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Acute Toxicity of Hexavalent Chromium in Adult *Channa punctatus* (Bloch, 1793) with regard to changes in Erythrocytic and Leucocytic Profiles

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Abstract: **The tannery industries and drainage from urban and agricultural areas add chromium as one of the heavy metals to the aquatic environment and thereby imposing negative effects on the aquatic fauna. In the present study, the haematological alterations produced on short-term exposure to sublethal concentration of chromium (25 mg L^{-1}) were investigated in fresh water air-breathing fish, *Channa punctatus* (Bloch) for 24h, 48h and 72h respectively. The 96h LC_{50} of chromium salt, potassium dichromate was determined to be 34.59 mg/L. The results revealed statistically significant decrease in erythrocytic parameters like TEC (Total Erythrocytic Count), Hb (Haemoglobin) content and Haematocrit (Hct %) values in all experimental groups compared to the control with an increase of exposure periods. On the contrary, leucocytic parameters such as TLC (Total Leucocytic Count), Leucocrit (Lct %) and neutrophil populations were significantly increased during acute exposure. Acute lymphocytopenia was also recorded. The absolute corpuscular values like MCH (Mean Cell Haemoglobin) and MCHC (Mean Cell Haemoglobin Concentration) also exhibited significant increase in fishes of experimental groups while MCV (Mean Cell Volume) exhibited a fluctuating pattern. The depression of erythrocytic parameters clearly indicated that the fishes became anaemic due to acute toxicity of chromium.**

***Keywords: Channa punctatus*, hexavalent chromium, erythrocytic parameters, leucocytic parameters, neutrophilia, lymphocytopenia.**

I. INTRODUCTION

A wide range of adverse effects on freshwater fishes have been observed due to various heavy metals entering aquatic ecosystem through effluents which are regularly discharged from industries, tanneries, sewage treatment plants [1]. Heavy metals are important pollutants because they are not eliminated from the aquatic ecosystem by natural processes easily. The wastewater generated by tanneries is a major source of chromium (Cr^{6+}) and their indiscriminate introduction in the aquatic ecosystem pose a serious threat to the growth and survival of the fish population [2]. The chromium discharged into water get into food chain very easily from the environment. By entering into biological systems, it can perturb the biochemical processes leading to health abnormalities. A considerable amount of experimental data on chromium toxicity to aquatic life was reviewed [3] but the data on chromium toxicity to Indian freshwater air-breathing teleosts are scarce and are mostly limited to the effects on biochemical, immunological or enzymological profiles [4], [5]. Blood is an excellent bio-indicator which can be used as a sensitive index in understanding various physiological processes in fish. The present work was undertaken to investigate the short-term toxicity of chromium (Cr^{6+}) to adult freshwater spotted murrel, *Channa punctatus* (family: Channidae) with regard to the alterations in the erythrocytic parameters viz.TEC, Hb content, Hct %, MCH, MCHC and MCV and leucocytic parameters like TLC, Lct % and DWBC% (Differential count of WBC). The morphological abnormalities of erythrocytes were also analyzed under light microscopic study.

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II. MATERIALS AND METHODS

A. Collection of specimens and acclimatization

The adult fresh-water air-breathing *Channa punctatus* (Bloch) of both sexes (13.78 ± 0.33 cm in length and 39.36 ± 2.35 g in weight) were procured from clean and unpolluted local freshwater pond sources in Kolkata and transported to the laboratory of Department of Zoology, Maulana Azad College, Kolkata-700013, West Bengal. They were treated with 0.05% $KMnO_4$ solution for 2 minutes to avoid dermal infection, if any and kept in glass aquaria filled with clean dechlorinated tap water (pH: 7.2 ± 0.05; water temperature: 23 ± 2° C; total hardness: 225.8 ± 5 mg L^{-1}as $CaCO_3$; dissolved oxygen: 3.8 ± 1 mg L^{-1}) under continuous aeration. The fish were acclimatized for 5 days prior to experimentation. The physico-chemical parameters of tap water were monitored using standard procedures of APHA [6]. Fish were fed with commercial dry pellets during the acclimatization period only. Approximately 50 % of water in aquaria was renewed daily in order to remove unutilized food or metabolic waste products to minimize the level of ammonia excreted as well as maintaining the dissolve oxygen level.

B. Detection of LC50 dose

Analytical grade potassium dichromate ($K_2Cr_2O_7$) by BDH (India) was used as a metal toxicant in the present experiment and for the determination of LC_{50} dose of chromium at 96 h using probit analysis [7]. Six test concentrations of narrow range viz. 25, 30, 35, 40, 45 and 50 mg L^{-1} respectively and a control (without chromium) were selected to estimate the LC_{50} dose at 96 h. 8 fish specimens were placed in each of the aquarium and triplicates were maintained for each of the six treatment groups as well as for control. The required concentrations were prepared in distilled water and maintained in respective aquaria by renewing the water every day. Both sexes of fish were used during experiments. Dead fishes were removed from the aquaria immediately. The behavioural pattern and percentage of mortality was recorded at 96 h interval for each of the test concentrations. The LC_{50} value of $K_2Cr_2O_7$ for adult *C. punctatus* was estimated to be 34.59 mg L^{-1} at 96 h exposure period.

C. Experimental design

For short-term study, the live fish samples were divided into four groups each containing 8 individuals. One Group was kept as control, and other three groups were exposed to chosen sublethal dose of $K_2Cr_2O_7$ (25 mg L^{-1}) for 24, 48 and 72 h respectively after determining 96h LC_{50} value. The whole exposure medium was changed every day in both the control as well as treatment groups to maintain the desired concentration of chromium salt. After stipulated exposure periods, blood was collected from control and experimental fishes by severing the caudal peduncle of fish without using anaesthesia for haematological investigations. After collection the blood was immediately transferred to glass vials containing 3.8 % Sodium citrate solution (anticoagulant).

D. Estimation of erythrocytic and leucocytic parameters

Total Erythrocytic Count (TEC) and Total Leucocytic Count (TLC) were determined using Neubaur's improved double chamber haemocytometer (Fein-OPTIK, Blankenburg, G.D.R.) using Hayem's solution as diluting fluid. Haemoglobin (Hb) percentage was determined using Sahli's Haemometer. The Haematocrit and Leucocrit values were estimated by microhaematocrit method using Wintrobe's tube [8]. Differential Count (DWBC %) of leucocytes was made by staining thin air-dried blood film with Leishman's stain. The count of leucocytes and morphology of erythrocytes were observed under oil immersion magnification in a research microscope (Magnus MLX-DX, Olympus India Pvt. Ltd.). The red cell indices, such as Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Cell Haemoglobin Concentration (MCHC) were calculated from TEC, Hb % and Haematocrit values using standard formulae [9], [10].

E. Statistical analysis

The results were expressed as mean and standard error (mean ± S.E.). Student's *t*- test was used to distinguish between means of significant differences [11]. Treatments were taken to be significantly different where $P < 0.05$ and highly significant where $P < 0.01$.

III. RESULTS

The majority of the fishes exposed to sublethal concentration (25 mg L^{-1}) of $K_2Cr_2O_7$ especially for 48 and 72 h exhibited abnormal behaviour like loss of equilibrium, erratic swimming and loss of appetite. The exposed fishes were found to swim to the water surface and excess mucous secretion was observed from the body. The fishes also stood motionless in the bottom of the aquarium, hanging in a vertical orientation.

In the present study, significant alterations in the haematological parameters were observed in fishes exposed to sublethal concentration of chromium during the exposure period, viz. 24, 48 and 72 h along with some morphological changes in RBC of exposed fishes. The TEC ($\times 10^6$ mm^{-3}) and haemoglobin values (g %) exhibited a highly significant decrease ($P < 0.01$) especially after 48 h and 72 h exposure periods compared to control whereas the Haematocrit values exhibited a sharp decline ($P < 0.01$) from the onset of the exposure period compared with control (TABLE I). As shown in TABLE II, MCV values were significantly decreased ($P < 0.05$) after 24 h exposure but a sharp increment was observed after 72 h ($P < 0.05$). There were significant fluctuations in the MCH values throughout the exposure periods but after 72 h of exposure, a significant increase in the value ($P < 0.05$) was found. MCHC values were found to be very high ($P < 0.01$) especially after 24 and 48 h of exposure periods.

Several cellular and nuclear abnormalities of peripheral RBCs were also studied in fish population exposed to sublethal dose of chromium. Three types of nuclear abnormalities viz. nuclear extrusion (Fig.1), notched nuclei (Fig.2) and bilobed nuclei (Fig.3) were observed along with four types of cellular abnormalities such as spindle-shaped cells (Fig.3), budding erythrocytes (Fig.1), vacuolated cells (Fig.4) and deformed erythrocytes (Fig.5). These morphological abnormalities were found to increase after 72 h of exposure.

Highly significant increase ($P < 0.01$) in TLC ($\times 10^3$ mm^{-3}) and Lct % values were observed initially and at the end (TABLE III). A significant decrease ($P < 0.05$) of both leucocytic parameters was observed in the middle of the exposure periods. The differential count of leucocytes showed highly significant alterations throughout the exposure periods (TABLE IV). A gradual increase in neutrophil and monocyte population was observed throughout the experimental period which was highly significant ($P < 0.01$) after 72 h compared to control. On the contrary, a significant decrease ($P < 0.05$) was observed in eosinophil (EO) population especially after 48 h of exposures. The populations of large lymphocytes (LL) and small lymphocytes (SL) also showed the opposite trend to that of the neutrophil (NEU). Both lymphocytes (SL and LL) exhibited a steep decline ($P < 0.01$).after 72 h of exposure (Lymphocytopenia). No significant deviation was found in the basophil cell population (B) throughout the short-term exposures.

IV. DISCUSSION

The present study reveals that the fishes exposed to hexavalent chromium showed significant decrease in erythrocytic parameters during experimental period. This result indicates anaemic condition associated with erythropenia. The reduction in TEC coupled with low haemoglobin count may be due to disruptive metabolic and haemopoietic activities of the fish exposed to sublethal concentration of $K_2Cr_2O_7$. The incidence of erythropenia in fishes under heavy metal exposure was reported earlier [12], [13], [14].The impairment of haemopoietic organs by heavy metals resulted slower erythropoiesis and subsequent reduction in TEC [15], [16], [17], [18]. The reduced RBC count coupled with low haemoglobin count also may be due to destructive action of chromium on erythrocytes. The damage of RBC in *Catla catla* exposed to $K_2Cr_2O_7$ was described [19]. Accumulation of several heavy metals in kidney, the major erythropoietic organ in fishes has been reported by various authors [20], [21]. Haemolysis of RBC after dichromate exposure in a marine fish, *Dicentrachus labrax* was reported [22]. Drop in TEC and haemoglobin percentage were also reported in the fish, *Heteropneustes fossilis* under exposure of nickel sulphate for 15 days [23]. Impairment of iron uptake in the intestine and defective iron metabolism might be one of the reasons behind the reduced percentage of haemoglobin in the experimental fishes exposed to sublethal doses of heavy metals [24]. The present study is in the line with the earlier works which suggests that the decrease in the level of TEC, haemoglobin and haematocrit value is due to the haemodilution mechanism because of gill damage or impaired osmoregulation and increased destruction in the circulating RBCs [25], [26], [27], [28], [29], [30], [31]. Contrastingly, significant increase in TEC and haemoglobin content has been reported after exposure to copper in *Cyprinus carpio* [32] which suggested that hypoxic condition induced by heavy metal accumulation stimulated the spleen for erythropoiesis and release of stored and immature erythrocytes into the circulation [33].

The anaemic condition of the experimental fish groups were further detected by Haematocrit study. Significant changes were recorded in the mean value of MCV, MCH and MCHC and similar results have been reported in *Labeo rohita* which was exposed to chromium [34]. Cells released from the affected spleen would have lowered MCV values initially, as observed in the present study. This present result is well in agreement with the observation [35], which suggested that the initial decrease in the MCV value may be due to high percentage of immature RBCs in the circulation released from the spleen. A similar kind of observation was reported in *Cyprinus carpio* after cadmium exposure [36]. However, an increasing trend on the MCV and MCH values were more or less observed in the present study. These haematological alterations might be due to haemopoietic or erythrocyte mobilization response to hypoxia induced by heavy metals [14]. Moreover, the elevation in MCV and MCH value may also be due to release of large erythrocytes into the circulation [37], [38]. The observed low concentration of MCHC after 48 and 72 h indicates a decrease in haemoglobin synthesis due to toxic action of $K_2Cr_2O_7$.

Increase in the TLC and Leucocrit (Lct %) values as observed in present study might be due to the adaptation to meet stressful toxic condition by exposed organisms. This observation is in agreement with the earlier reports [39], [40], [41],[42],[43]. In the present experiment, leucocytes showed an initial rise due to elevated levels of neutrophil and monocytes followed by leucopenia (especially lymphocytopenia) after 48 h of chromium exposure. This observation is in the line with the earlier observation [12] where similar kind of results were reported in leucocytes of teleosts under the exposure of sublethal dose of chromium. The increase in number of WBCs appears to be associated with the increased circulatory levels of granulocytes, especially neutrophils, which may play an important role in immunological defence system during heavy metal exposure [33]. This observation suggests the development of certain degree of tolerance during toxicant stress condition. The elevation in neutrophil population (neutrophilia) can be correlated with the earlier findings [44] where *Brycon amazonica* were exposed to low concentration of phenol in laboratory condition. This condition indicates hypersensitivity to toxic effluents [45]. On the contrary, the drop in lymphocyte population is probably due to the mobilization of lymphocytes from the peripheral blood to accumulate in the lymphoid tissue [45], [46]. According to some other reports, this decrease in lymphocyte count can also be correlated with the elevated level of corticosteroid hormone, whose secretion is a nonspecific immune response in fishes to any environmental stressor [47], [48].

7 types of erythrocytic abnormalities were observed in the present study. The increase in frequency of erythrocytic abnormalities depends upon the exposure time of heavy metal toxicant. In intracellular condition, chromium undergoes reduction from Cr^{+6} to Cr^{+3} and generates reactive oxygen species (ROS) as highly reactive free radicals which react and disrupt the DNA. Similar works reported by other authors suggested that the blebbed and lobed nuclei are caused by nuclear budding during interphase and this entire mechanism occurs probably by elimination of amplified genes from the nucleus [49], [50]. These abnormalities may also be raised due to formation of free radicals under oxidative stress by heavy metal toxicants [51]. According to some previous reports, binucleated cells and notched nuclei are formed by the aneugenic effect of heavy metals which exerts their action by creating aneuploidy which is produced due to failure in tubulin aggregation [52],[53]. The abnormal shapes of erythrocytes are probably produced by hypoxic condition which results in depression of ATP [54].The vacuolated condition of erythrocytes is most probably due to the interruption in the lipid solubility of erythrocyte membranes caused by heavy metals, ultimately leading to the apoptosis [55]. These changes in erythrocytes of fishes induced by chromium are non-reversible and these cytotoxic damages lead to the mortality of fishes.

V. CONCLUSION

Fish haematology has now become an important biomonitoring tool for the assessment of aquatic pollution as it creates a direct link between the environmental condition and physiological status of organisms. In present study, changes observed in the behavioural and haematological parameters of adult *C. punctatus* exposed with chosen sublethal dose (25 mg L^{-1}) of hexavalent chromium were described. The non-specific immune responses to heavy metal over the short-term exposure periods can be interpreted as initial increase in WBC, perhaps due to the increase in circulating neutrophils and monocytes coupled with stress-mediated reduction in leucocytes (especially lymphocytes) in the middle. The depression in most of the erythrocytic parameters was observed which possibly reflect the relative magnitude of stress due to exposure of chromium. The result obtained from this study clearly indicates that the industrial effluents containing heavy metals have

potential to alter the physiological as well as biochemical processes in the exposed organisms. The formation of erythrocytic abnormalities also reveals the genotoxic potential of chromium. Thus, these haematological parameters can be treated as good biomarker for monitoring the impact of industrial effluents containing heavy metals in aquatic environment. Being an edible freshwater fish, there might be a chance of accumulation of heavy metals in many non-target organisms mainly in human. Thus, a continuous monitoring with regard to the discharge of the industrial effluents into the water body is mostly important and it is suggested that the effluents should be passed through the treatment plant before being discharged into the aquatic body.

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APPENDICES - A

TABLE I: Changes in erythrocytic parameters (TEC, Hb and Hct) of freshwater fish, *Channa punctatus* exposed to sublethal dose of hexavalent chromium (25 mg L^{-1}).

Erythrocytic parameters	Control	Exposure period		
		24h	48h	72h
TEC ($\times 10^6$ mm^{-3})	3.07 ± 0.11	2.66 ± 0.41	2.15 ± 0.03 **	1.43 ± 0.13**
Hb (g %)	11.3 ± 0.35	10.12 ± 0.47	8.63 ± 0.46**	7.5 ± 0.11**
Hct (%)	24.43 ± 0.04	15 ± 0.73**	14 ± 0.57**	14.25 ±0.65**

Values are expressed as Mean ± S.E., n = 8, ** = highly significant at *P* < 0.01 level. TEC = Total Erythrocytic Count, Hb = Haemoglobin,

Hct = Haematocrit.

TABLE II: Changes in erythrocytic parameters (MCV, MCH and MCHC) of freshwater fish, *Channa punctatus* exposed to sublethal dose of hexavalent chromium (25 mg L^{-1}).

Erythrocytic parameters	Control	Exposure period		
		24h	48h	72h
MCV (fl)	79.91 ± 2.25	63.21 ± 3.02*	65.16 ± 9.02	102.88 ± 7.58*
MCH (pg)	37.09 ± 2.08	45.53 ± 6.86	40.22 ± 2.47	54.92 ± 5.41*
MCHC (gL^{-1})	46.28 ± 1.42	68.55 ± 5.0**	61.81 ± 2.79**	53.24 ± 2.81

Values are expressed as Mean ± S.E., n = 8. * = significant at *P* < 0.05 level, ** = significant at *P* < 0.01 level. MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Cell Haemoglobin Concentration.

TABLE III: Changes in leucocytic parameters (TLC and Lct %) of freshwater fish, *Channa punctatus* exposed to sublethal dose of hexavalent chromium (25 mg L^{-1}).

Erythrocytic parameters	Control	Exposure periods		
		24h	48h	72h
TLC ($\times 10^3$ mm^{-3})	32.33 ± 0.73	47.75 ± 0.66**	37.54 ± 1.32*	58.12 ± 1.04**
Lct (%)	1.48 ± 0.01	1.75 ± 0.03**	1.45 ± 0.08*	2.9 ± 0.18**

Values are expressed as Mean ± S.E., n = 8.* = significant at p < 0.05 level, ** = significant at p < 0.01 level. TLC: Total Leucocytic Count, Lct: Leucocrit.

TABLE IV: Changes in the relative population of leucocytes (DWBC %) of freshwater fish, *Channa punctatus* exposed to sublethal dose of hexavalent chromium (25 mg L^{-1})

DWBC%	Control	Exposure Periods		
		24 h	48 h	72 h
NEU	34.55 ± 2.38	35.31 ± 1.13	38.52 ± 0.91	47.66 ± 2.93**
EO	1.88 ± 0.17	2 ± 0.11	1.4 ± 0.17*	1.84 ± 0.22
B	3.8 ± 0.49	3.97 ± 0.36	3.56 ± 0.12	3.1 ± 0.29
LL	18.09 ± 3.32	11.5 ± 0.86	13.16 ± 1.32	6.64 ± 0.39**
SL	39.92 ± 6.17	33.75 ± 2.14	34.62 ± 2.01	28.83 ± 3.14**
M	9.65 ± 0.66	10.81 ± 0.96	14.23 ± 1.64**	17.29 ± 1.08**

Values are expressed as Mean ± S.E., n = 8. * = significant at p < 0.05 level, ** = significant at p < 0.01 level. NEU: Neutrophil, EO: Eosinophil, B: Basophil, LL: Large Lymphocyte, SL: Small Lymphocyte, M: Monocyte.

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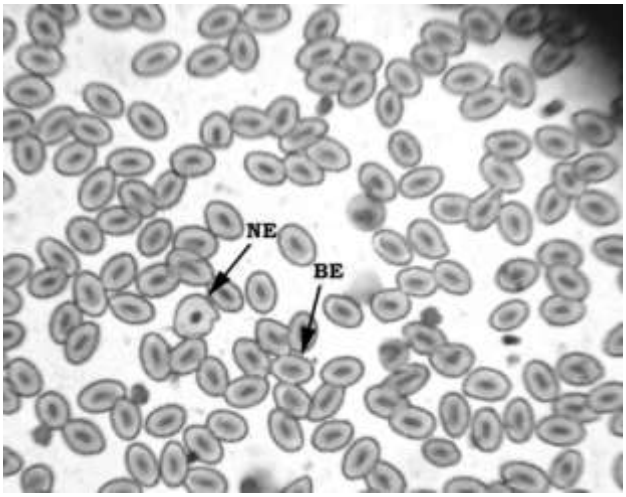

Fig 1: Peripheral blood smear (× 450) of *C. punctatus* exposed to sublethal dose (25 mg L^{-1}) of chromium (VI) at 72 h showing nuclear extrusion (NE) and budding erythrocytes (BE)

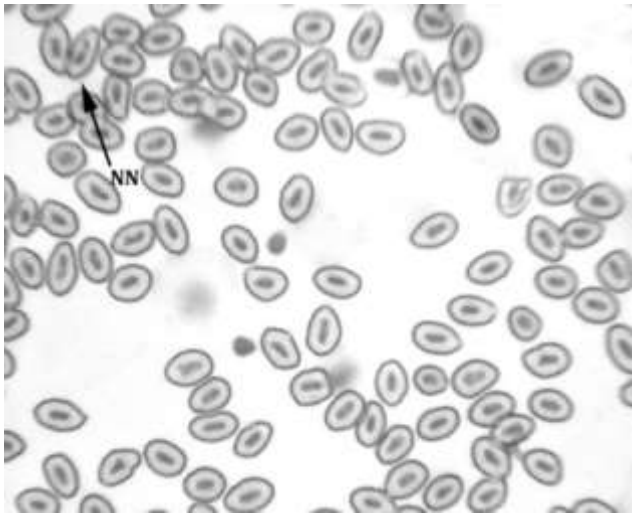

Fig 2: Peripheral blood smear (× 450) of *C. punctatus* exposed to sublethal dose of chromium (VI) at 72 h showing notched nucleus (NN)

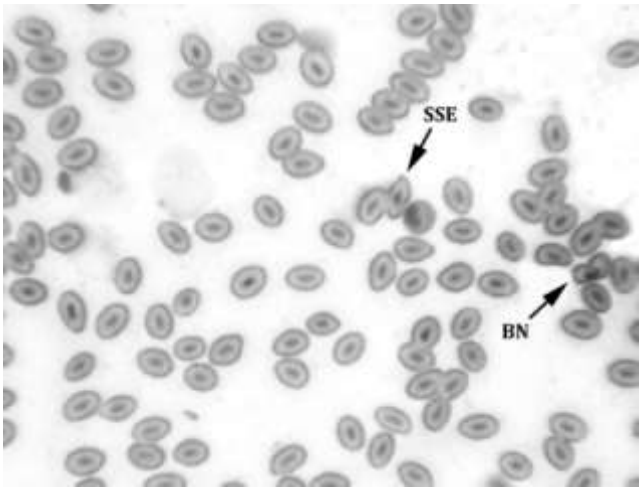

Fig 3: Peripheral blood smear (× 450) of *C. punctatus* exposed to sublethal dose of chromium (VI) at 72 h showing spindle-shaped erythrocyte (SSE) and bilobed nucleus (BN)

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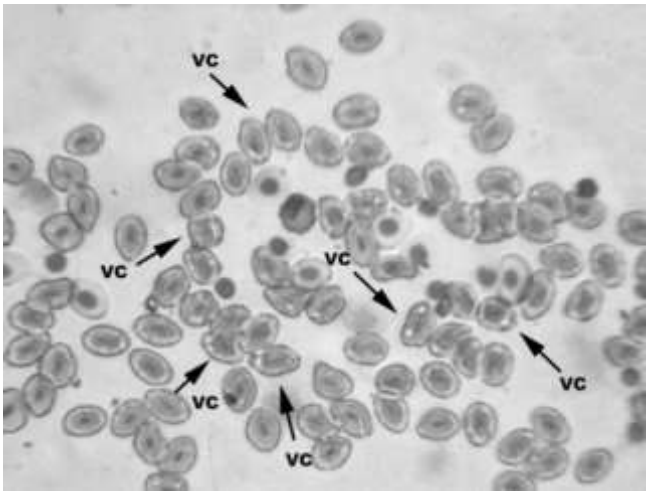

Fig 4: Peripheral blood smear (× 450) of *C. punctatus* exposed to sublethal dose of chromium (VI) at 72 h showing vacuolated cells (VC)

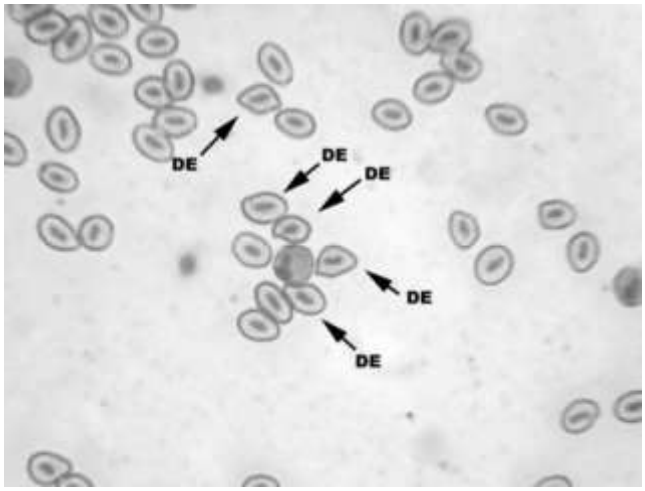

Fig 5: Peripheral blood smear (×450) of *C. punctatus* exposed to sublethal dose of chromium (VI) at 72 h showing deformed erythrocytes (DE)