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Light and Scanning Electron Microscopic Studies on Chromium-Induced Anemia in a Murine Model

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Abstract Blood hemoglobin level, hematocrit value and erythrocyte count were reduced by 17.5, 17.4 and 15.9%, respectively, as compared to the controls, in Swiss mice treated intraperitoneally with hexavalent chromium (4 mg of potassium dichromate per Kg for 5 day per week) for 2 weeks. Echinocytic transformation of 33.8% erythrocytes, as revealed by both light and scanning electron microscopy, indicated the anemia to be hemolytic in nature. Leucopenia was apparent after 2 weeks (mean leucocyte count: 4.91 thousand c mm^{-1}), but not 1 week of treatment (mean count: 6.43 thousand c mm^{-1}), However, cytochemical studies indicated that chromium did not interfere with iron utilization for hemoglobin synthesis and also, did not cause denaturation of already synthesized hemoglobin. The study hints to the necessity of periodic monitoring of blood in workers of chromium-dependent tanneries of Kolkata, India.

Keywords Chromium · Anemia · Echinocyte · Leucopenia

The heavy metal chromium in the form of its hexavalent salts has long been marked as an environmental pollutant by toxicologists as well as physiologists. Hexavalent chromium has been identified as a potent inorganic carcinogen. Lung cancer has often been found to affect the workers of different chromium-based industries (Mendelson 1994). Several reports indicate strong clastogenic (chromosome damaging) and mutagenic potentials of hexavalent chromium salts, and such actions have been considered to be responsible for the carcinogenic effect of chromium. The clastogenic potential in chromium salts was confirmed by the observation of chromosomal aberrations in cultured human lymphocytes (Stella et al. 1982) and in bone marrow of mice treated with chromium in drinking water (Goldina et al. 1989). A clastogenic effect of chromium has also been indicated by the formation of micronuclei in mouse bone marrow and blood cells (De Flora et al. 2006). Hexavalent chromium has also been reported to exert considerable hepatotoxic and nephrotoxic actions in animal models (Krishbaum et al. 1981; Ueno 1992). It has also been reported to adversely affect the reproductive function in both sexes of laboratory mice (Elbtieha and Al-Hamood 1997) and oocyte maturation as well as survival in fish (Mishra and Mohanty 2005).

However, little information is available on the effect of hexavalent chromium on the blood picture of animals, while some other heavy metals like arsenic and lead are known to cause marked anemia in laboratory mice (Guha and Sarkar 2000; Morse et al. 1972). The present study involves a thorough examination of the blood picture of a murine model chronically treated with hexavalent chromium, in order to determine if chromium causes anemia or other hematological disorders.

Different hematological parameters like blood hemoglobin level, total counts of erythrocytes and leucocytes, hematocrit value and differential counts of leucocytes have been compared between the experimental (chromium-treated) and the control groups of mice. Moreover, both light and scanning electron microscopy have been exploited to study chromium-induced changes in erythrocyte surface topography. Cytochemical staining techniques have been used to determine if chromium causes denaturation of

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hemoglobin and interferes with iron utilization for hemoglobin synthesis. Further, some human blood samples were incubated in the presence of either hexavalent chromium or physiological saline to determine the immediate effect of chromium on the morphology of human erythrocytes.

The rationale behind undertaking the present study is that hexavalent chromium appears to be a major environmental pollutant in the tannery-belt of the Greater Kolkata, India. An indiscriminate discharge of a huge quantity of chromium-rich effluent by the tanneries in the Bantala Leather Complex, where the construction of suitable chromium-treatment plants is still pending, may adversely affect the health of the tannery workers as well as the people living around the tanneries (Singh 2011). It is, therefore, necessary to obtain as much information as possible on any adverse physiological effects of hexavalent chromium.

Materials and Methods

Adult (8 weeks old) inbred and healthy, male, albino mice of the Swiss strain were divided into control and experimental groups. Only male mice were taken in view of the fact that the tannery workers of our state are generally males. The mice were maintained in polypropylene cages with sawdust bedding in a well-ventilated and well-lighted animal room under strict vigil of an Institutional Animal Ethical Committee, which in turn is registered with the Committee for the Purpose of Control and Supervision on Experiments on Animals, Ministry of Environment and Forest, Government of India (Registration No. 796/03/ac/CPCSEA). The mice were provided with food pellets and filtered tap water ad libitum. The experimental mice were intraperitoneally injected with an aqueous solution of potassium dichromate ($K_2Cr_2O_7$) (Merck, Germany; purity: 99.5%) at a dose of 4 mg Kg^{-1} body weight for 5 day per week, for a total period of 2 weeks. The selected dose of $K_2Cr_2O_7$ was nearly one-tenth of its LD_{50} value (39 mg Kg^{-1}) for mice (Ueno 1992). The control mice were injected with physiological saline (0.9%) for a similar tenure.

After 1 and 2 weeks of chromium treatment, 5 experimental and 5 control mice were anaesthetized under ether vapor and blood samples were collected in heparinized vials by cardiac puncture. Blood hemoglobin (Hb) was determined by the acid-hematin method; total counts (TC) of erythrocytes and leucocytes were determined by refined visual methods using a hemocytometer having an improved Neubauer counting chamber, while hematocrit (Ht) values of blood samples were determined by the capillary method (Dacie and Lewis 1984). Differential counts (DC) of leucocytes were determined from microscopic examination of blood smears fixed in methanol and stained with 10% Giemsa's stain buffered to pH 7.0 with 0.1 M phosphate buffer (Dacie and Lewis 1984). Erythrocyte morphology was examined under the oil-immersion objective of a microscope and the abnormal morphs of erythrocytes, whenever observed were named as per an international system of nomenclature of erythrocytes (Bessis et al. 1986). Some of the methanol-fixed but unstained blood smears were subjected to acid-ferrocyanide reaction for microscopic detection of non-heme iron granules within erythrocytes (Dacie and Lewis 1984), while others were subjected to crystal violet staining for detection of denatured hemoglobin (Heinz bodies) within erythrocytes (Beutler 1986).

For scanning electron microscopic study of erythrocyte surface topography, thin smears of heparinized blood samples drawn over clean cover-slips, were fixed in 2% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.0, at 4°C for 4 h, followed by a thorough washing in the buffer alone (Hayat 1975). The cover-slips were air-dried, cemented on metal stubs with a silver-adhesive, coated with a 100Å thick layer of gold by the help of an ion-coater and observed under a Hitachi S-520 Scanning electron microscope at an accelerating voltage of 15 kV.

Moreover, in order to examine the immediate effect of hexavalent chromium on human erythrocyte morphology in vitro, blood samples donated by the present authors and four of their students were incubated with $K_2Cr_2O_7$ in Eppendorf tubes for 2 h at 37°C. The experiment was designed after Winski and Carter (1998) who incubated human blood samples with arsenic salts for examining arsenic effect on erythrocyte surface topography. Each blood sample was used for a 'control' and an 'experimental' incubation. A 'control' incubation mixture contained 20 μL of blood and 80 μL of physiological saline (0.9%). On the other hand, an 'experimental' incubation mixture contained 20 μL of blood and 80 μL of 5 mM $K_2Cr_2O_7$ made isotonic with physiological saline (by adding 147 mg of $K_2Cr_2O_7$ and 753 mg of sodium chloride in 100 mL of deionized water). The incubated blood samples were smeared on glass slides, fixed in methanol and stained with 10% Giemsa's stain for microscopic examination.

All reagents used in the study were of analytical grade. All experiments were performed in triplicate, and significant differences of blood parameter means between the control and experimental (chromium-treated) groups of mice (results of all 3 sets of experiments were considered together) were determined by Fisher's two-tailed t test (Goon et al. 1981).

Results and Discussion

At the end of the first week of $K_2Cr_2O_7$ treatment, blood Hb level, Ht value and TC of erythrocytes were found to be

considerably decreased in the experimental mice, as compared to those in the controls ($p < 0.001$ in all cases). Moreover, a conspicuous change was noted in the morphology of many of the erythrocytes, which transformed into the so-called 'echinocytes' with crenated cell membranes (Fig. 1). The erythrocytes of the control mice were almost exclusively round, discoid 'normocytes' having smooth cell membranes (Fig. 2). Scanning electron microscopic examination of erythrocytes under high magnification (2,000×) revealed that the echinocytes had 8–14 short projections from their cell membranes (Fig. 3), while the normal erythrocytes were simply biconcave discs (Fig. 4). Such echinocytes with short projections were designated as 'type I' echinocytes at par with the standard system of nomenclature of erythrocytes (Bessis et al. 1986). Both light and scanning electron microscopy revealed the transformation of a small percentage of erythrocytes into the so-called 'poikilocytes' looking like tennis rackets (Figs. 1, 3). A small number of deeply stained, basophilic reticulocytes were also observed (Fig. 1). However, at the end of the first week, TC and DC of leucocytes did not differ significantly between the experimental and the control groups of mice ($p > 0.05$ in either case; Table 1).

At the end of the second week of $K_2Cr_2O_7$ treatment, further reductions in blood Hb level, Ht value and TC of erythrocytes were noted. The percentages of echinocytes, poikilocytes and reticulocytes had increased considerably from week 1 (Table 1). Moreover, anisocytosis, or erythrocyte size heterogeneity, was quite apparent; microcytes (4–5 μm in diameter) were scattered among normocytes (7–8 μm in diameter) in blood smears (Fig. 1).

Interestingly, cytochemical studies (acid-ferrocyanide reaction and crystal violet staining) did not reveal the presence of either non-heme iron granules or denatured hemoglobin in erythrocytes of the experimetnal mice after both 1 and 2 weeks of $K_2Cr_2O_7$ treatment. On the other hand, human blood samples incubated with 5 mM $K_2Cr_2O_7$ for 2 h revealed a shrunken and deformed appearance of more than half of the erythrocytes (Fig. 5).

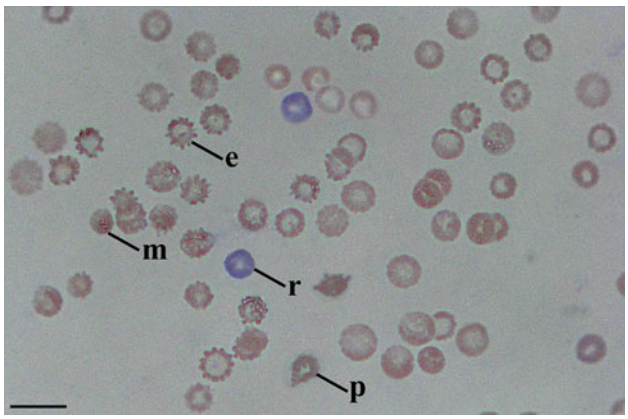

Fig. 1 Echinocyte (e), poilkilocyte (p), microcyte (m) and reticulocyte (r) in blood smear of a chromium-treated mouse. (×1,000, Bar = 15 μm)

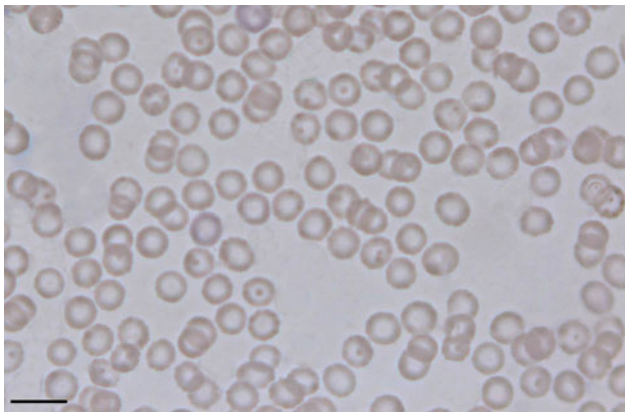

Fig. 2 Normocytes in blood smear of a control mouse. (×1,000, Bar = 15 μm)

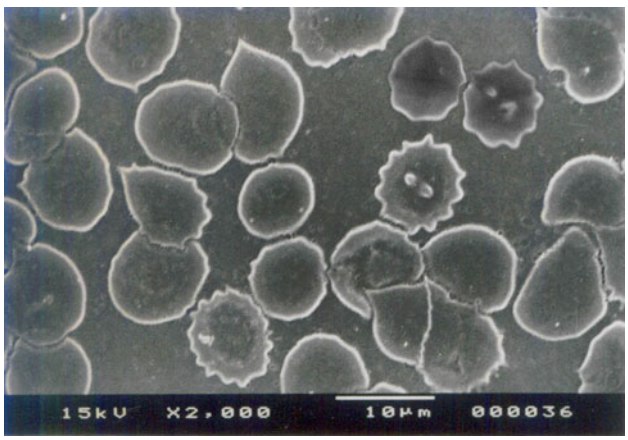

Fig. 3 Echinocyte, poilkilocyte and microcyte (under scanning electron microscope) in blood smear of a chromium-treated mouse. (×2,000, Bar = 10 μm)

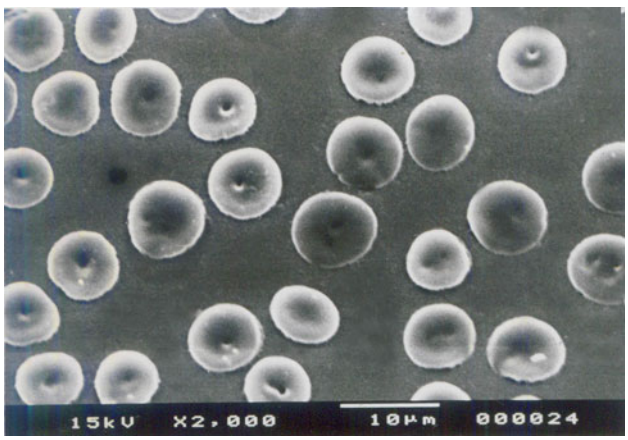

Fig. 4 Normocytes (under scanning electron microscope) in blood smear of a control mouse. (×2,000, Bar = 10 μm)

Table 1 Hematological findings in $K_2Cr_2O_7$-treated and control Swiss mice (15 mice per group)

Blood parameter under study	Groups of mice		Groups of mice	
	1 week after treatment	Respective controls	2 weeks after treatment	Respective controls
Blood Hb (mg dL^{-1})	10.52 ± 0.34*	12.41 ± 0.43	10.25 ± 0.41*	12.42 ± 0.32
Erythrocyte (million c mm^{-1})	5.01 ± 0.33*	5.64 ± 0.27	4.76 ± 0.23*	5.66 ± 0.35
Ht value	0.42 ± 0.01*	0.46 ± 0.01	0.38 ± 0.01*	0.46 ± 0.01
Echinocyte (%)	25.57 ± 3.46*	0.28 ± 0.06	33.85 ± 5.23*	0.23 ± 0.07
Poikilocyte (%)	3.37 ± 1.06*	0	4.60 ± 1.06*	0
Microcyte (%)	5.76 ± 2.32*	2.43 ± 0.73	10.28 ± 2.37*	2.57 ± 0.73
Reticulocyte (%)	2.80 ± 0.75*	0.25 ± 0.05	3.40 ± 1.02*	0.22 ± 0.07
Leucocyte (thousand c mm^{-1})	6.47 ± 0.43	6.55 ± 0.51	4.91 ± 0.28*	6.43 ± 0.34
Lymphocyte (%)	75.50 ± 2.99	76.83 ± 2.11	84.33 ± 2.87*	75.83 ± 2.41
Neutrophil (%)	19.67 ± 2.87	17.67 ± 2.43	10.67 ± 3.14*	19.00 ± 3.26
Monocyte (%)	3.50 ± 0.50	3.67 ± 0.74	3.67 ± 0.74	3.67 ± 0.74
Eosinophil (%)	1.33 ± 0.47	1.83 ± 0.69	1.33 ± 0.47	1.50 ± 0.50
Crystal violet staining	−ve	−ve	−ve	−ve
Acid-ferrocyanide staining	−ve	−ve	−ve	−ve

* Statistically significant from respective controls ($p < 0.001$)

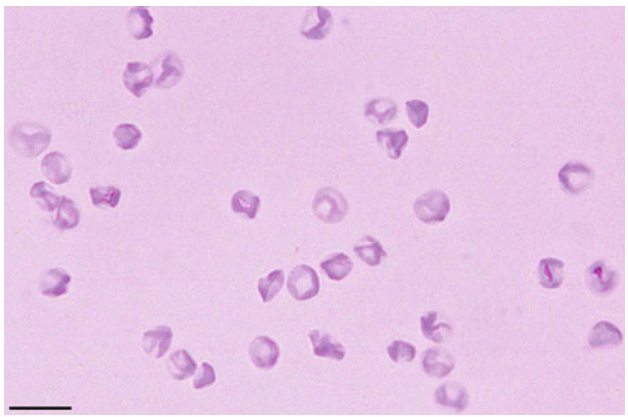

Fig. 5 Shrinkage and deformation of human erythrocytes following incubation with $K_2Cr_2O_7$. (×1,000, Bar = 15 μm)

The findings of decreased blood Hb level and erythrocyte count clearly indicate that hexavalent chromium induces anemia in the murine model under study. Echinocytic transformation of a large proportion of erythrocytes along with the finding of a few poikilocytes indicate that chromium induces deformation and fragility of erythrocytic membranes, resulting in hemolysis; echinocytosis is considered by modern hematologists to be an indicator of hemolysis to follow (Firkin et al. 1990). The presence of a few reticulocytes along with some microcytes in blood smears indicates that the treated mice may have attempted to compensate for the loss of erythrocytes by enhancing the rate of erythropoiesis, but did not have time to synthesize sufficient quantities of Hb. This possibly resulted in the formation of microcytes instead of normocytes.

The negative results obtained with cytochemical tests, on the other hand, indicate that chromium probably does not interfere with iron utilization for hemoglobin synthesis, and also does not cause any denaturation of already synthesized Hb. The present study further reveals that chromium gradually exerts a cytotoxic action on both lymphopoietic and myelopoietic tissues, resulting in a marked leucopenia in the treated mice. However, the bone marrow may be more sensitive than the lymphopoietic tissues to the cytotoxic action of chromium, resulting in a marked depletion in the DC of neutrophils along with an apparent but not absolute rise in the DC of lymphocytes. Additionally, the shrunken appearance of human erythrocytes incubated in presence of $K_2Cr_2O_7$ adds support to the ability of chromium to deform mammalian erythrocytes, in general.

In conclusion, it may be stated that the present findings on anemia in a murine model following chronic treatment with hexavalent chromium, strongly hints to the necessity of periodic monitoring of blood from tannery workers of our state, who are continually being exposed to chromium on account of their occupation.

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