Aberrations in the Chromosomes of *Cirrhinus mrigala* (Hamilton) upon Exposure to Butachlor

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ABSTRACT

Background: *Cirrhinus mrigala*, one of the important fish widely consumed in India, was used for karyological observations and to evaluate the toxic effect of butachlor, an extensively used herbicide in rice fields in terms of chromosomal aberration test.

Methods: Fishes were collected from "National Fish Seed Farm" Jyotisar with mean body weight of 20-50g. The experimental fishes were kept in two treatments each with replicate of two. There were 15 fish each in the control group (T_1) without exposure to butachlor and in T_2 where fishes were exposed to butachlor.

Results: Karyotype revealed the 2n=50 chromosome from the somatic cell. Chromosomal aberrations were reported after 24 hrs, 48 hrs, 72 hrs, and 96 hrs from kidney cell preparation in fishes exposed to 1.0 ppm, sublethal concentration of butachlor. Frequencies of chromosomal aberration revealed a significant ($P<0.05$) time-dependent response. Stickiness and clumping appeared at 24 and 48 hrs of exposure, end to end joining appeared after 72 hrs and chromosomal fragmentations were observed after exposure for 96 hrs.

Conclusion: These studies clearly revealed the genotoxic potential of butachlor even at low dose level (1.0 ppm) and suggest that butachlor interferes with cellular activities in fishes at genetic level, inducing chromosomal aberrations. Therefore, the results of these investigations suggest a serious concern towards the potential danger of butachlor for aquatic organisms and the environment suggesting judicious and careful use of this pesticide in agricultural area. These aberrations in chromosome from kidney cell preparation illustrate the risk that butachlor possesses.

Keywords: Chromosomal Aberrations, Clumping, End to End joining, Karyotype, Stickiness.

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INTRODUCTION

India is primarily an agricultural country. With increase in human population in geometric ratio, the problem to feed its population is becoming more and more difficult every year. Thus, efforts are being made continuously to increase the agricultural produce. The most important concern associated with agricultural production is the problem of pests (weeds and insects). Inspite of extensive efforts, all the pests cannot be controlled/ manipulated through biological control. Hence, use of chemicals, pesticides/insecticides/herbicides, is indispensable in modern agricultural technology to control pests for production of more food and management of public health, especially in developing countries.

Aquatic environment that covers more than two-thirds of the earth is inhabited by more than 28,000 fish species [1]. Aquatic environment remains the ultimate recipient of an increasing number of agrochemicals. Many of these chemicals have the ability to interact with DNA and can lead to gene mutation or genetic disease syndromes [2, 3] in the aquatic organisms, particularly fishes.

Butachlor, 2-chloro-N-(2, 6-diethylphenyl) acetanilide, is an important herbicide used mainly in rice paddy fields to control perennial grasses and some broad-leaf weeds. It is estimated that in Asia alone it is used more than 1000,000,000 lbs/year [4].

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Although some studies have demonstrated the mutagenicity of butachlor in *Salmonella* sp. [5], *Channa punctatus* [6] and Catfish [4], yet there is paucity of toxicological information on it. *Cirrhinus mrigala* (Hamilton) is one of the important carp widely consumed in India that is also cultured in village ponds situated near rice fields in mono or polyculture. Studies on the genotoxicity evaluation of butachlor formulations in fish are extremely scarce [7]. Therefore, attempts have been made in the present study to determine the genotoxic effects of butachlor on *Cirrhinus mrigala* using the kidney for chromosomal aberration test.

MATERIALS AND METHODS

Experimental Herbicide

Butachlor (50%EC), bearing the trade name Machete manufactured by Monsato India Limited, Mumbai, was used in the present investigation.

Experimental Design

Specimens of farm reared freshwater fish *Cirrhinus mrigala* (Hamilton; class: Teleostomi; order: Cypriniformes; family: Cyprinidae), with mean body weight of 20-50 g were procured from local fish farmer. Fishes were acclimated in plastic tubs of 80L capacity in laboratory conditions where temperature was maintained at 25±1ºC and lighting schedule at 12 hrs of light alternating with 12 hrs of darkness (LD:12:12). The water in the plastic container was renewed daily with stored tap water that was free from chlorine. Proper aeration was continuously provided in all plastic containers to maintain the optimum dissolved oxygen by an oil free air blower through plastic pipe via air store regulators attached to each aquarium to adjust pressure of air. Before stocking, the fishes were acclimatized for 5-7 days and disinfected by potassium permanganate ($KMnO_4$) solution. To maintain hygienic condition and prevent pollution caused by remaining food and faeces, the plastic containers were cleaned everyday prior to feeding time in morning by siphoning out of the excreta and 80% of the water was exchanged to prevent sudden increase in water temperature because the experiment was conducted during summer months. The dead fishes, if any, were removed and recorded for calculating the survival rate. Proper biosafety measures were taken for the disposal of treated water and fish as per norms.

Chromosome Preparation

The experimental fishes were kept in two groups each with replicates of two: 15 fish in the control group (T_1) without the exposure to butachlor and 15 in T_2 where fishes were exposed to butachlor. Then, 0.05% colchicine was injected below the dorsal fin to the test fish at the rate of 0.1 ml/40 gm of the body weight and left undisturbed for 2 hrs. After 2-3 hours, the kidney tissue of the test fish was dissected out. The tissue was kept in saline solution (0.56% KCl at room temperature for 20-25 minutes) to allow for proper swelling of cells. The tissue suspension was fixed in chilled Cornoy's fixative (3 part methanol, 1 part acetic acid). The slides were prepared by air-drying method. The air-dried slides were stained for 20-40 minutes in 2% Giemsa solution. These were differentiated in distilled water and air dried. The slides were screened under light microscope. The photomicrographs of selected stages were taken under 10×100X using Olympus digital camera.

Karyotype Preparation

Computer printed photomicrographs of well spread metaphase plates were used for the preparation of karyotypes. Individual chromosomes were cut out and grouped into homologous pairs on the basis of their length, arm ratio and morphology. These were then arranged tentatively in order of decreasing length and centromeric position. All the chromosomes were readjusted after morphometric measurement and finally pasted on a white sheet.

Morphometric Analysis

The chromosomes from photographs were measured with the help of dial type vernier caliper. For the morphometric analysis, actual length of chromosome, mean length of the chromosomes, mean total

haploid length, arm ratio (AR), and centromeric index (CI) were calculated.

Chromosomal Aberration Test (CAT)

Chromosome aberrations are the abnormalities of chromosome that occur during the cell division due to physical, chemical, or physiological factors.

Treated Group

Fishes in group 2 were exposed to a sublethal concentration of butachlor i.e. 1.0 ppm for 24, 48, 72 and 96 hrs. This dose was selected according to Farombi *et al.* (2008)[8]. After 24, 48, 72, and 96 hrs, 0.05 % Colchicine was injected below the dorsal fin to the test fish and the kidney tissue of the test fish was dissected out. Slides were prepared by air-drying method and stained for 20-40 minutes in 2% Giemsa solution. These were differentiated in distilled water and air dried. The slides were screened under light microscope.

Statistical Analysis

Data for percent aberration were analyzed by ANOVA followed by Duncan's multiple range tests using SPSS software version 11.5 for windows.

RESULTS

Karyotype Preparation

The present investigations were carried out on mrigal, *Cirrhinus mrigala* (Hamilton). Preparations from kidney cells were utilized for karyological observations. The somatic metaphase plates showed the presence of 50 chromosomes (Figure 1). The karyotype revealed the following features:

(i) 50 chromosomes were observed in karyotype.
(ii) Sex chromosomes were not distinguishable in the *Cirrhinus mrigala*.
(iii) The fundamental number of arms (NF) was found to be 80.
(iv) Karyotype was comprised of 15 pairs of metacentric chromosomes.
(v) 10 pairs of acrocentric chromosomes.

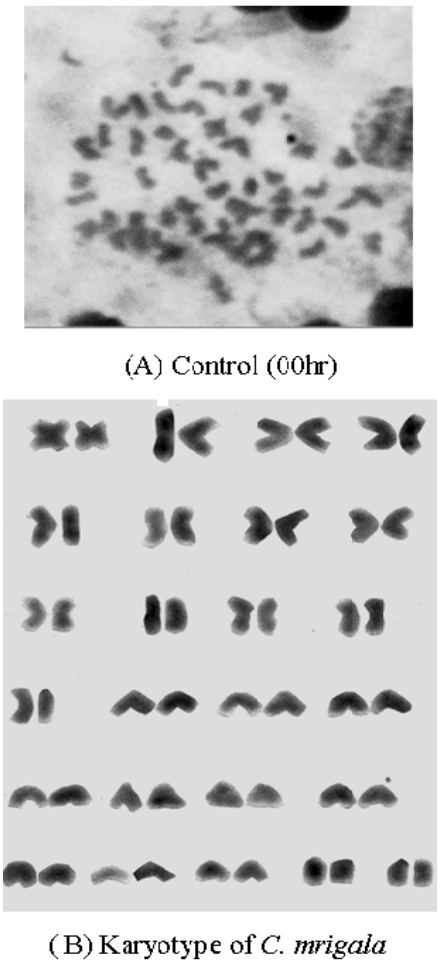

Figure1. (A) Metaphase stage (control), (B) karyotype of the chromosomes of *C. mrigala* without butachlor exposure.

Total haploid mean length of chromosomes in *Cirrhinus mrigala* was calculated to be 21.526 µm (Table 1). Percentage relative length (RL%) of the largest chromosome pair, which is the first pair of metacentric chromosome, was calculated to be 6.254 while this value for the smallest chromosome pair, which is the 25th metacentric chromosome pair, was calculated to be 2.534 (Table 1). Arm ratio (AR) and centromeric index (CI) for the biarmed chromosomes were also calculated. Centromeric index (CI) of the first pair of chromosome (metacentric) was recorded to be 47.113 (Table 1). CI of the 6th chromosome pair was calculated to be 46.194 and centromeric index of the 18th chromosome pair was calculated to be 50.000.

Table 1. Morphometric data of somatic chromosome of *Cirrhinus mrigala* (Hamilton).

Chromosome morphology	Centromeric index(CI)	Arm ratio(AR)	Percentage relative length(RL%)	Total length (TL) µm	Long arm µm(q)	Short arm µm(p)	Chrom. pair number
Metacentric	47.113	1.126	6.254	1.851±0.053	0.979	0.872	1.
Metacentric	48.814	1.048	5.882	1.744±0.021	0.893	0.851	2.
Metacentric	49.498	1.024	5.789	1.719±0.008	0.868	0.851	3.
Metacentric	47.921	1.091	5.301	1.575±0.032	0.820	0.755	4.
Metacentric	46.228	1.157	4.766	1.420±0.052	0.762	0.658	5.
Metacentric	46.194	1.159	4.417	1.312±0.050	0.706	0.606	6.
Metacentric	48.563	1.059	4.440	1.320±0.019	0.679	0.641	7.
Metacentric	48.179	1.075	4.138	1.266±0.040	0.673	0.593	8.
Metacentric	47.660	1.098	3.975	1.179±0.027	0.617	0.562	9.
Metacentric	49.428	1.023	4.068	1.206±0.007	0.610	0.596	10.
Metacentric	51.083	1.048	3.929	1.164±0.017	0.596	0.568	11.
Metacentric	47.678	1.097	3.743	1.113±0.025	0.582	0.531	12.
Metacentric	48.407	1.065	3.650	1.082±0.017	0.558	0.524	13.
Acrocentric			3.696	1.080±0.024			14.
Acrocentric			3.696	1.037±0.053			15.
Acrocentric			3.510	1.034±0.012			16.
Acrocentric			3.487	1.023±0.031			17.
Acrocentric			3.417	1.006±0.029			18.
Acrocentric			3.278	0.992±0.014			19.
Acrocentric			3.371	0.979±0.003			20.
Acrocentric			3.301	0.964±0.017			21.
Acrocentric			3.278	0.951±0.021			22.
Acrocentric			3.255	0.925±0.004			23.
Metacentric	48.559	1.059	2.813	0.837±0.012	0.431	0.406	24.
Metacentric	48.623	1.056	2.534	0.751±0.010	0.386	0.365	25.

Chromosomal formula = 15m+10a Total haploid mean length = 21.526µm
Fundamental arm number = 80

Chromosomal Aberration Test (CAT)

On the basis of the observations made on controls, the following abnormalities (shown in Figure 2) were encountered after treatment of fish with the herbicide, butachlor. The percent contribution of various abnormalities with respect to the duration of exposure has been depicted in Table 2.

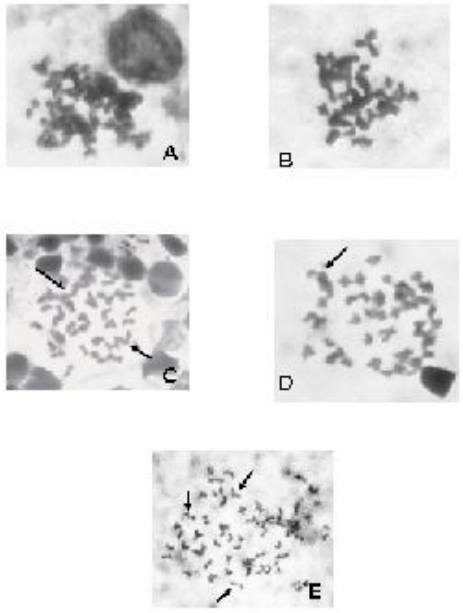

Figure.2 . Abnormalities in chromosomes of *C. mrigala* on exposure to butachlor (1.0 ppm), (A) Stikiness and clumping (24 hrs), (B) Stikiness and clumping (48 hrs), (C) End to end joining (72 hrs), (D) Break and Gap (72 hrs), (E) Fragmented chromosome (96 hrs).

Table 2. Anomalies observed in chromosomes of *Cirrhinus mrigala* on exposure to butachlor.

No. of Aberration per aberrant metaphases	Aberrant metaphase	Percent Aberration ±S.E	ABBERATIONS						No. of examined metaphases	Duration of exposure
			Total	AC	FC	BG	EEJ	SC		
1.66	3	2.88^{B}±1.00	13	_	3	_	2	8	150	Control
2.12	16	6.79^{AB}±2.20	51	2	4	8	15	22	150	24hrs
2.33	18	8.53^{A}±1.87	64	5	6	14	19	20	150	48hrs
2.5	14	7.06^{AB}±0.89	53	7	8	10	13	15	150	72hrs
2.38	13	6.26^{AB}±1.20	47	11	16	4	7	9	150	96hrs

Means with the same letter in the superscript in the same column are not statistically significant (P<0.05).

SC = Stickiness / clumping
EEJ = End to end joining
BG = Break and gap
FC = Fragmented chromosome
AC = Attenuated chromosome

Stickiness/Clumping (SC)

Stickiness among a few chromosomal ends or clumping of all the chromosomes was observed as a result of severe effect in certain cells. Out of the 150 metaphases examined, stickiness was observed only in 8 cells in positive control. With respect to time the number of cells showing stickiness increased from 0-24 hrs and then decreased at 48, 72, and 96 hrs.

i. End To End Joining (EEJ)

In this abnormality, one end of a chromosome joins another due to the effect of the toxicant. In the present study, the frequency of end to end joining was highest at 48hrs and thereafter declined after 72 and 96 hrs. However, the frequency was high in comparison to the control groups (Table 2).

ii. Break And Gap (BG)

A break and gap is considered to be present when the distance between the chromosome and its separated part is very small. The present results depicted no such gap in control group; however, the frequency of break and gap increased up to 48 hrs of exposure and thereafter decreased at 96 hrs.

iii. Fragmented Chromosome (FC)

In this case, the chromosomes get separated in the form of fragments. A single break gives fragmented chromosome and it may either be aligned or unaligned with the main chromosome. A continuous increase in the frequency of fragmented chromosome was observed with increase in the exposure duration. The highest number of fragmented chromosome was observed in metaphases when observed after 96 hrs of exposure (Table 2).

iv. Attenuated Chromosome (AC)

A disturbance in the condensation of chromosomes at some sites, resulting in thinning of chromatid is included under attenuation. The number of attenuated chromosomes also increased in proportion to increases in duration of exposure. The highest attenuated chromosomes were observed in the group of fishes exposed to butachlor for 96 hrs (Table 2).

Analysis of overall data of percent aberrations (Table 2) showed no significant (P<0.05) variations at different durations of exposure; however, abnormalities in all cases were maximum till the 48 hrs of exposure. The highest aberrant metaphases were observed on 48 hrs of exposure.

DISCUSSION

Cytogenetic analysis of chromosomes has been employed as an important biological tool to estimate the effect of genotoxic agents, like agrochemical butachlor, on fish. Karyotype of control *Cirrhinus mrigala* in the present study showed 2n = 50; 15 pairs of chromosomes were metacentric and 10 acrocentric. Chromosomes of *C. mrigala* have been studied by Manna and Prasad (1971) [9] and Zhang and Reddy, (1991)[10]. These studies have also reported the diploid number 50. However, Manna and Prasad (1971)[9] from Kallyani (West Bengal) reported 18 pairs of acrocentric, 4 pairs of submetacentric,

and 3 pairs of metacentric chromosomes. Such a difference may be geographic as the *C. mrigala* collected in the present study were farm reared. Chromosome aberration test (CAT) in the present study signifies the genotoxic effect of a widely used herbicide, butachlor, on an economically important food fish, *C. mrigala*. Anitha *et al*. (2000) [11] also showed the importance of aberration in studying the genotoxic effect of heat shock at different temperatures on gold fish, *Carassius auratus.*

The organochlorine compounds persist as such in water bodies for longer periods of time. There is thus a continuous exposure of the aquatic fauna to these contaminants. Butachlor is an organochlorine compound and has greater inhibitory effects on photosynthesis and respiration of macrophytes, undesirable grasses, and broadleaf weeds in rice fields [12-14]. However, along with run-off, this herbicide enters in the nearby fish ponds and its effect on bottom fauna and fishes in ponds have also been reported [15]. In the present study, stickiness and clumping, end to end joining, break and gap appearance of the fragmented and attenuated chromosomes were some of the effects of butachlor observed during chromosomal aberration test. Rishi and Grewal also reported that chromosome aberration test results show the constancy of effect over various durations in dichlorvos on *Channa punctatus* [16]. Biochemistry of butachlor shows that it inhibits cell division by blocking protein synthesis [8]. In the present study, although butachlor was used at low concentration (1.0 ppm), the exposure for 24, 48, 72, and 96 hrs showed chromosomal aberrations, indicating the toxic effects of butachlor. An increase in chromatid break and chromosomal exchange due to fluoride was also been reported by Chaurasia *et al*. (2007) [17]. Similar results have also been reported by Yadav and Trivedi (2009)[18] in *Channa punctatus* and Rita and Milton (2008)[19] in *Orechromis mosambicus* on exposure to chromium. Disruption of DNA synthesis, DNA repair or protein synthesis directly by some other mechanism might be the reasons for these aberrations. According to Mattar *et al*. (1992)[20], chromosomal aberration results from abnormalities in DNA duplication during S-phase. Also, in the present study, butachlor might have interfered with nucleotide synthesis leading to malformation of DNA molecules as the ultimate lesions responsible for aberration formation and DNA stand break [21]. End to end joining of chromosomes observed during the present study indicated that butachlor damages telomeres, thereby interfering with their protective function.

Although the frequencies of stickiness and clumping decreased after 48 hrs, but fragmented and attenuated chromosomes showed the persistence of this chemical in aquatic environment. Biswas and Manna (1989, 1992) [22, 23] and Rishi and Grewal, 1995 [16] have also reported similar results. Rishi and Grewal, 1995 [16] have also reported that chromosomal aberrations become less in intensity with increasing duration supporting the present results that total aberration decreased after 96 hrs of exposure in comparison to 48 hrs post treatment analysis showing that butachlor is less genotoxic with passage of time. Polynomial curve was drawn adding trend line to data clearly depicting a decline in percent chromosomal aberration after 72 hrs and 96 hrs (Figure. 3).

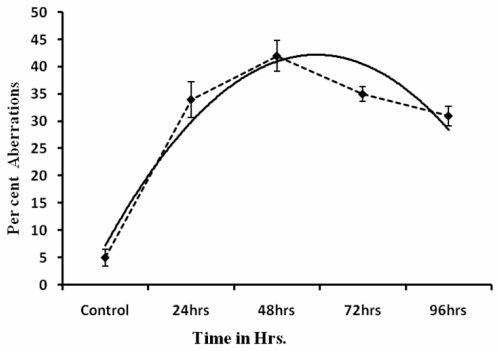

Figure 3. Polynomial fit curve showing trend line for chromosomal aberration with respect to time (24, 48, 72, and 96 hours of butachlor exposure).

According to Yiru *et al*. (1996) [24], butachlor dissipates rapidly from water with half life of 1 day and residues remain below detectability within 8 days. This might be the reason of decline in percent chromosomal

aberration after 2 days i.e. on 72 hrs (3 days) and 96 hrs of butachlor exposure.

CONCLUSION

These studies clearly reveal the genotoxic potential of butachlor even at low dose level (1.0 ppm) and suggest that butachlor interferes with cellular activities in fishes at genetic level inducing chromosomal aberrations. Therefore, the results of these investigations suggest a serious concern towards the potential danger of butachlor for aquatic organisms and the environment suggesting judicious and careful use of this pesticide in agricultural area.

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