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Assessment of Genotoxic Effects of Butachlor in Fresh Water Fish, *Cirrhinus mrigala* (Hamilton)

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Abstract: The present study was undertaken to evaluate the toxic effects of butachlor, a widely used herbicide on Indian major carp *C. mrigala* in terms of micronucleus assay and growth performance. Fish were exposed to 1.0 ppm, a sublethal concentration of butachlor and micronucleus assay was carried out after 24, 48, 72 and 96 h. Peripheral blood was collected and smear was prepared using Giemsa stain to analyse micronuclei. A positive control was also maintained. Growth performance of fish was studied for a period of 60 days during which fish were fed on a diet containing 40% protein. Growth parameters were calculated and carcass composition was analysed according to standard procedures. Frequencies of micronuclei reveal a significant ($p<0.05$) time dependent response, being higher at 48 h of exposure. Broken Egg (BE) and multiple micronuclei appeared after 72 and 96 h, respectively showing the genotoxic effect of the chemical. Fish growth studies also depicted a growth repressing effect due to butachlor. On exposure to 60 days a significant ($p<0.05$) decrease in fish growth was observed in terms of live weight gain, length gain and growth % gain in b.wt. Significantly ($p<0.05$) low accumulation of carcass protein and low gross energy in the butachlor exposed fishes were observed. Muscle glycogen levels were also significantly ($p<0.05$) low in the group of fishes exposed to butachlor indicating that this herbicide persists in the aquatic system for a long period of time. These results together with those related to mutagenicity demonstrate the risk that butachlor possess.

Key words: Broken egg, carcass protein, growth performance, micronucleus assay, herbicide

INTRODUCTION

The synthetic chemicals such as insecticides, pesticide, herbicides are used as knights in armour to control pests or weeds in modern agriculture technology for the production of more food and management of public health. These chemicals even when applied in restricted areas are washed and carried away by rains and floods to nearby aquatic system, thereby affecting aquatic biota, specially fish, which is important due to their nutritive value (Chattopadhay *et al*., 2006; Farombi *et al*., 2008; Ndimele *et al*., 2010). These chemical affect not only the physiology and survival of aquatic organisms including fish but also can interact with their genetic material which may lead to the mutations and/or carcinogenesis (Goksoyr, 1991; Da-Fonseca *et al*., 2008; Nwani *et al*., 2010).

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

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estimated that in Asia only, it is used more than 1000,000000 lbs year^{-1} (Ateeq *et al*., 2002). Although, some studies have demonstrated the mutagenicity of butachlor in *Salmonella* sp. (Hsu *et al*., 2005), induction of stomach tumours in rats (Thake *et al*., 1995) and induction of micronuclei in cat fish erythrocytes (Ateeq *et al*., 2002) and accumulation in body tissue in fresh water fish *Channa punctatus* (Tilak *et al*., 2007), yet there is paucity of toxicological information available on it. *Cirrhinus mrigala* (Hamilton) is one of important carp fish widely consumed in India and is also cultured in village ponds situated near rice fields in mono or polyculture. Junl *et al*. (2006) studied acute toxicity of different pesticides (triazophos, acephate and isocarbophos) on *Cirrhinus mrigala*. However, the studies concerning the genotoxic effects of butachlor on this fish are lacking. To address the issue of genotoxicity in terms of micronucleus assay of this widely used herbicide butachlor on Indian major carp *C. mrigala* and to study the magnitude and scope of these effects, present studies were undertaken.

MATERIALS AND METHODS

Experimental Herbicide

Butachlor (50% EC) bearing trade name Machete (1978) manufactured by Monsanto India Limited, Mumbai was used for present investigation. This study was conducted from 2008 to 2010.

Fish and Treatment

Specimens of freshwater fish *Cirrhinus mrigala* (Hamilton; Class: Teleostomi; Order: Cypriniformes; Family: Cyprinidae), with mean body weight of 20-50 g were procured from local sources. Fishes were acclimated in plastic tubs of 80 L capacity in laboratory conditions where temperature was maintained at 25±1°C and lighting schedule at 12 h of light alternating with 12 h of darkness (LD:12:12). The water in 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 solution of potassium permagnate ($KMnO_4$). To maintain hygenic 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 in summer months. The dead fishes if any, were removed and recorded for calculating the survival rate. After acclimatizing them to laboratory conditions, the fishes were kept in two groups. Group 1 served as control. Fishes in group 2 were exposed to sublethal concentration of butachlor i.e., 1.0 ppm for 24, 48, 72 and 96 h. This dose was selected according to Farombi *et al*. (2008). After 24, 48, 72 and 96 h, peripheral blood of one fish from each group was collected by caudal vein and in some cases by heart puncture and micronuclei assay were performed.

Micronucleus Assay

Micronuclei are some extra nuclear bodies that are formed in mitosis from acentric chromosomal fragment or chromosomes that are not included in either daughter nucleus. Micronucleus assay is widely used in fishes since, it is simple economic and reliable technique to detect effect of chemical. After exposure the peripheral blood was collected from caudal vein or by heart puncture. A smear was prepared on clean glass slide. The smear was

fixed with methanol for 5 min, air dried and stained with 2% Giemsa. The slides were scanned and analysed for 1000 cells/individual with micronuclei.

Fish Growth Studies

In order to evaluate the effect of butachlor on fish growth, the fishes were continued to kept under control and butachlor exposure for 60 days. During the experimental period (60 days) fish in both the groups were fed on a formulated diet containing 40% protein level containing soybean (Autoclaved at 15 lbs, 121°C for 15 min for the removal of antinutrient factors) as the major protein source. Fish were fed at 4% b.wt. day^{-1} in two installments at 0900 and 1700 h. The feeding trial for control (no toxicant) and treated (Butachler at 1.0 ppm) groups was continued for 60 days. Individual weights of the fish were recorded with the help of top pan balance (Make AFCO SETEX-1200), length of fish was measured using a simple centimeter scale. Weight gain, Specific Growth Rate (SGR) and growth percentage gain was calculated using standard formulae (Garg *et al*., 2002).

Proximate Analysis of Body Carcass and Muscle Glycogen

At the beginning of the experiment some fishes were taken from the reserve and their weight and length was recorded. After evisceration the fishes were cut into pieces. These pieces were weighed and put in hot air oven (60°C for 12 h) for drying to determine the moisture content. The dried sample in aluminium foil was stored in desiccator for proximate analysis for crude protein, fat, ash and NFE following the standard procedures (AOAC, 1995). The same protocol was followed at the end of the experiment (60 days) to estimate final carcass quality. Gross energy was calculated according to Henken *et al*. (1986). Muscle glycogen of treated fish at the end of 60 days was determined according to Thimmaiah (1999).

Statistical Analysis

Student t-test was used for determining the significant differences between different treatments, using SPSS version 11.5 for windows.

RESULTS

Induction of Micronuclei (MNi)

The frequencies of micronuclei induced in the peripheral blood erythrocytes, determined at concentration 1ppm of butachlor are shown in Table 1. The criteria of Tolbert *et al*. (1992) was followed for the identification of micronuclei. Different types of micronuclei (MNi) were observed (Fig. 1a-d) and these were categorized into following groups:

- Small MNi
- Large MNi
- Two micronuclei in a cell
- Broken Egg (BE)
- Multiple MNi

The number of micronuclei at 48 h were maximum (22.5±1.0) and thereafter decreased. After 24 and 48 h of exposure to butachlor no stage of Broken Egg (BE) have been found. The BE started appearing after 72 h (04) and after 96 h 1 or 2 stages of BE have been found. Multi-micronuclei were observed only after 96 h of exposure to butachlor (Table 1).

Table 1: Frequency of micronuclei in 1000 blood cells of *Cirrhinus mrigala* treated with butachlor at different duration

Duration of exposure (h)	Slide No.	No. of blood cells examined	Total No. of cell with micronuclei	Total No. of broken egg	Total No. of cell with multiple micronuclei	Mean±SE
Control	1	1000	3	-	-	2.50±0.353
	2	1000	2	-	-	
24 h	1	1000	8	-	-	9.50±1.010
	2	1000	11	-	-	
48 h	1	1000	21	-	-	22.5±1.060
	2	1000	24	-	-	
72 h	1	1000	11	3	-	6.75±1.672
	2	1000	9	4	-	
96 h	1	1000	7	2	3	4.00±1.201
	2	1000	9	1	2	

Significant at $p<0.05$

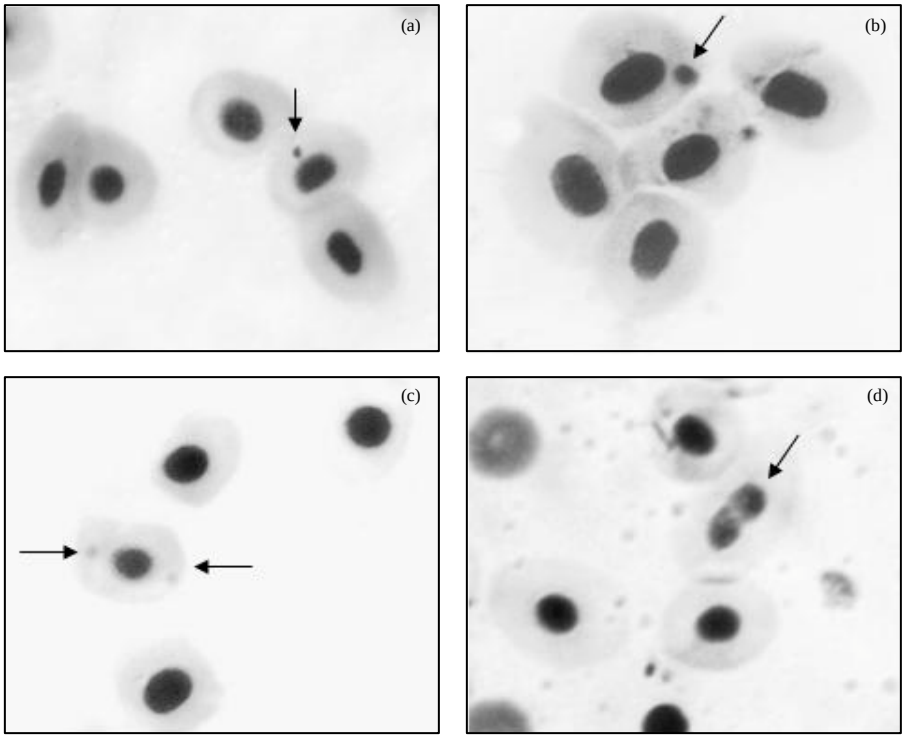

Fig. 1: Micronuclei and broken egg in the blood cells of *Cirrhinus mrigala* on exposure to butachlor. (a) small micronucleus, (b) large micronucleus, (c) two micronuclei in a cell and (d) broken egg

Effect of Butachlor on Fish Growth

The growth responses of the experimental fish in control and treated groups are shown in Table 2, survival was high (100%) in both the groups. The growth of fish in terms of weight gain, length gain and growth (%) gain wt. in body wt. were significantly ($p<0.05$) high in control group where no toxicant was present where as a clear cut growth repressing effect was observed in group of fishes which were treated with Butacholr at 1.0 ppm.

Table 2: Growth performance of *C. mrigala* when exposed to Butacholr for 60 days

Parameters	Control	Treatment
Initial weight (g)	38.5±0.10	38.60±0.17
Initial length (cm)	12.4±0.10	12.30±0.10
Final weight (g)*	59.5±0.21	47.25±0.14
Final length (cm)*	19.2±0.49	14.90±0.31
Weight gain (g)*	20.9±0.10	8.65±0.03
Length gain (cm)*	6.8±0.35	2.60±0.21
Growth % gain in BW (%)*	54.3±0.14	18.30±0.10

*Significantly ($p<0.05$) different (student's t-test)

Table 3: Proximate composition of fish carcass after 60 days of exposure to butachlor (1.0 ppm)

Parameter	Initial	Control	Treatment (butachlor 1.0 ppm)
Moisture (%)	71.90±0.10	72.4±0.247	70.70±0.56
Crude protein (%)	13.10±0.23	15.2±0.31*	10.70±0.14*
Crude fat (%)	5.10±0.07	5.31±0.04	5.22±0.02
Crude ash (%)	4.30±0.21	4.29±0.04*	5.47±0.01*
NFE (%)	5.45±0.67	2.67±0.13*	7.90±0.38*
Gross energy	3.13±0.72	6.15±0.06*	5.94±0.10*
Muscle glycogen	2.00±0.14	2.40±0.09*	1.30±0.07*

*Significantly ($p<0.05$) different (student's t-test)

Effect of Butachlor on Fish Carcass Composition and Muscle Glycogen

Initial and final carcass composition in control as well as treatment group with respect to proximate nutrients of test fish is shown in Table 3, crude protein (%) was found to be significantly ($p<0.05$) higher (15.2±0.31) in the carcass of fish kept under control where as the protein (10.07±0.14) content decreased in the group of fishes exposed to butachlor. The values were even lower than the initial values. Gross energy also followed the similar trend where as crude ash was higher in butachlor treated fishes. No significant variations were observed in moisture and fat content. Significant ($p<0.05$) decrease in muscle glycogen level was also observed in group of fishes treated with butachlor. The values were even lower than the initial group.

DISCUSSION

Among the various toxic effects of agrochemicals, large-scale impact on the genetic materials is of great concern. Hence, the potential long-term genetic hazards of pesticides/herbicides for man and other organisms cannot be ignored (Durham and Williams, 1972; Waters *et al*., 1980; Sharma, 1984). The biological magnification leads to the presence of residues of pesticides in human food and their subsequent consumption by humans. As the fishes are directly exposed to contaminants in water, these can act as good bio-indicators for the state of pollution in water bodies (Manna and Biswas, 1986).

The present study signifies the effect of a widely used herbicide butachlor on an economically important food fish *C. mrigala* in terms of micronuclei test and growth performance. The frequency of micronuclei in the present study significantly ($p<0.05$) increased from 0 to 48 h post treatment, thereafter there was appearance of broken egg followed by multiple micronuclei stage confirming the effect of butachlor on nucleus. Actually, butachlor is an organochlorine compound and have greater inhibitory effect on photosynthesis and respiration of macrophytes, undesirable grasses and broadleaf weeds in rice fields (Jones and Winchel, 1984; Jones *et al*., 1986; Delistraty and Hershner, 1984). 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 (Sarkar, 1991). Cavas and Konen (2005) have also reported that butachlor inhibits the cell division and thus result in

micronuclei. Thus it can be confirmed that this herbicide cause genetic damage in erythrocytes of *C. mrigala.* Reports on the impact of butachlor addressing micronuclei in *C. mrigala* are not available. However, Rishi and Grewal (1995) reported that micronucleus assay results show constancy of effect over various duration in dichlorvos on *Channa punctatus*. According to Kirsch-Volders *et al*. (2006), MN assay is a multi endpoint test of genotoxic responses to clastogens. The frequency of micronucleus (MN) is extensively used as a biomarker of genomic stability (Norrapa and Falak, 2003). Micronuclei result from chromosome breakage or interference with the mitotic apparatus and such events are thought to be related to carcinogenesis (Bishop, 1987). It is used as an indicator of genotoxic exposition since, it is associated with chromosome aberrations (Remirez and Saldanha, 2002), supporting the view that appearance of micronuclei, Broken egg and Multiple micronuclei in the present studies might be due to the genotoxic effect of butachlor. In the present study, total number of micronuclei initially increased up to 48 h of exposure thereafter decreased at 72 or 96 h of exposure. This decrease is due to its effect in the form of broken egg and appearance of multiple micronuclei which are considered to be associated with severe chromosomal aberration (Remirez and Saldanha, 2002). Although, there was appearance of micronuclei showing genotoxic effect of chemical used even at low concentration, yet, the survival was not significantly affected. Therefore, in the present study the fishes were continued to kept under butachlor exposure for 60 days and its effect on growth was also evaluated. A growth depressing effect was further observed when fish were exposed to the chemical for 60 days. Although, same feed at the same ration level was fed to the control and exposed fishes, yet the growth rate was very low in group of fishes exposed to butachlor, very clearly revealing that butachlor might have decreased the digestibility causing improper feed and dietary protein utilization and decrease in fish growth. Thus inhibition of cell division might be the reason contributing to growth depression. The herbicide butachlor has been observed to cause a substantial reduction in the fish carcass protein content, even less than the initial value in *C. mrigala* in the present study. Singh and Bhati (1994) have also observed similar results in *Channna punctatus* when exposed to 2, 4-D. There might be two reasons for decrease in carcass protein, first may be simply a disturbance in the enzyme systems decreasing digestibility, the second may be based on effect of chemical on genetic material since, it is well known that butachlor affect the rate of cell division to a considerable extent. An increase in ash content further confirmed low dietry protein utilization. Muscle glycogen levels were also low in butachlor exposed fishes in comparison to control, though they were fed on similar diets again showing utilization of accumulated glycogen because of low feed utilization. These studies clearly reveal the genotoxic potential of butachlor even at low dose level 1.0 ppm. Since, fishes can respond to mutagens at low concentration of toxicant in a manner similar to higher vertebrates (Goksoyr, 1991; Al-Sabti and Metcalfe 1995). Moreover, as compared to mammals, the DNA repair was reported to be slower in fishes (Walton *et al*., 1984; Maccubin *et al*., 1991; Espina and Weiss, 1995). Therefore, these investigations suggest a serious concern towards the potential danger of butachlor to aquatic organisms and its use in agriculture practices.

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