Research Article

Genotoxic Effects of Chlorpyrifos in Freshwater Fish *Cirrhinus mrigala* Using Micronucleus Assay

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The genotoxicity of pesticides is an issue of worldwide concern and chlorpyrifos is one of the largest selling organophosphate agrochemicals that has been widely detected in surface waters of India. The studies on long term genotoxic biomarkers are limited; therefore, present study was carried out to analyze the incidence of nuclear anomalies in the blood cells of fresh water fish *Cirrhinus mrigala* using micronucleus (MN) assay as a potential tool for assessment of genotoxicity. Acute toxicity of chlorpyrifos was evaluated by exposing fingerlings to different doses of chlorpyrifos (1/20, 1/10, and 1/5 of LC_{50}) and LC_{50} was calculated as 0.44 mg L^{-1} using probit analysis. Blood samples were taken on days 2, 4, 8, 12, 21, 28, and 35. In general, significant effects for both concentration and duration of exposure were observed in treated fish. It was found that MN induction was highest on day 14 at 0.08 mg L^{-1} concentration of chlorpyrifos. It was concluded that chlorpyrifos is genotoxic pesticide causing nuclear anomalies in *Cirrhinus mrigala*.

1. Introduction

Occupational exposure to pesticides is a common and alarming worldwide phenomenon. Various industrial and agricultural activities increase pollution, particularly in the aquatic environment, which is contaminated by various toxic chemicals from the discharge of waste waters and agricultural drainage [1]. These are responsible for multiple effects at the organisms, including humans, affecting organ function, reproductive status, species survival, population size, and ultimately biodiversity. Chronic sublethal effects are also clearly relevant if the concentrations of these chemicals are below the acute threshold. Presently, over 100 organophosphates representing a variety of chemical, physical, and biological properties are being used for agricultural purposes [2].

Chlorpyrifos (O,O diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is one of the earliest developed organophosphate, introduced in India in 1965 [3]. It is nonsystemic broad spectrum, general use pesticide that disrupts the nervous system function by inhibiting cholinesterase enzyme that normally terminates nerve transmission by cleaving the neurotransmitter acetylcholine and resultant cholinesterase

accumulation [4, 5]. It is an active ingredient in the Dursban, Lorsban, and Predator brand of pesticides [6]. Recently with the prohibition of those high toxic organophosphorus insecticides in the production of vegetables and the food crops, the usage of chlorpyrifos has rapidly increased.

Low persistence of such compounds in aquatic ecosystems raised concerns about their potential to cause adverse effects on nontarget populations especially the fish [7]. Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate, and store waterborne pollutants. The main application of fish used as a test model is to determine the distribution and effects of chemical contaminants in the aquatic environment, evaluating monitoring systems that use aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. The micronucleus assay, developed by Schmidt [8], is an *in vivo* and *in vitro* short-time screening method, is sensitive, and is an extensively used tool for detecting mutagenic and genotoxic effects of chemicals in the environment.

Although the toxic effects of chlorpyrifos on fish have been studied earlier [9], data pertaining the genotoxicity of chlorpyrifos in aquatic organisms is less, especially in terms

TABLE 1: Determination of LC_{50} ($mg L^{-1}$).

Test chemical	Regression equation	95% confidence limit for doses		Median lethal concentration (LC_{50}) ($mg L^{-1}$)
		Upper limit	Lower limit	
Chlorpyrifos	$y = 11.87x - 0.533$ $R^2 = 0.881$	0.369	0.519	0.44

of long term exposure in fish. Therefore, the present study was conducted to investigate the mutagenic and genotoxic effects of chlorpyrifos using micronucleus assay in erythrocytes of *Cirrhinus mrigala* (one of the important food fish) exposure *in vivo*.

2. Materials and Methods

2.1. Chemicals and Labware. Absolute alcohol (methanol) with Cas number 67-56-1, Giemsa stain with Cas number 51811-82-6, and glass slides with product code CG001 (thickness 25.4×76.2) were purchased from LINCO, Laboratory Instruments and Chemicals, Ambala Cantt, Haryana, India. Commercial formulation of chlorpyrifos product (50% EC) named as chlorpyrifos (manufactured by M/s. Cheminova India Ltd., 242/P, G.I.D.C Panoli 394116, District Bagraich, Gujarat) was purchased from the local market. It was observed that chlorpyrifos of this grade is mostly employed in the fields.

2.2. Experimental Specimen. Fingerlings of *Cirrhinus mrigala* (with mean weight 14 ± 0.04 g) were obtained from Sultan Singh fish farm, Nilokheri, Karnal, Haryana, and shifted to glass aquaria in the laboratory. These specimens were acclimatized at $28^\circ C$ in large sized plastic tubs disinfected with potassium permanganate and washed thoroughly to prevent the fungal infection for 20 days prior to experimentation. During laboratory condition, fishes were fed with diet containing 40% protein (dietary ingredients, $g Kg^{-1}$: groundnut oil cake: 650 g, rice bran: 42 g, processed soyabean: 276 g, wheat flour: 32 g, and mineral mixture: 10 g) at 4% BW in two installments a day. Tap water free from chlorine was renewed every day with these physiochemical characteristics (Temp $32^\circ \pm 0.02^\circ C$, pH 7.3 ± 0.33 , and DO $5.5 \pm 0.33 mg L^{-1}$).

2.3. Determination of Acute Toxic and Sublethal Concentration. Stock solution of the pesticide was prepared by dissolving chlorpyrifos in tap water. Working chlorpyrifos concentration doses starting from 0.0005, 0.0006, 0.0007, and $0.0008 mg L^{-1}$ were prepared in form of stock solution and then the working concentration dose was increased after checking dead specimen at 24, 48, 72, and 96 h. To determine the acute LC_{50} value of chlorpyrifos, five groups of fish specimens, each containing 10 individuals, were selected at random and placed in aquaria. Glass aquaria with capacity of 40 L were filled with 30 L of water. The temperature of the water was regulated at $30^\circ \pm 1^\circ C$. The electric conductivity and the pH of water were $565 \pm 0.43 \mu mho cm^{-1}$ and 7.3 ± 0.33 , respectively. The above-mentioned concentrations were added to different aquaria containing specimen keeping

TABLE 2: Selection of different doses of pesticide chlorpyrifos for experiment.

Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)	Treatment 4 (T4)
No pesticide	$0.02 mg L^{-1}$ (1/20 of LC_{50})	$0.04 mg L^{-1}$ (1/10 of LC_{50})	$0.08 mg L^{-1}$ (1/5 of LC_{50})

one as control receiving no pesticide. Acute LC_{50} (96 h) value for chlorpyrifos for *Cirrhinus mrigala* was calculated as $0.44 mg L^{-1}$ (Table 1). Using the LC_{50} value, three nominal sublethal concentrations, namely, Sublethal 1 (1/20 = $0.02 mg L^{-1}$), Sublethal 2 (1/10 = $0.04 mg L^{-1}$), and Sublethal 3 (1/5 = $0.08 mg L^{-1}$), were prepared (Table 2).

2.4. In Vivo Sublethal Exposure Experiment and Micronucleus Assay. The micronucleus (MN) test was performed using the procedure of [8] with slight modifications in the protocol [10]. The *Cirrhinus mrigala* fingerlings were exposed to the three above-mentioned sublethal concentrations of chlorpyrifos in a static bioassay system. Dechlorinated tap water was used to maintain the control. Physicochemical properties of the water (pH, temperature, DO, electrical conductivity, and others) were analyzed at the beginning and the end of experimental period. In order to maintain the desired concentration, water was renewed on alternative day with freshly prepared solution during the entire period of experiment.

Blood samples were collected on days 2, 4, 8, 12, 14, 21, 28, and 35 from the two specimens for each concentration per interval with a preheparinized syringe (1 mL) either from caudal vein or from heart puncture and MN assay was performed according to Ali et al. [7]. A total of about 3000 erythrocytes (1000 cells from each slide and three slides from each sample were studied) were examined for each specimen under microscope (Olympus CX 41, oil immersion lens, 100/1.25). The criteria for a cell to be considered as micronucleated are as follows: it is required to have a round, smooth perimeter membrane, no connection with the main nucleus, and staining intensity similar to that of nucleus and to bear smaller size than one-third of main nucleus [11]. The nuclei that appear to be broken but still connected to main nuclei with a thin nucleoplasmic bridge or the nuclei that appeared cinched were designated as broken egg [12, 13]. MN frequency was calculated according to the formula suggested by Ali et al. [7].

2.5. Statistical Analysis. Statistical analysis of the data was carried using IBM SPSS statistics 20. ANOVA was employed to compare mean difference between different concentrations

TABLE 3: Incidence of micronuclei as nuclear anomaly in peripheral erythrocytes of *Cirrhinus mrigala* exposed to different concentrations of chlorpyrifos.

Exposure time	Number of cells with MN	Micronuclei						
		T1	Number of cells with MN	T2	Number of cells with MN	T3	Number of cells with MN	T4
		MN Freq (%) $\pm$ SE		MN Freq (%) $\pm$ SE		MN Freq (%) $\pm$ SE		MN Freq (%) $\pm$ SE
Day 2	2	0.066 $\pm$ 0.03 ^{Ac}	7	0.233 $\pm$ 0.03 ^{Cbc}	12	0.401 $\pm$ 0.05 ^{Db}	21	0.701 $\pm$ 0.11 ^{Da}
Day 4	3	0.100 $\pm$ 0.03 ^{Ac}	10	0.333 $\pm$ 0.03 ^{Cc}	23	0.766 $\pm$ 0.08 ^{BCb}	40	1.333 $\pm$ 0.12 ^{Ca}
Day 8	2	0.060 $\pm$ 0.03 ^{Ad}	18	0.610 $\pm$ 0.05 ^{Bc}	32	1.066 $\pm$ 0.12 ^{Ab}	51	1.733 $\pm$ 0.17 ^{BCa}
Day 14	1	0.033 $\pm$ 0.02 ^{Ad}	26	0.866 $\pm$ 0.08 ^{Ac}	36	1.200 $\pm$ 0.11 ^{Ab}	72	2.400 $\pm$ 0.11 ^{Aa}
Day 21	2	0.066 $\pm$ 0.03 ^{Ac}	25	0.833 $\pm$ 0.13 ^{Ab}	33	1.100 $\pm$ 0.11 ^{Ab}	65	2.166 $\pm$ 0.08 ^{Aa}
Day 28	2	0.066 $\pm$ 0.03 ^{Ad}	21	0.700 $\pm$ 0.05 ^{ABc}	30	1.00 $\pm$ 0.05 ^{ABb}	53	2.166 $\pm$ 0.08 ^{Ba}
Day 35	1	0.033 $\pm$ 0.03 ^{Ac}	16	0.533 $\pm$ 0.03 ^{Bb}	21	0.700 $\pm$ 0.05 ^{Cb}	41	1.366 $\pm$ 0.14 ^{BCa}

Values with different capital alphabet superscripts differ significantly ($P < 0.05$) within the same column.

Values with different small alphabet superscripts differ significantly ($P < 0.05$) within the same row.

with time duration and between durations within concentration. Statistical significance was settled at probability value of $P < 0.05$.

3. Results

3.1. Physicochemical Properties of Test Water. The temperature of the test water during experimental period was recorded around $26.3^\circ \pm 0.36^\circ\text{C}$ and pH ranged from 7.3 to 7.5 ± 0.03 . The DO concentration was found to be in the normal range $5.64 \pm 0.03 \text{ mg L}^{-1}$. The electrical conductivity of the water ranged from 566.7 ± 0.03 to $861 \pm 1.77 \mu\text{mho cm}^{-1}$; total hardness and alkalinity ranged from 350 ± 1.15 to 398 ± 0.06 and from 30.05 ± 0.5 to $43.4 \pm 0.77 \text{ mg L}^{-1}$, respectively.

3.2. Micronuclei Induction. Micronuclei (MN) induced by chlorpyrifos in peripheral erythrocytes were generally dot shaped and were close to the main nucleus with size and shape and varied among cells. Mostly each affected erythrocyte had a single micronucleus while a few cells contained more than one micronucleus and some had larger sized micronuclei. The frequency and number of MN and other nuclear anomalies as broken egg in the peripheral erythrocytes in the *Cirrhinus mrigala* exposed to varying doses of chlorpyrifos for varying period of time and in control are summarized in Tables 3 and 4. Evidently all the treated groups of specimens had higher frequency of erythrocytes with MN and nuclear anomaly as broken egg (Figures 1(a)–1(d)), as compared to the control. The frequency of MN in peripheral erythrocytes increased progressively with increase in period of exposure and concentration of chlorpyrifos. Throughout the experimental period the induction of the MN and its frequency was found significantly higher in T4 in comparison to T3, T2, and T1. Values were compared between durations within concentration as well as concentrations within duration. It was found that treatment 1 showed no significant variations within different periods whereas treatments 2, 3, and 4 showed significant differences within the same row.

When all the treatments were compared between concentrations within the duration, they showed a significant ($P <$

0.05) difference. The highest MN frequency of $2.4 \pm 0.11\%$ was observed on day 14 in sublethal treatment T4 and $1.2 \pm 0.01\%$ in T3 and lowest frequency of MN (0.03 ± 0.03) was observed in T1 throughout the study period, whereas nuclear anomalies as broken egg were observed only during days 14, 21, 28, and 35 and sublethal treatments 2, 3, and 4 when compared showed significant differences. Highest frequency of broken egg was observed on day 14 (1.733 ± 0.14) and least on day 28 in T3 (0.2 ± 0.12).

4. Discussion

A number of studies on genotoxicity of chlorpyrifos or its formulations were reported in literature. Based on 96 h LC_{50} , it has been observed that *Cirrhinus mrigala* is more sensitive to chlorpyrifos than *Oncorhynchus mykiss* ($24 \mu\text{g L}^{-1}$) [14]; *Nile tilapia* (1.57 mg L^{-1}) [15]; *Oreochromis mossambicus* ($25.7 \mu\text{g L}^{-1}$) [16]; and *Cyprinus carpio* ($580 \mu\text{g L}^{-1}$) [17], which rated chlorpyrifos as highly toxic to *Cirrhinus mrigala* as the value determined was 0.44 mg L^{-1} . This value is slightly lower than LC_{50} 96 h of chlorpyrifos in *Channa punctatus* reported by [7]. Studies carried out on some amphibians have shown that LC_{50} 96 h for anurans exposed to chlorpyrifos was calculated as 1 mg L^{-1} and 0.56 mg L^{-1} in *Rana limnocharis* [18, 19]. The variation might be due to the difference and hardness of the test species and optimum water quality parameters.

This study was purposed to assess the extent of nuclear anomalies in test species during the long term exposure to more or less similar concentration of pesticide, as the test species experience similar conditions in the field. Further, for micronuclei assay, the sampling time was selected as appropriate for the end point measured. The exposure duration has mostly been taken for 4 to 5 weeks or more with weekly sampling [20–22].

Additional blood sampling has been considered useful on days 2–4 after exposure to toxicant [23]. Keeping these points in consideration, specimens were exposed for 4–5 weeks with initial sampling on days 2, 4, and 8 and thereafter at weekly

TABLE 4: Incidence of broken egg as nuclear anomaly in peripheral erythrocytes of *Cirrhinus mrigala* exposed to different concentrations of chlorpyrifos.

Exposure time	Number of cells with BE	Broken egg				Number of cells with BE	Number of cells with BE	Number of cells with BE	Number of cells with BE
		T1 BE Freq (%) $\pm$ SE	T2 BE Freq (%) $\pm$ SE	T3 BE Freq (%) $\pm$ SE	T4 BE Freq (%) $\pm$ SE				
Day 2	—	—	—	—	—	—	—	—	—
Day 4	—	—	—	—	—	—	—	—	—
Day 8	—	—	—	—	—	—	—	—	—
Day 14	—	—	22	0.733 $\pm$ 0.08 ^{Ab}	28	0.933 $\pm$ 0.08 ^{Ab}	52	1.733 $\pm$ 0.14 ^{Aa}	—
Day 21	—	—	17	0.566 $\pm$ 0.03 ^{Ab}	23	0.766 $\pm$ 0.12 ^{Aab}	26	0.866 $\pm$ 0.12 ^{Ba}	—
Day 28	—	—	—	—	7	0.230 $\pm$ 0.12 ^{Ba}	9	0.450 $\pm$ 0.12 ^{Ca}	—
Day 35	—	—	—	—	9	0.300 $\pm$ 0.05 ^{Ba}	—	—	—

Values with different capital alphabet superscripts differ significantly ($P < 0.05$) within the same column.

Values with different small alphabet superscripts differ significantly ($P < 0.05$) within the same row.

FIGURE 1: Micronuclei and broken egg in the blood cells of *Cirrhinus mrigala* after exposure to chlorpyrifos. (a) erythrocytes; (b) smaller micronuclei; (c) large micronuclei; (d) broken egg.

interval. The micronucleus test performed showed the alteration in cell morphology, presence of nuclear anomalies as broken egg and large size micronuclei, alteration in cell morphology besides micronuclei confirming the effect of chlorpyrifos on the nucleus. Similar results have been reported by several other authors, either in humans [24], in tadpoles [18], or in fish [25]. All the test concentrations of chlorpyrifos

induced a significantly higher number of micronuclei compared to the control. Further, the MN induction increased significantly ($P < 0.05$) from days 2, 4, and 8 to 14 and was found to decrease from day 14 to days 21, 28, and 35. Maximum frequency of broken egg and micronuclei was also observed on day 14 thus showing maximum effect of chlorpyrifos on chromosome breakage on day 14, clearly confirming that

this can cause genotoxic damage in erythrocytes of *Cirrhinus mrigala*. Highest induction in number of MN increased from days 2 to 14 and thereafter decreased after day 14. This decrease may be due to formation of broken egg which is considered to be associated with severe chromosomal aberrations [25]. Moreover, Nepomuceno et al. [26] suggested that frequencies of micronuclei tend to even out or reduce after sometime because fish might promote some defensive mechanisms to reduce the toxicants in the body in order to stabilize the micronuclei frequencies. Thus induction clearly demarcates genotoxicity even at all sublethal concentrations.

Ali et al. [7] studied the MN induction in *Channa punctatus* on exposure to chlorpyrifos on days 1, 3, 5, 7, 14, 21, 28, and 35 and observed maximum MN induction (1.62%) on day 14. Concentration dependent increase and time dependent decrease in MN induction due to malathion had also been reported by [27]. However, in some studies, both concentration and time dependent increase in MN induction have also been reported due to chemical exposure in fish [6]. Hoofman and De Raat [28] and Das and Nanda [29] reported a concentration and time dependent increase in MN induction in fish erythrocytes, corroborated to the present work. Yadav et al. [25] reported that frequency of MN in *Cirrhinus mrigala* after exposure of butachlor increased significantly from 0 to 48 h; thereafter broken egg appeared. Yin et al. [30] reported that chlorpyrifos induced significantly higher number of MN that increased with concentration and duration in anurans. Time dependent responses have also been observed in amphibians exposed to radiation [31, 32].

Although the MN test has been found to be a sensitive assay to evaluate genotoxic compounds in fish under controlled conditions as an index of cumulative exposure [30], it might suffer variations according to tests organisms [33]. Further, as the preexisting mature (and nondividing) erythrocytes would predominate in the blood, the detection of induced MN in mature blood cells will be at a low frequency in the beginning of the exposure. However, with the progression of the experiment, a greater number of the dividing cells (polychromatic erythrocytes) would be expected to predominate in the blood and, therefore, a latency period is normally required between treatment and subsequent MN peak [33].

Nwani et al. [34] studied the MN induction in fresh water fish *Channa punctatus* on exposure of carbosulphan and reported significantly ($P < 0.05$) higher number of MN than the control and frequency increased with concentration and durations. A dose dependent increase in induction of MN in peripheral blood of *Heteropneustes fossilis* has been reported by [29] in response to paper mill effluents. Abdul-Farah et al. [35] reported time dependent increase in the MN induction in peripheral blood of *Channa punctatus* exposed to PCP and 2,4-D supporting the present results for *Cirrhinus mrigala* due to chlorpyrifos toxicity.

5. Conclusion

The results of the present investigation on the genotoxic potential of chlorpyrifos raised a severe concern about the potential danger to aquatic organisms especially to fish and

indirectly to humans. However, studies are needed to explore the biological consequences of nuclear anomalies in aquatic organism after chlorpyrifos exposure and to formulate the future strategies for safeguarding aquatic organism and the environment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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