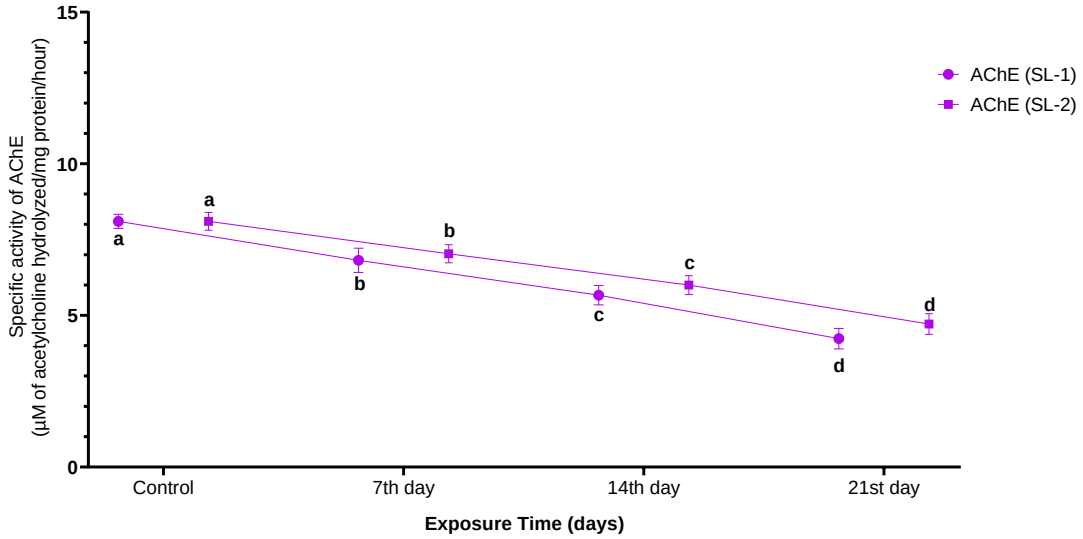

Fig. 3. Changes in the AChE content in muscle tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of acetylcholine hydrolyzed/mg protein/hour. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote nonsignificance. Since P<0.05 there is a significant decrease in the AChE level of muscle tissue of *C.carpio* with the different days of exposure in both the exposure concentrations(Fig.3).

3.4. Acetylcholine(Ach) level in Muscle

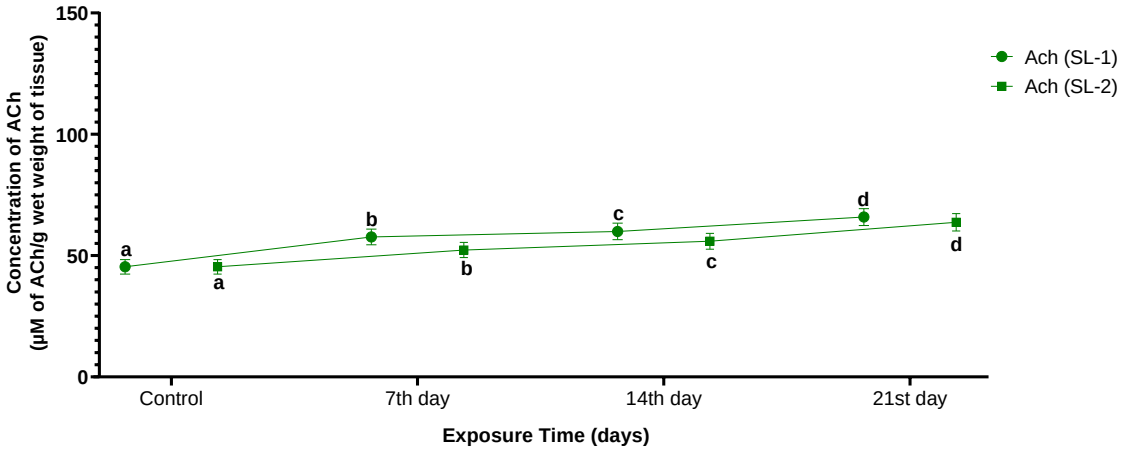

Fig. 4. Changes in the ACh content in muscle tissue of *Cyprinus carpio* exposed to sublethal concentrations of chlorpyrifos for 7, 14, and 21 days.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of ACh/g wet weight of tissue. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant increase in the ACh level of muscle tissue of *C.carpio* with the different days of exposure in both the exposure concentrations(Fig.4).

3.4. Alkaline single-cell gel electrophoresis or Comet assay

Chlorpyrifos intoxication resulting in DNA damage in gill cells of *C.carpio* is shown in (Fig. 5). During electrophoresis pesticidal intoxication results in the migration of damaged DNA fragments more quickly towards anode than intact DNA, which causes a tail to form. In the present study length of the tail formed was too long while the head is smaller in size, thus damaged DNA streaks or comets were measured as %tail DNA in erythrocytes and gill cells of control as well as exposed groups, and measurement of percentile DNA to assess the DNA damage is considered as suitable parameter [29].

Using the Comet test, fish specimens subjected to different sublethal dosages of Chlorpyrifos showed significantly greater levels of DNA damage ($P < 0.05$) as indicated by (%) tail DNA, than control samples. thus, indicating the genotoxic potential of Chlorpyrifos to aquatic organisms. There was a significant effect ($P < 0.05$) was observed from the sub-lethal concentrations and period of exposure in the gill and erythrocytes. The highest DNA damage was observed at the highest SL-I of CPF intoxication followed by SL-II. In gill tissue, there is an increase in the induction of DNA damage from day 7 which was significant up to 21 days and was maximum on day 21 of exposure (Fig.7) at both the sublethal concentrations, there is a significant increase in the DNA damage in the tissues as the duration of exposure increased whereas, in erythrocyte, increase in DNA damage was observed on day 14 and was significant up to day 21 and maximum count of comets were observed on 21st day of exposure at both the sublethal concentrations(Fig.6). With respect to DNA damage comparison between gill cells and blood erythrocytes revealed a significant impact of Chlorpyrifos on the induction of DNA damage was comparatively higher in gill cells than in erythrocytes at both sub-lethal concentrations and exposure periods. Maximum DNA damage as (%) tail DNA in both gill and erythrocytes was observed in SL-I concentration followed by SL-II. Thus DNA damage gradually increased with the dose-response relation (Fig.5).

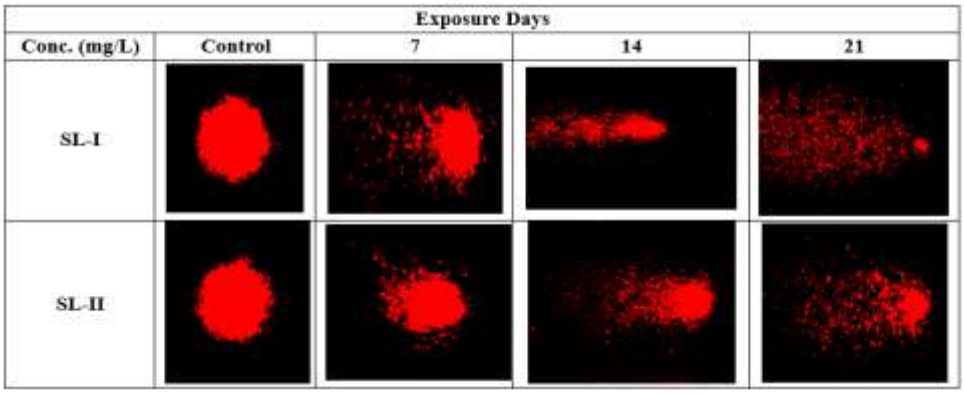

Fig. 5. Comet images of damaged DNA in Gill cells of *Cyprinus carpio* exposed to SL-I and SL-II of Chlorpyrifos at 7, 14, and 21 days of exposure.

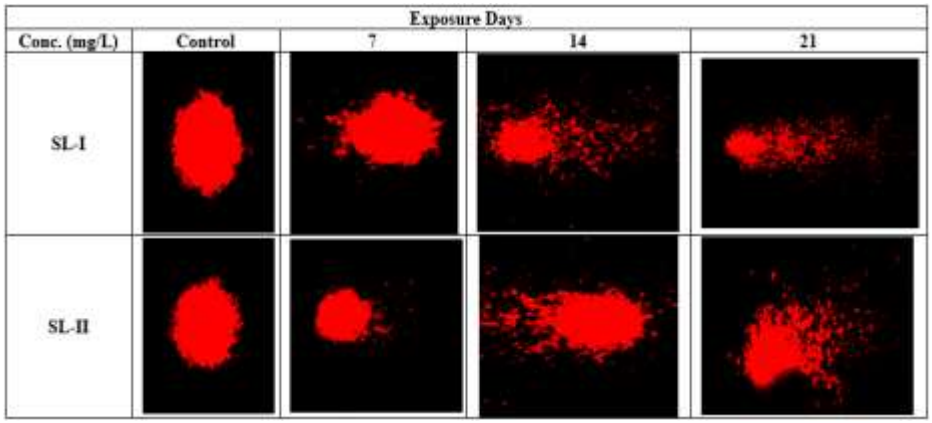

Fig. 6. Comet images of damaged DNA in Blood cells of *Cyprinus carpio* exposed to SL-I and SL-II of Chlorpyrifos at 7, 14, and 21 days of exposure.

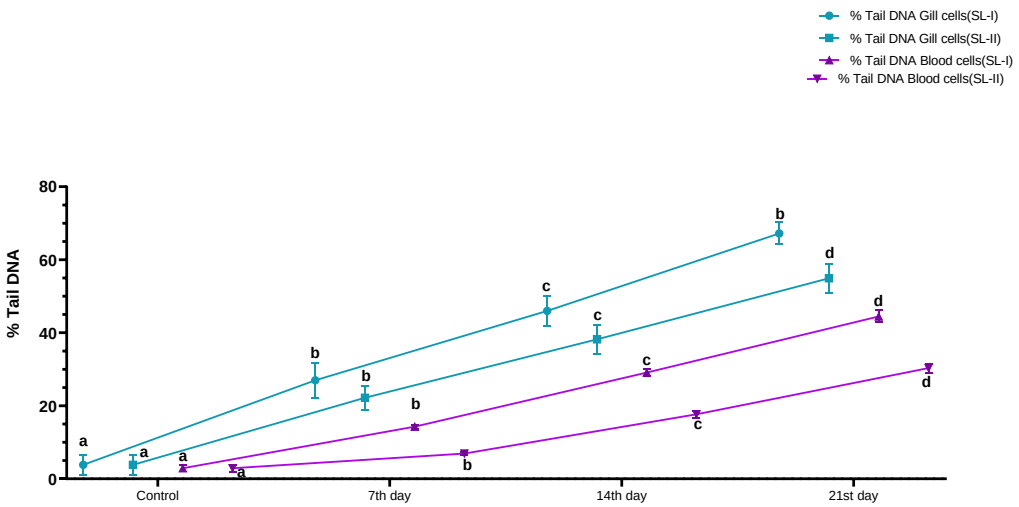

Fig. 7. DNA damage (% Tail DNA) of erythrocytes and Gill cells of *Cyprinus carpio* exposed to SL-I and SL-II of chlorpyrifos at 7, 14, and 21 days of exposure.

Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed in terms of Percentage Tail DNA damage. Different alphabets within each tissue between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance. Since P<0.05 there is a significant increase in the DNA damage(% Tail DNA)of blood and gill tissue of *C.carpio* with the different days of exposure in both the exposure concentrations(Fig.7).

3.5. Micronucleus assay

Chlorpyrifos induces a significant increase in MN frequency in blood erythrocytes of *Cyprinus carpio* after exposure to two test concentrations of chlorpyrifos at different time intervals is presented in (Fig. 8). In the present study significant (P < 0.05) increase in MN (t-test) was found in the treatment groups than in the control. In our findings, MN was often formed like dots that lie closer to the main nucleus in some cells or lie at the corner or boundary of the cell and each cell was differing in size and shape (Fig. 9(a,b,c)). An increase in concentration and time intervals is responsible for an increase in Micronuclei which is recorded at SL-I concentration, in both concentrations of chlorpyrifos frequency of MN increased with an increase in dose and duration of exposure. micronuclei induction and binucleated nucleus, lobed nucleus, and nuclear anomalies were observed on day 7th and were linearly increased from day 14 to 21 of the exposure period of both sublethal concentrations. Higher MN formation and binucleated nucleus, lobed nucleus, and other nuclear anomalies were observed on day 21 of SL-1 concentration followed by SL-2. In the present analysis, a significant (P < 0.05) increase in the Mn frequency and other nuclear abnormalities in erythrocytes is greater in number than the control increase in frequency and is dependent on an upsurge of concentration and exposure durations in both concentrations. The result infers the genotoxicity and mutagenicity of CPF on aquatic organisms. Chlorpyrifos's genotoxic and mutagenic potential was amply demonstrated in the current investigation and is verified by previous findings [12].

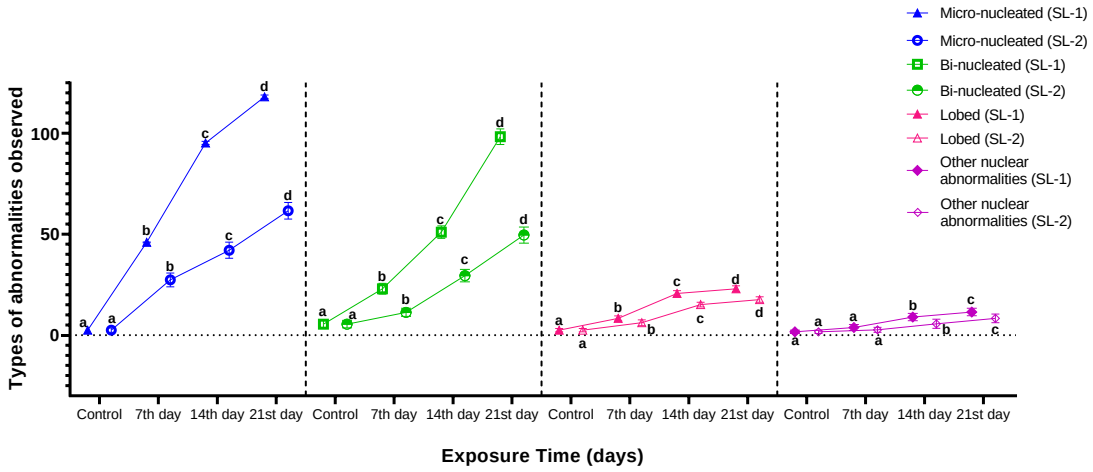

Fig. 8. Variations in frequency of MN and other nuclear anomalies induced by SL-I and SL-II of Chlorpyrifos in *C.carpio* erythrocytes at 7, 14, and 21st days of exposure.

Each datum represents the Mean ± SD, of six individuals (n=6). Various forms of nuclear abnormalities are investigated, and the mean number of abnormalities per 1,000 erythrocytes and the related standard deviation are shown. N = number of slides (1,000 cells per slide) examined for each concentration of exposure. Different alphabets within each tissue

between exposure times denote significance at a 5% level ($P<0.05$) and the same alphabets denote non-significance. Since $P<0.05$ there is a significant increase in the micronuclei induction and other nuclear anomalies at both exposure concentrations(Fig.8).

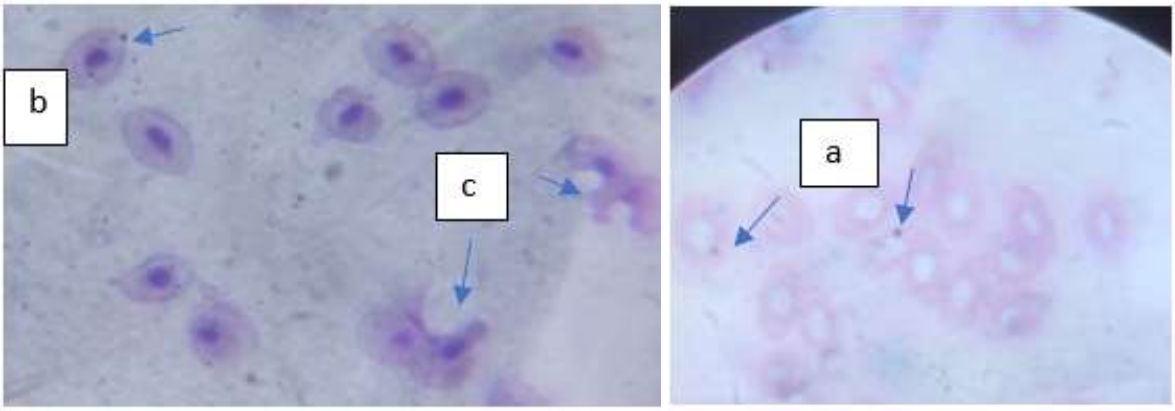

Fig. 9. (a),(b) Micronuclei formation in the erythrocytes after exposure to chlorpyrifos (c), lobed and notched micronuclei.

4. DISCUSSION

Organophosphorus insecticides often break down more quickly and are less persistent in the environment. The frequent finding of chlorpyrifos residues in aquatic biota, soil, sediment, and water has increased because of its extensive exploitation and usage in agriculture. For assessing hazardous pollutants and the toxic potentiality of pesticides in water samples, fish is a useful model organism [35]. The current study shows an inhibition in the AchE activity and a significant increase in MN and DNA damage in chlorpyrifos-treated fish indicating the chemical's genotoxic and mutagenic potential in the freshwater fish *C. carpio* as well as the Common carp's potential utility in determining the pesticide pollution of freshwater bodies.

According to the current study, the concentration and length of exposure have an impact on the level of AchE activity in the brain and muscle tissue of *C. carpio* exposed to chlorpyrifos. Greater inhibition of AChE activity was produced at higher sublethal-I CPF concentrations thus it is a dose and time-dependent increase in AchE activity which regulates the cholinesterase in the nervous system. Chlorpyrifos may damage the active serine hydroxyl group of AChE, causing the enzyme structure to be altered[36]. Consequently, the enzyme is rendered inactive and the hydrolysis of the hydroxyl group is hindered. As a result, inhibiting AChE permanently causes acetylcholine to build up in the brain, This significant buildup of Ach in synaptic clefts of nerves is attributable to numerous neurotoxic effects and halted cholinergic transmission[37]. Catecholamines may rise under these circumstances, and chlorpyrifos may interrupt vital functions like the energy metabolism of nerve cells [38]. Thus, AChE is an enzyme biomarker in fish exposed to pesticides [39].

In the present study, the level of AchE activity in the brain, and muscle tissues of fish, *C. carpio* exposed to chlorpyrifos signifies the greater inhibitory effect of pesticide on the AchE activity in both brain and muscle tissues (Fig 1 and 3). Difference in the AchE inhibition in these organs is due to the interaction between CPF and its metabolites with AchE in various organs and the degree of coherence and intervene of AchE inhibition in these organs. Inhibition of AchE activity owing to effects of their active oxygen analogue chlorpyrifos-oxon. Cytochrome P450 (CYP) initiates a desulfuration reaction that transforms the parent component, chlorpyrifos, into the oxon form . The detoxification metabolism of chlorpyrifos to TCP (3,5,6-trichloro-2-pyridinol) via a dearylation process using the same enzymes competes with the formation of oxon. Through the metabolism of chlorpyrifos-oxon, a-esterase (PON1) also aids in the production of TCP. The extent of enzyme inhibition is determined by the ratio of the toxication/detoxication reactions, which can be used to assess metabolic processes. The inactivation of AchE at neural junctions, which frequently happens by phosphorylation of the enzyme active site, follows initial metabolic activation to form the CPF oxon, which results in the toxicity of CPF.CPF exerts a considerable effect on AchE activity in *Cyprinus carpio* attributed to the changes in the biochemical synthesis which influences the metabolism of acetylcholine. In Inconsonance with the decrease in the AchE activity, there is a corresponding increase in the Ach content of both tissues (Fig 2 and 4) suggesting a decrease in the cholinergic transmission and consequent accumulation of Ach in the tissues. In the present study, chlorpyrifos regulates nerve impulses by shunting the influx of sodium channels as a result multiple nerve impulses occur in place of a single nerve impulse, and these impulse release Ach neurotransmitters to activate more neurons, causing an accumulation of abundant ACh within the nerve impulse thus results in impaired neurophysiological activity attributed to multiple neurotoxic effects and reduced cholinergic transmission. Similar results were obtained in tissues and other fish species [40].

The greater inhibition of AchE activity with a simultaneous rise in Ach content in the tissues implies stronger inhibition of central nervous system integratory activity and thus accumulation of Ach in the brain tissue [41]. Damage to the central nervous system may have resulted in the uncontrolled release of hormones, and an animal's toll may be conceivable due

to the degradation of numerous biochemical and physiological activities [42]. A comparable corroborative rise in Ach content as a result of a drop in tissue AchE levels was documented in different fish species [43,44,45].

In the present work, it is evident that CPF is a potent neurotoxin and an AchE inhibitor because of the phosphorylation of serine by CPF at the site of the AchE catalytic domain resulting in the inhibition of AchE activity [36]. Pesticides bind the active site and prevent Ach breakdown resulting in synaptic transmission blockage in cholinergic neuronal cells overall inhibitory activity of AchE activity potentially alters the metabolism, cell function, and signal transduction [13]. Acetylcholine, a neurotransmitter molecule, is deactivated by AchE. Neurotransmitters are required to transmit nerve impulses flowing from one nerve cell to the next across the synaptic gap. If AchE is blocked, Ach accumulates and nerve impulses cannot be halted, which leads to uncontrolled muscle contraction resulting in paralysis, and also causes behavioral abnormalities, and widespread disruption in neuronal physiology, ultimately leading to the organism's death.
Among the various methods available for evaluating xenobiotics' ability to cause genotoxicity and Mutagenicity. Genotoxic biomarkers such as comet and Mn assay are the most recommended methods to evaluate different structural and functional modifications in chromosomes. Both micronucleus (MN) test and single cell gel electrophoresis (SCGE) or comet assay are sensitive, rapid, and widely employed techniques and important genotoxic biomarkers for assessing genomic instability and have proved to be an effective tool for assessing the interaction between susceptibility of aquatic animals with the exposure of genotoxic contaminants and DNA damage. Both assays were preferred in aquatic toxicological studies owing to their sensitivity in detecting the cytogenetic effects and DNA damage after acute and chronic exposure to various genotoxins. In the current study, blood and gill cells from chlorpyrifos-exposed fish *Cyprinus carpio* showed a significant increase in MN and DNA damage. Several previous studies [12,46] have proved that chlorpyrifos is a positive mutagen. According to our findings, fish blood and gill cells can be a sensitive and consistent type for assessing the genotoxic effects of environmental contaminants.

Increased MN may be caused by chlorpyrifos-induces oxidative stress resulting in ROS generation which interferes with the macromolecules like DNA resulting in clastogenic and molecular damages. In the present work, CPF induces DNA -strand breaks, which result in asymmetrical or symmetrical chromatid network formation and due to a lack of spindle attachment during the anaphase segregation process, resulting in failure of chromosome exchange or fragments in the daughter nuclei at the end of telophase of the cell cycle. Micronuclei are the result of chromosomal abnormalities caused by preceding mitotic divisions in erythrocytes[47]. The formation of a micronucleus as a result of a cell cycle error suggests that it may play a role in tumor formation, leading to a cancerous state [48]. Chlorpyrifos intoxication results in increased MN frequency in erythrocytes which is attributed to the interaction of its metabolites with chromosomes by targeting nucleophilic sites on DNA. It was observed that. The dose-dependent rise in the MN induction may be owing to a higher dose of chlorpyrifos inducing more double-strand DNA breaks and their lower chance of being properly repaired. increased MN frequency in erythrocytes is used to evaluate the aquatic toxicity due to pollutants that exhibit clastogenic and aneugenic properties[49]. Similar findings were observed in different fishes this assay was used to determine the genotoxicity of xenobiotics [50.]

Comet assay or Alkaline single-cell gel electrophoresis (SCGE), the method is highly advantageous over other cytogenetic techniques. The findings of the present study showed that the CPF induces a concentration-dependent increase in DNA damage by producing comet and a duration-dependent increase in the damage was observed resulting in Variations in the DNA damage among two tissues of fish exposed to sublethal concentrations of CPF.
gill cells of *Cyprinus carpio* exposed to two sublethal concentrations of CPF for 21 days, had shown significantly higher DNA damage than erythrocytic cells. DNA damage was seen to worsen throughout the exposure period. These variations between the tissues are specific it could be due to a varied number of alkali-labile sites in the DNA of different tissues and further physiological activities of particular tissues about detoxification of the toxicants Gill cells generally exhibited severe DNA damage because the gills of the most relevant and appropriate organ that is directly and continuously exposed to the DNA-damaging pesticide present in water [51] and also physiological activity associated with the detoxication of toxicants or repairing mechanism thus the increased DNA damage in gill cells may be justified by their applicability for experiments on genotoxicity has also been established before using the rainbow trout, shellfish [52]. Whereas DNA-damaging genotoxic chemicals enter the circulatory system lymphocytes come into contact our findings are in agreement with the previous studies indicating higher DNA damage was observed in gill cells than blood erythrocytes in *Cyprinus carpio* exposed to CPF[12].
The DNA damage reported in this study might have resulted from pesticide toxicity or their metabolites interacting with DNA, causing single-strand breaks, double-strand breaks, DNA adduct formation, and DNA-DNA and DNA-protein cross-links due to loss in excision repair mechanism [53].CPF metabolites or phosphorous groups of organophosphates are found to be a good nucleophilic assault, this could lead to DNA phosphorylation, a type of DNA damage, Therefore, it is likely that CPF could result in changes in the DNA of *C. Carpio* resulting in the formation of comets.

In the present investigation, it was found that CPF intoxication induces oxidative stress resulting in loss of cellular physiology and cellular survival [54], and thus reduces the antioxidant enzymes, glutathione (GSH) and glutathione-dependent enzymes, the major enzyme involved in eliminating the toxic content, due to pesticide toxicity GSH level is reduced which results in the formation of GSH conjugates with xenobiotics which allows them to covalently combine

with cellular proteins, DNA or RNA thus causes DNA damage. On the other hand, Reduced GSH levels may induce Reactive oxygen species (ROS) due to xenobiotic-induced toxicity which causes lipid peroxidation of bio-membranes, affecting their permeability and integrity and possibly causing DNA damage[55].

CPF induces DNA damage in *Cyprinus carpio* and may be attributed to the formation of reactive oxygen species (ROS). Increased ROS formation results in loss of metabolism due to the reaction between ROS and DNA resulting in DNA strand breaks[56]. increased level of ROS in fish results in a depressed antioxidant defense system attributed to cell apoptosis, mutation, and, ultimately, DNA damage [57]. it was also found that intermediates generated during chlorpyrifos catabolism may potentially interact with intact DNA molecules, causing structural damage. It has also been proposed that labile methyl groups in pesticides would be an excellent substrate for electrophilic assault, resulting in the methylation of the DNA resulting in DNA lesions [58].

Chlorpyrifos's genotoxic potential was amply demonstrated in the current investigation by a substantial genotoxic effect of chlorpyrifos that was detected from the time of exposure as well as concentrations. The current findings were in agreement with earlier research on a variety of fish species, including *Cyprinus carpio* [59].

The present findings increased significantly ($P < 0.05$) higher DNA damage and MN induction than control due to chlorpyrifos intoxication for both sublethal concentrations. It was discovered that the percentage of tail DNA damage was concentration and time-dependent, indicating that CPF was genotoxic to *C. carpio*. these results are in agreement with the findings of [12].
The advantage of using comet and MN biomarkers in aquatic toxicology is to assess DNA damage in different tissues of the same animal. both these methods are helpful in screening the genotoxic nature of chemicals and their consequences of DNA damage and also reduced repairing the property furthermore these assays are used to assess the status of aquatic organisms and the quality of water [60]. Our results are in agreement with the previous studies conducted on mutagenic and genotoxic potentials of different organophosphate pesticides in different organisms [61,62,63]. As per the result obtained it is evident that chlorpyrifos is a neurotoxic and genotoxic agent to fish *Cyprinus carpio* at sublethal concentrations. The results show a considerable concern about the possible risks that chlorpyrifos poses to aquatic life. As a result, it should be used carefully in residential, industrial, and agricultural applications.

CONCLUSION:

According to the aforementioned findings and discussion, AchE, comet, and micronucleus assay are sensitive biomarkers to diagnose pesticide toxicity to fish and to evaluate different structural and functional modifications in chromosomes and enzymatic regulation during neurotransmission. Our findings demonstrated that chlorpyrifos has the potential to cause neurotoxicity and genotoxicity. The findings of this study could help to assess the toxicant impacts at the sub-lethal concentration on freshwater species. These could serve as a biomarker of CPF toxicity in the aquatic environment and also help predict the possible mechanisms of toxicity in humans because of their diagnostic significance. It would be very advantageous in assessing the associated environmental risk of these pesticides being moderately toxic it exerts hazardous impact on non-target species, thus precautions should be taken while using even low concentrations of chlorpyrifos, and prohibiting or restricting chlorpyrifos usage is preferable.

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Conflict of Interest

On behalf of all authors, the corresponding author states that no relevant financial or non-financial interest to disclose.

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