



## RESEARCH ARTICLE

### Butachlor Induced Clinico-Hematological and Cellular Changes in Fresh Water Fish *Labeo rohita* (Rohu)

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#### ABSTRACT

The present study was conducted to explore the clinico-hematological and genotoxic effects in fresh water fish (*Labeo rohita*) exposed to various concentrations of butachlor (chloroacetanilide herbicide). For this purpose 30 fish weighing about 500-600 g were randomly allocated to five equal groups (A-E) and treated with various concentrations (0, 0.25, 0.50, 0.75 and 1.00 mg/L) of butachlor. Blood samples were collected from each fish after 48, 72 and 96 h of post treatment and fresh thin blood smears were made for cellular abnormalities. All the fish kept in group A did not reveal any clinical and behavioral ailments. However, various clinical signs such as rapid breathing, gasping at the water surface, restlessness, opercular movement, loss of equilibrium and erratic swimming were observed at higher concentrations (0.75 and 1.00 mg/L) of herbicide. The results showed that the erythrocyte, hemoglobin, hematocrit and lymphocyte values were significantly ( $P < 0.05$ ) decreased while significantly increased total leukocyte counts were recorded. Significantly increased incidence of different morphological and nuclear abnormalities such as pear shape erythrocyte, microcyte, tear shape erythrocyte, erythrocytes with micronuclei, lobed, blebbed and notched nuclei and cells with nuclear remnants were observed. The results suggested that butachlor poses serious clinico-hematological and mutagenic effects in fish and may be hazardous to the other species when those are exposed to this herbicide.

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#### INTRODUCTION

Varieties of environmental pollutants are responsible for multiple serious effects on the organisms including human population, ecosystem levels, reproductive status, organ functions, population size and biodiversity (Chang *et al.*, 2011; Hussain *et al.*, 2013). Among pollutants which induce the carcinogenic and mutagenic effects, the pesticides, insecticides and herbicides are the most problematic compounds and may exert damage beyond that of individual. Pesticides, insecticides and herbicides are frequently used in developing countries in agro production system to enhance the crop productivity and to eradicate the variety of parasites from livestock population (Ateeq *et al.*, 2006; Hussain *et al.*, 2012). The extensive use of these synthetic chemicals has increased dramatically over the last two decades and accumulates in agricultural products, surface and ground water and soils (Farombi *et al.*, 2008).

Residues of these chemicals in water, food crops, fish, and ecosystem can pose serious threats to organisms, predators and human population (Ahmad *et al.*, 2012; Praveena *et al.*, 2013; Ali *et al.*, 2014; Auon *et al.*, 2014; Isa *et al.*, 2014).

Pesticides at high concentrations reduce growth, reproduction and the survival of fish and induce many perceptible effects on different aquatic population (Hussain *et al.*, 2013). Pesticides are recognized as the major water pollutants globally which interfere with the aquatic environment (Naveed *et al.*, 2011). Herbicides are frequently applied during the pre- and post-harvest of different cereal crops to remove the broad leaf weeds. Herbicides obstruct the synthesis of various useful cellular pigments in plants by interfering a variety of mechanisms including inhibition of photosynthesis, mitosis, cell division, leaf formation, root growth and enzyme function (William *et al.*, 1995). Butachlor (2-chloro-2', 6'-diethyl-N-butoxymethylaceta-nilide) being an important member of

chloroacetanilide herbicides, is widely and regularly used in modern agriculture in Asia which has significant role in the elimination of useful microorganisms from the field (Ateeq *et al.*, 2002; Bibi *et al.*, 2014).

The extent of huge range of xenobiotically induced effects at cellular levels such as structures or functions and biochemical components in organisms are considered useful tools to monitor the early responses to environmental stress (Bhatkar, 2011). The morphological and different nuclear abnormalities in erythrocytes are considered the main biomarkers to assess the pollution-related toxic effects and demonstrate the associations between mutagenicity and chronic health effects (Celik *et al.*, 2005). The micronucleus (MN) assay is known to be one of the most reliable and useful technique due to its simplicity for the identification of genomic alterations in different environmental organisms (Hussain *et al.*, 2014). This assay is technically easier and rapid than the microscopic analysis of chromosomal aberrations and can be widely utilized as a mutagenic biomarker in environmental biomonitoring programs (Bolognesi and Hayashi, 2011). Previously scanty information is available in published literature about the hematological and mutagenic effects due to butachlor herbicide in birds (Hussain *et al.*, 2014). Therefore, the present research work was conducted to know the hematological and morphological/nuclear alterations in erythrocyte of fish exposed to different levels of butachlor.

## MATERIALS AND METHODS

A total of 30 fresh water fish weighing 500-600g having almost similar age and clinically free from any gross ailment were obtained from local hatchery. These were kept in 70 L aquarium containing fresh water (16 to 23°C), fed daily and the water was changed daily. After 5 days acclimatization, these were randomly divided into five equal groups (A-E). The fish were treated with butachlor (50% w/w; M/S Ali Akbar Enterprises, Pakistan) as follow: A (0 mg/L), B (0.25 mg/L), C (0.50 mg/L), D (0.75 mg/L) and E (1.00 mg/L) for 96 h.

All the fish were observed for clinical and behavioral changes daily. Blood samples from the caudal vein were collected from all fish after 48, 72 and 96 h of post treatments with anticoagulant (EDTA; 1 mg/ml). The collected samples were analyzed for various hematological parameters (Natt and Herrick, 1952).

Duplicate blood smears were prepared from each fish and stained with Wright-Giemsa stain. The frequency of nuclear abnormalities was studied using light microscope (Nikon, Tokyo, Japan) at the Department of Pathology, University of Agriculture, Faisalabad, Pakistan. A total of 2000 erythrocytes/smear/fish were observed (Hussain *et al.*, 2014).

Data thus collected were analyzed using statistical analysis (SAS, 2004). Mean±SE values for hematological parameters and different nuclear abnormalities of erythrocytes were compared using analysis of variance. All the means in different groups were compared by Tukey's test.

## RESULTS

**Clinical signs and behavioral alterations:** The fish kept in groups A and B did not indicate any behavioral and clinical

abnormalities during the experiment. Mild to moderate clinical signs such as rapid breathing, gasping at the water surface, restlessness, opercular movement, loss of equilibrium and erratic swimming were observed at higher concentration (0.75 mg/L). The maximum intensity of these clinical signs was observed in fish exposed to higher concentration of herbicide in group E (1.00 mg/L) throughout the present experiment.

**Hematological parameters:** Erythrocyte indices significantly reduced at 72 and 96 h post exposure in fish of groups C-E as compared to control group (Table 1). The erythrocyte counts were significantly ( $P<0.05$ ) decreased in fish kept in groups D-E at 48 h and in groups C-E after 72 and 96 h post treatment as compared to control group (Table 1). The hemoglobin concentration decreased significantly in fish of groups C-E at 96 h post exposure as compared to non-treated fish. Hemoglobin values were also reduced in fish of group D after 72 h and in group C at 96 h post exposures. The pack cell volume reduced significantly in fish kept in groups D and E at 48 h of post treatment and in groups C-E after 72 and 96 h of post treatment (Table 1). The leukocyte count was significantly increased in groups D-E after 48 h of post treatment and in groups C-E after 72 and 96 h. Serum total proteins levels significantly decreased in groups D-E at 72 h while in groups C-E of treatment. The values of neutrophil, monocyte and mean corpuscular volume increased significantly in group D-E throughout the experiment. The lymphocyte values significantly decreased in groups D-E throughout the experiment. Mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin decreased and increased in groups C-E throughout the experiment (Table 1).

### Genotoxic and Cellular abnormalities in erythrocyte:

The results on the frequency of heteropycnotic nuclei and different other nuclear changes in erythrocytes of fish (Fig. 1-6) are presented in Table 2. The results revealed that various nuclear abnormalities significantly increased at different levels of butachlor herbicide when compared to fish of control group. It was also observed that the percentile rate of erythrocyte with heteropycnotic nuclei, lobed nuclei, blebbed nuclei, nuclei, cells with micronuclei and cells bearing broken nuclei (Fig. 1-6) was significantly increased in groups D-E throughout the experiment. The incidence of erythrocyte with notched nuclei and cells with nuclear remnants were significantly increased in fish present in groups C-D at all experimental sampling. The incidence of various nuclear changes observed in erythrocytes of fish such as heteropycnotic nuclei, lobed nuclei, blebbed nuclei, cells with micronuclei, cell bearing broken nuclei, notched nuclei and cells with nuclear remnants was increased significantly in fish exposed to butachlor. The results also showed that the frequency of erythrocytes with condensed and fragmented nuclei increased (Fig. 1-6) significantly in group E after 48 and 72 hours and in groups C-E after 48, 72 and 96 hours of post treatment. The results on different morphological alterations in erythrocytes are presented in Table 3. The incidence of different morphological alterations in erythrocyte such as microcyte, pear shape erythrocyte, dividing erythrocyte, leptocyte, tear shaped erythrocyte and stomatocyte (Fig. 1-6) was significantly increased in groups D-E throughout the present experiment.

**Table 1:** Various hematological parameters of fish administered different levels of butachlor

Parameter/Hours	Groups				
	A	B	C	D	E
Erythrocyte ( $10^6/\text{mm}^3$ )					
48	4.25±0.01	4.14±0.01	4.08±0.01	3.46±0.03*	3.37±0.01*
72	4.12±0.02	3.77±0.01	3.65±0.01*	3.06±0.01*	2.96±0.02*
96	4.03±0.06	3.68±0.01	3.17±0.07*	2.91±0.02*	2.74±0.02*
Hemoglobin (g/dl)					
48	6.84±0.02	6.74±0.01	6.67±0.01	6.44±0.04	5.57±0.03*
72	6.95±0.05	6.63±0.01	6.29±0.06	5.84±0.10*	5.45±0.03*
96	7.20±0.04	6.40±0.00	5.57±0.01*	5.29±0.02*	4.96±0.08*
Pack cell volume (%)					
48	34.56±0.22	32.49±0.27	31.14±0.31	28.75±0.23*	27.55±0.18*
72	31.92±0.58	26.92±0.24	20.65±0.29*	18.99±0.25*	18.08±0.03*
96	31.36±0.24	25.49±0.28	19.33±0.61*	17.19±0.33*	17.25±0.11*
Mean corpuscular volume (fl)					
48	91.87±1.55	96.5±0.50	100.67±1.02	107.72±2.12*	113.4±1.33*
72	92.62±1.32	99.31±0.30	102.67±1.21	110.62±1.88*	117.32±0.70*
96	94.17±1.28	100.22±0.94	106.91±0.69	113.92±1.08*	119.7±1.05*
Mean corpuscular hemoglobin concentration (g/dl)					
48	29.84±0.49	26.30±0.39	25.71±0.23*	22.86±0.10*	21.98±0.06*
72	29.81±0.23	25.59±0.32	24.75±0.42*	21.60±0.17*	20.26±0.10*
96	29.91±0.16	25.28±0.25	23.36±0.30*	20.38±0.15*	19.20±0.09*
Leukocyte counts ( $10^3/\text{mm}^3$ )					
48	20.65±0.30	20.29±0.43	23.64±0.19	25.36±0.11*	26.50±0.17*
72	19.64±0.17	21.59±0.58	23.07±0.39	25.62±0.18*	27.04±0.11*
96	20.05±0.24	22.72±0.24	24.04±0.21	26.03±0.02*	27.48±0.06*
Neutrophil (%)					
48	11.25±0.39	12.17±0.36	13.12±0.11	14.51±0.23*	15.11±0.21*
72	12.05±0.25	13.05±0.22	13.5±0.11	15.3±0.14*	16.27±0.17*
96	11.95±0.20	13.52±0.37	14.52±0.06	15.6±0.21*	17.32±0.14*
Lymphocyte (%)					
48	22.98±0.45	21.10±0.11	20.84±0.28	17.20±0.11*	16.39±0.03*
72	22.89±0.44	20.58±0.24	20.17±0.05	17.04±0.10*	15.84±0.16*
96	20.58±0.21	19.87±0.22	19.51±0.19	16.47±0.08*	15.24±0.04*
Monocyte (%)					
48	4.19±0.05	4.40±0.03	4.44±0.04	4.54±0.02	5.17±0.03*
72	4.34±0.10	4.46±0.04	4.53±0.02	5.66±0.07*	5.84±0.01*
96	4.28±0.01	4.55±0.02	4.62±0.02	5.99±0.10*	7.22±0.19*

Values (mean±SE) in a row bearing asterisk are significantly ( $P \leq 0.05$ ) different from control group.

## DISCUSSION

Pesticides are extensively used in agriculture to control pests and livestock sector to remove parasitic infestation which act as major to environment and all other species living in the same ecology. Extensive use of organophosphorus pesticide due to less toxicity to mammals, increase selectivity towards pests and moderate environmental persistence poses serious risks that not only damage human health but also environment (Naz *et al.*, 2011). In present study various clinical signs including rapid breathing, gasping at the water surface, restlessness, opercular movement, loss of equilibrium and erratic swimming were observed. The respiratory signs in fish in this study could be due to edema of gills resulting impaired functioning. Previously respiratory signs have also been reported in fish due to butachlor (Guo *et al.*, 2010; Nwani *et al.*, 2013). The clinical and behavioral changes observed in present like abnormal swimming patterns also are similar to the observations of different studies in fish exposed to butachlor (Bekeh *et al.*, 2011; Altinok *et al.*, 2012). The clinical signs and behavioral alterations in fish are the indicators of potential toxic effects of pesticides (Pandey *et al.*, 2011).

In present study hemoglobin concentrations, pack cell volume, erythrocyte counts, serum total proteins and lymphocyte was significantly decreased while total leukocyte, mean corpuscular hemoglobin and mean corpuscular volume increased significantly in exposed fish. As blood parameters are considered as the best

biomarker to assess the patho-physiological status of different organisms exposed to any toxicants (Maheswaran *et al.*, 2008; Hussain *et al.*, 2012). The decrease values of hemoglobin concentration, pack cell volume and erythrocyte in present study could be due to inability of fish to deliver sufficient oxygen to hematopoietic tissues suggesting poor physical activity (Nussey, 1995; Hussain *et al.*, 2014). In present study decreased values of hemoglobin contents, erythrocyte and serum total proteins might be due to impair/exhaustion of both metabolic and hemopoietic activities of fresh water fish exposed to butachlor (Sawhney and Johal, 2000). The hematological indices such as MCV, MCHC and MCH are important biomarkers to diagnose anemia in animals (Sharaf *et al.*, 2010; Ghaffar *et al.*, 2014). Abnormalities in these hematological parameters (increase MCH and MCV, decrease of MCHC) could be due to toxic effects of butachlor also supported by lower values of erythrocyte, hemoglobin and pack cell volume in present study. Moreover, increased values of MCV and MCH in present study could also be due to higher number of immature erythrocyte suggesting macrocytic anemia (Carvalho and Farnandes, 2006). The significant lower values of the MCHC in present experimental study may be related to decrease synthesis of hemoglobin and swelling of erythrocyte. Similar results also have been reported due to toxicants (Kori-Siakpere, 2011). These hematological alterations in fish in present study could also be due to impaired exchange of gases by gills and linings of operculum (Bhatkar, 2011). The increased values of total

**Table 2:** Various nuclear changes (%) observed in erythrocytes of fish given various doses of Butachlor

Parameters/hours	Groups				
	A	B	C	D	E
<b>Heteropycnotic Nuclei</b>					
48	0.24±0.01	0.25±0.01	0.28±0.01	0.36±0.01*	0.39±0.00*
72	0.25±0.00	0.26±0.01	0.28±0.01	0.37±0.00*	0.47±0.02*
96	0.26±0.00	0.27±0.00	0.31±0.00	0.41±0.01*	0.63±0.02*
<b>Lobed Nuclei</b>					
48	0.19±0.01	0.19±0.01	0.21±0.01	0.27±0.01*	0.302±0.01*
72	0.19±0.01	0.22±0.00	0.22±0.01	0.30±0.00*	0.36±0.00*
96	0.20±0.01	0.23±0.01	0.23±0.00	0.33±0.02*	0.49±0.01*
<b>Blebbled nuclei</b>					
48	0.12±0.01	0.13±0.02	0.13±0.01	0.24±0.03*	0.26±0.02*
72	0.13±0.00	0.14±0.01	0.14±0.02	0.26±0.03*	0.33±0.04*
96	0.15±0.01	0.15±0.00	0.16±0.01	0.31±0.00*	0.44±0.02*
<b>Cells with micronuclei</b>					
48	0.22±0.00	0.22±0.00	0.26±0.00	0.84±0.01*	0.92±0.02*
72	0.21±0.02	0.25±0.01	0.39±0.02	1.15±0.03*	1.43±0.02*
96	0.21±0.00	0.25±0.01	0.59±0.02	1.23±0.02*	1.79±0.11*
<b>Notched nuclei</b>					
48	0.18±0.00	0.23±0.00	0.29±0.01*	0.51±0.00*	0.75±0.01*
72	0.17±0.00	0.21±0.01	0.34±0.03*	0.56±0.00*	0.84±0.01*
96	0.16±0.00	0.23±0.01	0.43±0.05*	0.64±0.01*	0.92±0.01*
<b>Cells with nuclear remnants</b>					
48	0.13±0.01	0.16±0.02	0.17±0.01*	0.26±0.01*	0.31±0.04*
72	0.14±0.01	0.17±0.02	0.21±0.01*	0.29±0.02*	0.35±0.06*
96	0.15±0.02	0.17±0.02	0.26±0.01*	0.32±0.01*	0.44±0.14*

Values (mean±SE) in a row bearing asterisk are significantly ( $P \leq 0.05$ ) different from control group.

**Table 3:** Various morphological alterations observed in erythrocytes of fish given different concentrations of Butachlor

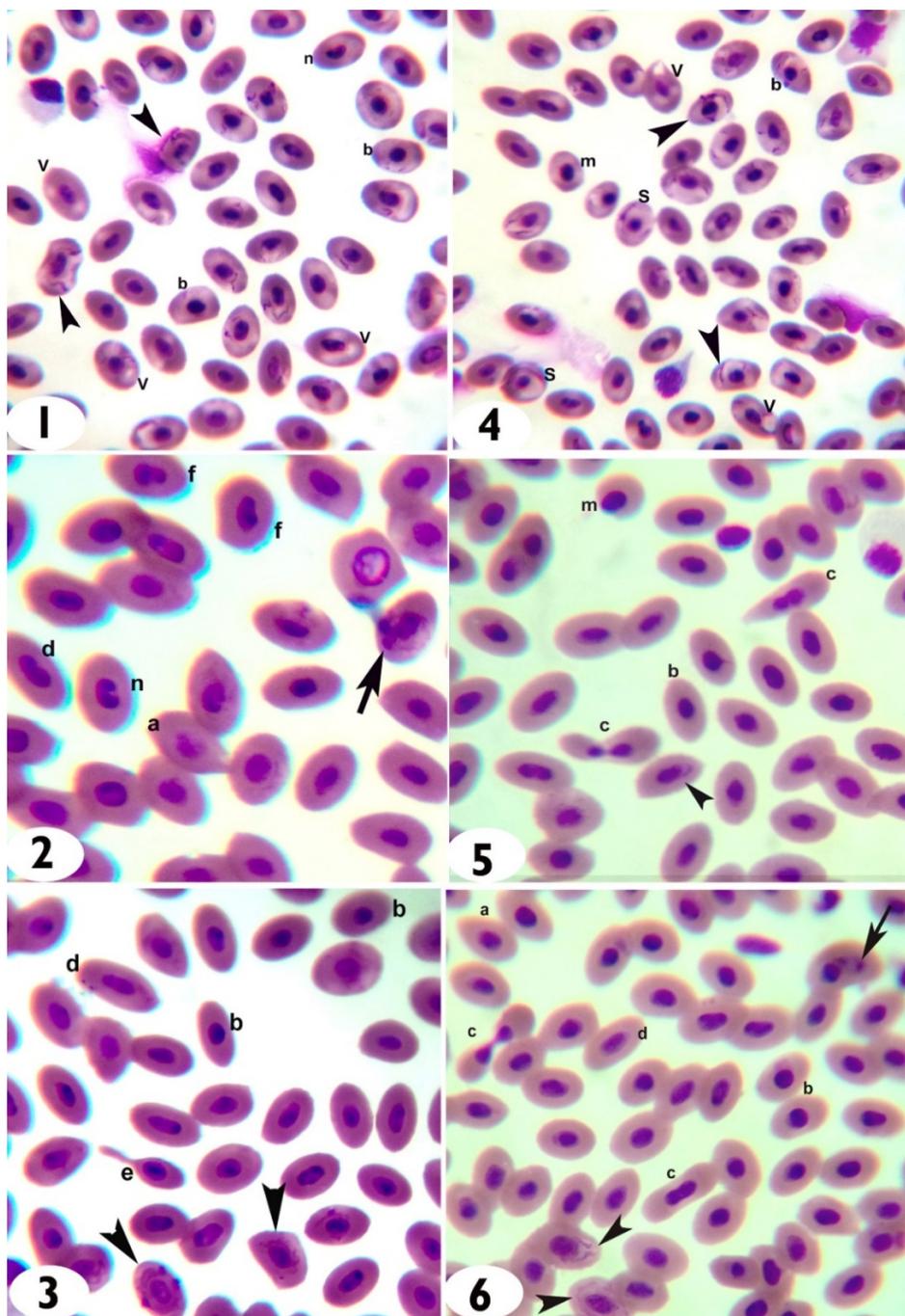
Parameters/hours	Groups				
	A	B	C	D	E
<b>Pear shape Erythrocyte (%)</b>					
48	0.44±0.01	0.46±0.00	0.50±0.00	0.64±0.00*	0.73±0.01*
72	0.44±0.00	0.48±0.00	0.51±0.02	0.7±0.01*	0.77±0.01*
96	0.40±0.00	0.48±0.00	0.52±0.02	0.73±0.01*	0.84±0.01*
<b>Microcyte (%)</b>					
48	0.32±0.00	0.35±0.00	0.36±0.00	0.41±0.01*	0.52±0.01*
72	0.34±0.00	0.37±0.00	0.40±0.00*	0.50±0.00*	0.59±0.01*
96	0.33±0.00	0.38±0.00	0.45±0.00*	0.56±0.01*	0.75±0.01*
<b>Dividing erythrocyte (%)</b>					
48	0.13±0.03	0.15±0.01	0.16±0.03	0.19±0.01*	0.30±0.01*
72	0.13±0.04	0.15±0.03	0.17±0.05	0.30±0.02*	0.39±0.01*
96	0.12±0.01	0.16±0.03	0.18±0.02	0.37±0.04*	0.45±0.06*
<b>Leptocyte (%)</b>					
48	0.12±0.04	0.14±0.03	0.15±0.02	0.24±0.01*	0.35±0.06*
72	0.13±0.03	0.15±0.05	0.17±0.05	0.30±0.02*	0.42±0.06*
96	0.15±0.02	0.17±0.04	0.19±0.04	0.32±0.01*	0.51±0.01*
<b>Tear shaped erythrocyte (%)</b>					
48	0.12±0.03	0.13±0.02	0.14±0.02	0.15±0.03*	0.27±0.04*
72	0.13±0.04	0.15±0.02	0.16±0.03	0.18±0.04*	0.37±0.01*
96	0.14±0.03	0.16±0.01	0.18±0.04	0.30±0.01*	0.43±0.03*
<b>Stomatocyte (%)</b>					
48	0.25±0.02	0.26±0.01	0.26±0.01	0.27±0.04*	0.39±0.05*
72	0.24±0.01	0.26±0.02	0.27±0.01	0.35±0.08*	0.48±0.09*
96	0.24±0.05	0.25±0.02	0.27±0.01	0.37±0.06*	0.54±0.01*

Values (mean±SE) in a row bearing asterisk are significantly ( $P \leq 0.05$ ) different from control group.

leukocyte counts, neutrophils in fish could be due to the inflammatory reaction following injurious stimuli. Increased monocyte values and decrease in lymphocyte count in present study could be due to sensitivity of the immune system against the stress condition due to butachlor herbicide.

In present study various cellular and nuclear changes including heteropycnotic nuclei, lobed nuclei, blebbed nuclei, pear shape erythrocyte, cells with micronuclei and cells bearing broken nuclei in erythrocytes of fish were significantly increased. Previously these pathological changes in fish due to butachlor have not been reported. However, different cellular and nuclear alterations due to herbicide in erythrocytes of birds have been observed (Hussain *et al.*, 2014). These nuclear and cellular changes

might be due to increase in production of reactive nitrogenous and oxygen species as results of oxidative stress to mitochondrion (Hussain *et al.*, 2012). The increased frequency of erythrocyte with lobed, notched, heteropycnotic nuclei, cell with nuclear remnants and micronuclei indicating mutagenic and genotoxic effects in present study could be due to higher production of caspase activated DNase which results in cleavage of nuclear and cytoskeletal proteins and aneuploidy (Hussain *et al.*, 2012). Previously various studies have reported that these nuclear alterations could be due to cytotoxic impacts of variety of toxic compounds which induce chromosomal abnormalities (Lim *et al.*, 2009; Sharaf *et al.*, 2010). Moreover, lobed, notched, vacuolated, condensed, fragmented and erythrocyte with blebbed nuclei could



**Fig. 1-6:** Blood smears of fish treated with butachlor indicating various morphological and nuclear changes of erythrocytes such as nuclear remnants (arrow heads), micronucleus (arrow), pear shaped (a), condensed nucleus (b), dividing erythrocyte (c), leptocyte (d), tear shaped erythrocyte (e), fragmented nucleus (f), microcyte (m), notched nucleus (n), stomatocyte (s) and cytoplasmic vacuolation (v). Wright-Giemsa stain:1000x

also be related to failure of tubuline polymerization due to toxic effects (Fernandes *et al.*, 2007; Campos-Pereira *et al.*, 2011). The various morphological alterations such as pear shape erythrocytes, tear shape erythrocytes, dividing erythrocyte, microcyte, leptocyte and stomatocyte might be due to concentration dependent increase of lipid peroxidation products in erythrocytes of exposed fish to butachlor. Moreover, it can be hypothesized that such types of abnormal erythrocytes may undergone morphological alterations in their plasma membrane affecting surface deformability and makes erythrocytes

more susceptible to burst when crossing small capillaries. Previously it is well established that many toxicants having the oxidative stress potential may assault DNA, resulting in clastogenic, morphological and molecular damages (Jha, 2008).

**Conclusion:** From the results of present experimental study it can be concluded that butachlor poses serious clinico-hematological and mutagenic effects in fish and may be hazardous to the other species when those are exposed to this herbicide.

**Author's contribution:** AG and RH perceived the idea and AG, RH and MA carried out the research work. AK and RH examined the blood smears. RH and RZA analyzed the data. AG and MA wrote the manuscript, finally edited by AK and all authors approved it.

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