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# Effects of dengue virus infection on peripheral blood cells of mice exposed to hexavalent chromium with drinking water

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***Background & objectives*: The occupational and non-occupational exposure to hexavalent chromium Cr (VI) is common. The effect of chromium compromises the immune response of the host. Dengue virus (DV) infection causes various changes in the peripheral blood cells. It is, therefore, possible that the chromium toxicity may affect the disease process during DV infection. The present study aims to study the effects of dengue virus infection on peripheral blood cells of mice fed Cr (VI) with drinking water.**

***Methods*: One group of mice was given *ad libitum* drinking water containing Cr (VI) and the other group used as the normal control mice was given plain water to drink. At the 3, 6 and 9 wk of Cr (VI) drinking, a set of mice from each group was inoculated intracerebrally (ic) with DV and studied at the 4th and 8th day post inoculation.**

***Results*: It was observed that Cr (VI) drinking led to reduction in lymphocytes, haemoglobin and the haematocrit values while the granulocyte, monocyte and platelet counts were increased. On the other hand, most of the parameters were decreased following inoculation of normal mice with DV. In Cr (VI)-fed mice the effects of DV infection were minimal. The most significant finding of these experiments was that the reduction in platelet counts following inoculation with DV was markedly less in Cr (VI)-fed mice than that in DV-inoculated normal control mice.**

***Interpretation & conclusion*: Cr(VI) compounds have been declared as a potent occupational carcinogen. On the contrary, Cr(III) salts such as chromium polynicotinate, chromium chloride and chromium picolinate, are used as micronutrients and nutritional supplements, and have been shown to exhibit health benefits in animals and humans. Whether therapeutic doses of chromium (III) compounds may be able to prevent the DV-induced fall in platelet counts, needs to be investigated.**

**Key words** Blood cells - Cr (VI) - dengue virus - leucocytes - lymphocyte - monocyte - platelet - polymorphonuclear cells - RBC

Hexavalent chromium [Cr (VI)] occurs naturally in the environment and is widely used in paints, metal finishes, steel including stainless steel manufacturing, alloy cast irons, chrome and wood treatment and leather tanning. Cr (VI) ingested with food such as vegetables, meat, *etc*., is reduced to Cr (III) before entering the blood stream. Cr (VI) is highly toxic to all forms of living organisms[1-3]. Chromium enters the body through the lungs, gastrointestinal tract, and to a lower extent through skin[4]. Non-occupational

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exposure occurs via ingestion of chromium containing food and water[5]. Regardless of route of exposure, Cr(III) is poorly absorbed whereas Cr(VI) is more readily absorbed[4]. Cr (VI) compounds that enter the bloodstream are transported into red blood cells (RBCs) via sulphate anion channels[6]. Cr(VI) is rapidly reduced to Cr(III) via unstable intermediates [Cr(V) and Cr(IV)] inside the RBC. The reduced product binds to haemoglobin and other intracellular proteins, resulting in elevation of total chromium levels in the RBC fraction of blood for several weeks[6-8]. The main routes for the excretion of chromium are via kidney/urine and the bile/feces[5,9].

During dengue virus (DV) infection various changes occur in the peripheral blood cells. The total leucocyte count in patients with dengue infection varies from mild leucopaenia to moderate leucocytosis with predominance of lymphocytes. The haematocrit value increases during the first three days but gradually decreases from day four to nine. The platelet count is usually normal during the first three days. Thrombocytopaenia in more than 80 per cent cases begins during febrile stage and reaches its lowest value during the haemorrhagic phase (dengue haemorrhagic fever, DHF) of illness[10]. Thrombocytopaenia is one of the simple diagnostic criteria proposed by World Health Organization (WHO) for diagnosis of DHF[11]. Constant haematological abnormalities occurring in DHF include bone marrow suppression, leucopaenia and thrombocytopaenia[12]. DV does not produce any clinical illness in any animal species and there is no animal model similar to human DHF. In absence of such a model, inoculated mice have been used extensively to answer several questions related to dengue infection[13-18].

DV infection is endemic all over India so is the occupational and non-occupational exposure to Cr (VI). The effect of chromium compromises the immune response of the host[20]. It is, therefore, possible that the chromium toxicity may affect the disease process during DV infection. The present experiments were, therefore, conducted to study the effects of dengue virus infection on peripheral blood cells of mice fed Cr (VI) with drinking water.

## Material & Methods

*Animals*: The study was carried out on Swiss mice weighing 25-30 g, aged 6-8 wk obtained from the animal breeding facility of the Industrial Toxicology Research Centre, Lucknow. Clearance from the Animal Ethical Commettee of the Institute was obtained for the use of animals. Mice were maintained on pellet diet.

*Plan of study*: One group of mice (n=72) were given *ad libitum* drinking water containing Cr (VI) and the second group (n=24) was given plain water to drink (Fig.1). At the 3, 6 and 9 wk of Cr (VI) drinking, a group of mice (12 animals in each group) were inoculated ic with dengue virus in doses of 1000 $LD_{50}$[13]. The mice were killed in groups at days 4 (6 animals) and 8 (6 animals) post virus inoculation (pi) and various investigations were done.

*Chromium treatment of mice*: Each group with 6 mice was given *ad libitum* drinking water containing 250 ppm chromium (VI) in form of potassium dichromate for 3, 6 and 9 wk. Oral $LD_{50}$ values for Cr(VI) compounds was 300 mg/kg for potassium chromate in the mouse[19]. From the daily consumption of water it was found that the average daily intake of Cr (VI) per mouse was 14.8 mg/kg.

*Dengue virus*: Dengue type 2 virus (DV), strain P23085 (kindly supplied by the Director, National Institute of Virology, Pune) and was used in the form of infected infant mouse brain suspension[13]. Mice were inoculated with 1000 $LD_{50}$ of DV intracerebrally (ic) in doses of 0.03 ml.

*Collection of blood sample*: Animals were sacrificed and blood samples were collected from the jugular vein in tubes containing 0.5 ml freshly prepared 10 per cent $K_3$ EDTA solution as anticoagulant. The samples were shaken gently.

*Haematological study*: The following estimations were performed immediately with the help of a fully automatic haematology analyzer (Bayer; Technicon H1*E, USA): total leucocyte count (TLC), differential leucocyte count (DLC), total RBC count, haematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet counts.

**Table I.** Leucocytes count in Cr (VI) fed mice after dengue virus (DV) infection

| Cr treated group | TLC ($10^3$/µl) | | | Lymphocytes (%) | | | Granulocytes (%) | | | Monocytes (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | DV4d | DV8d | Control | DV4d | DV8d | Control | DV4d | DV8d | Control | DV4d | DV8d |
| 0 wk | 7.0±0.9 | 3.1±0.5$^+$ | 5.1±0.9** | 83±1.0 | 87±5.0 | 80±3.0 | 11±0.9 | 11±5.0 | 18±2.0 | 3±0.1 | 2±0.5 | 3±1.0 |
| 3 wk | 5.2±1.0** | 4.2±1.2$^+$ | 4.6±0.9$^+$ | 70±8.0$^+$ | 45±3.0$^+$ | 42±5.0$^+$ | 21±8.0** | 48±3.0$^+$ | 50±5.0$^+$ | 9±1.0$^+$ | 7±1.0$^+$ | 8±2.0$^+$ |
| 6 wk | 6.8±0.9 | 5.7±1.2* | 5.9±1.4 | 66±6.0$^+$ | 50±3.0$^+$ | 47±5.0$^+$ | 23±5.0** | 42±3.0$^+$ | 45±5.0$^+$ | 11±2.0$^+$ | 8±3.0$^+$ | 8±3.0$^+$ |
| 9 wk | 5.8±1.0 | 3.4±0.7$^+$ | 4.3±1.3$^+$ | 50±60$^+$ | 40±12$^+$ | 30±6.0$^+$ | 35±7.0$^+$ | 50±15$^+$ | 62±6.0$^+$ | 15±3.0$^+$ | 10±1.0$^+$ | 8±2.0$^+$ |

Control, normal mice; DV4d, 4th day after infection DV; DV8d, 8th day after infection with DV
*P**<0.05, ***<0.01, $^+$<0.001 compared to controls

**Table II.** Red blood cell indices of Cr (VI) fed mice after dengue virus (DV) infection

| Chronium treated group | DV infected group | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RBC ($10^6$ µl) | | | MCV (fl) | | | Hct (%) | | | MCHC (g/dl) | | | Hb (g/l) | | |
| | Control | DV4d | DV8d | Control | DV4d | DV8d | Control | DV4d | DV8d | Control | DV4d | DV8d | Control | DV4d | DV8d |
| 0 wk | 8.3±0.3 | 5.2±0.5$^+$ | 5.9±0.3** | 46±0.3 | 44±0.8 | 44±0.5 | 38±0.1 | 22.3±2.0$^+$ | 24.2±0.2$^+$ | 30.2±0.4 | 30.9±1.4 | 30.8±0.6 | 12.8±0.1 | 6.9±0.6** | 7.4±0.0* |
| 3 wk | 11±1.0** | 9.1±1.0 | 11±1.4** | 39±2.0 | 40±0.7 | 41±4.2 | 40±3.6 | 35.3±4.5 | 45.0±9.0 | 29.9±1.4 | 32.2±0.5 | 29.1±5.4 | 12.0±0.7 | 11±1.3 | 12.7±3 |
| 6 wk | 8.2±0.2 | 8.2±1.0 | 7.4±0.5 | 50±2.3 | 50±3.0 | 53±3.0 | 39±3.0 | 40.0±1.0 | 39.3±1.4 | 29.0±2.0 | 29.0±2.0 | 28.1±1.0 | 11.3±0.2 | 11.0±0.5 | 11±0.7 |
| 9 wk | 7.0±0.5 | 8.0±0.8 | 6.2±0.6** | 37±1.0 | 36±0.9* | 35±1.0* | 25.9±2$^+$ | 28.0±3.0** | 22.0±2.0$^+$ | 35.0±1.0 | 33.6±0.5 | 37.2±4.0 | 9.02±0.7* | 8.94±0.9* | 7.6±0.2* |

Control, normal mice; DV4d, 4th day after infection with DV; DV8d, 8th day after infection with DV; RBC, red blood cell; MCV, mean corpuscular volume; HCt, haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width; Hb, haemoglobin
*P* *<0.05, **<0.01, $^+$<0.001 compared to controls

*Statistical analysis*: Two-way analysis of variance (Two-way ANOVA) was done to compare the mean values of the outcome variables (TLC, DLC, RBC count, Hb, Hct, MCV, MCHC, and platelet count) considering chromium exposure and dengue virus infection to animals as independent variables. Prior to this homogeneity of variance between the treated groups were ascertained. The two-group comparison was done by calculating least significant difference at 5 per cent level of significance.

## Results

*Effects of dengue virus inoculation on mice*: The mice remained apparently healthy up to the day 4 of DV inoculation. The back was arched and the fur was ruffled from day 5 pi. By the 8th day the mice became seriously ill with the development of hind limb paralysis and all of them died by the day 10 pi. Therefore, the experiments were conducted on days 4 and 8 post inoculation of the dengue virus. The clinical dengue illness in Cr (VI)-fed mice was similar to that in normal mice.

*Effects on leucocytes*: In normal control mice the total leucocyte count was $7\pm0.9x10^3/\mu l$ with 83 per cent lymphocytes and 11 per cent granulocytes. By giving Cr(VI) in drinking water for 3 wk the total count was reduced significantly ($P<0.01$) to $5.2\pm1x10^3/\mu l$, with 21 per cent granulocytes. At the 6th and 9th wk the total leucocyte counts recovered slightly but the percentage of granulocytes and monocytes was increased significantly ($P<0.01$, $P<0.001$) with consequent reduction in lymphocytes (Table I). When the normal mice were inoculated ic with DV, a reduction in the total leucocyte count was seen at the day 4. On day 8 there was little improvement in the counts. The predominant cells in the blood remained lymphocytes in all the groups (Table I). Total leucocyte counts were reduced at 3 and 9 wk in Cr(VI)-fed mice given DV as compared to normal controls. A marked increase in the percentage of granulocytes was seen at all the periods of Cr(VI) drinking with the consequent decrease in the lymphocytes (Table I).

*Effects on red blood cells*: The total red blood cell (RBC) count in normal mice was $8.3 \pm 0.3$ µl. By feeding Cr(VI) the red blood cell count increased significantly ($P<0.001$) to $11.6 \pm 1.7x10^6$ µl. This was associated with a slight reduction in the mean corpuscular volume and mean corpuscular haemoglobin. At the 6th and 9th wk there was not much change in total RBC count but the haematocrit ($P<0.001$) and the haemoglobin ($P<0.01$) concentration was reduced especially at the 9th wk (Table II). Normal mice inoculated with DV showed reduced total RBC counts ($P<0.001$), haematocrit value ($P<0.001$) and haemoglobin ($P<0.01$) concentration at day 4 of DV inoculation (Table II). These values remained low at day 8 pi also. When Cr(VI) fed mice were inoculated with DV the total RBC count remained more or less similar in the 3rd and the 6th wk to that in controls while at day 8 after DV of the 9th wk Cr exposure the reduction was significant ($P<0.01$) (Table II).

*Effects on platelets*: The platelet count in the blood of normal control mice was $370 \pm 15x10^6/\mu l$ with Cr(VI) drinking the total platelet count was significantly increased to $622 \pm 128 x10^6/\mu l$ ($P<0.001$)

**Table III.** Platelet counts during dengue virus (DV) infection in Cr (VI) fed mice

| Cr treated group | DV infected group | | | | | |
|---|---|---|---|---|---|---|
| | MPV | | | PDW | | |
| | Control | DV4d | DV8d | Control | DV4d | DV8d |
| 0 wk | 7.0±0.2 | 6.8±0.1 | 5.9±0.2* | 9.0±0.5 | 8.3±0.3 | 8.8±0.1 |
| 3 wk | 5.2±0.3* | 5.3±0.2* | 5.9±0.5* | 5.9±0.6** | 6.2±0.4* | 6.9±1.7* |
| 6 wk | 6.0±0.5 | 5.9±1.0* | 6.9±0.5 | 7.1±0.7* | 6.8±0.8* | 7.0±2.0* |
| 9 wk | 6.3±0.2 | 6.4±0.3 | 6.5±0.5 | 8.0±0.7 | 8.1±1.7 | 7.0±1.0* |

Control, normal mice; DV4d, 4th day after infection with DV; DV8d, 8th day after infection with DV; MPV, mean platelet volume; PDW, platelet distribution width

*P* *<0.05, **<0.01 compared to normal controls

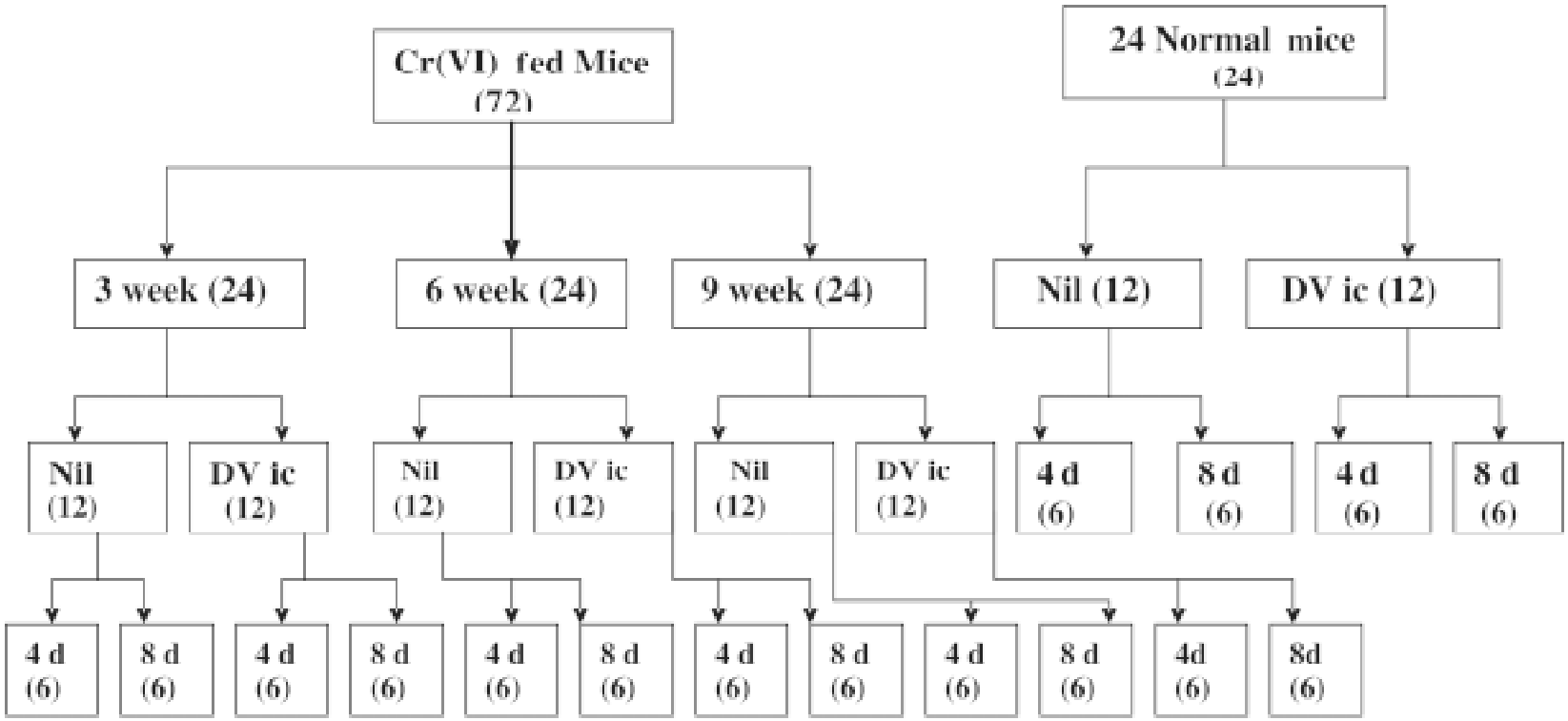


**Fig. 1.** Schematic presentation of the plan of study. DVic, mice inoculated with dengue virus intracerebrally; Nil, mice not given dengue virus; 4d, mice killed on the 4th day of the virus inoculation; 8d, mice killed on the 8th day of the virus inoculation; mice not given virus were also killed on the similar days. The number of mice in each group has been presented in parenthesis.

at the 3 wk to 736 ± 185 x$10^6$/µl ($P$<0.001) at the 9th wk compared to normal controls. When normal control mice were inoculated with the dengue virus, a significant reduction in the platelet count was seen both at days 4 and 8 being 185 ± 67 ($P$<0.01) and 254 ± 71x$10^6$ ($P$<0.05) respectively (Fig.2). When Cr(VI) treated mice were inoculated with dengue virus, the virus-induced reduction in platelet count was not observed at all the periods as the counts remained 401±120 to 628 ±70x$10^6$/µl. It was observed that by drinking Cr(VI) the mean platelet volume was slightly reduced. A greater change was observed in platelet distribution width by drinking Cr(VI) (Table III).

When normal mice were inoculated with DV slight change was observed in platelet distribution width and the mean platelet volume (Table III). Inoculation of dengue virus in Cr(VI) treated mice resulted in the decrease of mean platelet volume ($P$<0.01) especially at 3 wk (Table III).

## Discussion

The findings of the present study showed a marked reduction in lymphocyte percentage and increase in the granulocyte, monocyte and platelet counts in mice fed Cr(VI) with drinking water. Differential leucocyte counts revealed that percentage of lymphocytes decreased by 50 per cent while the percentage of polymorphonuclears and monocytes was increased gradually. Similar findings have been reported in fish exposed to chromium[21]. Rats exposed to atmosphere containing soluble potassium chromate have significantly increased levels of polymorphonuclear cells and monocytes[22]. A significant increase in polymorphonuclear cells has been reported in a 25 yr old women who drank a solution containing potassium dichromate[23]. The normal mice inoculated with DV showed a 48 per cent decrease in total leucocyte count. The number of polymorphonuclears was also reduced in the present study. Patients with dengue fever have mild leucopaenia to mild leucocytosis associated with lymphocytosis[11,24]. The interesting finding in the present study was that Cr(VI)-fed mice inoculated with DV showed marked reduction in number of lymphocytes, while the number of polymorphonuclears was not affected significantly.

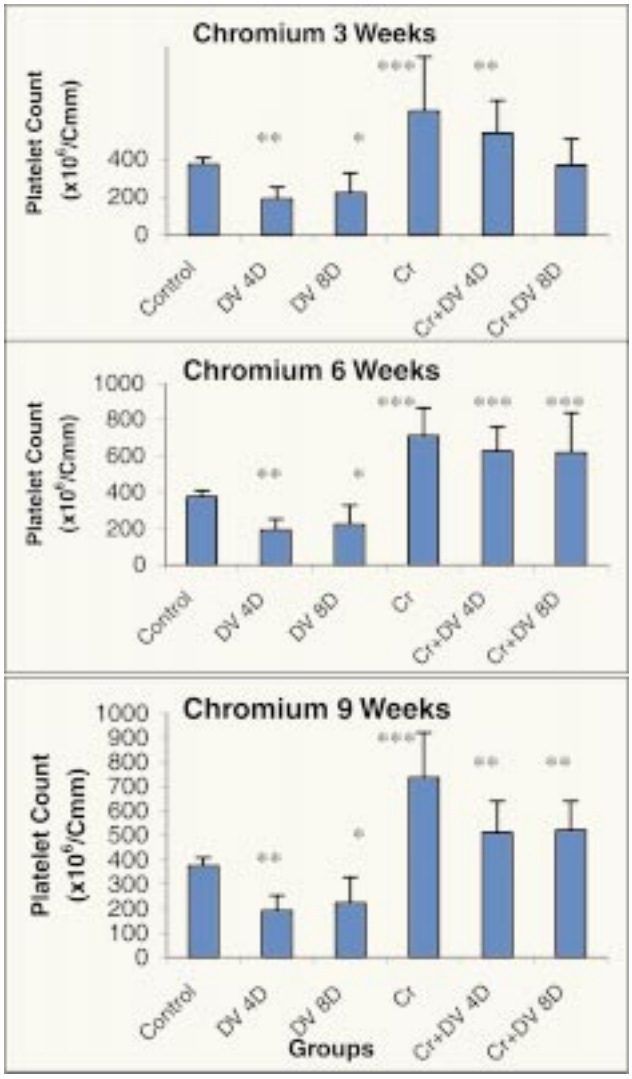


**Fig. 2.** Effects of subtoxic dose of Cr (VI) on platelets during dengue virus infection. Cr, chromium; DV, dengue virus; 4, 8 days after the virus infection. $P$ *<0.05, ** <0.01, ***<0.001 compared to normal controls.

At three week of Cr(VI) drinking the red blood cell count was increased by 29 per cent while at 6th and 9th wk it remained the same in our study. Glaser *et al*[25,26] reported that rats exposed to Cr(VI) for 18 months had increased red blood cell counts. A reduction in MCV and MCHC was observed at the end of 3rd wk of Cr(VI) drinking. Rats and mice fed with potassium dichromate showed slightly reduced MCV and MCH values at earlier periods and low doses of Cr (VI) but the decrease was marked with the higher doses given for longer periods[27-29]. In the present study, it was observed that after 9 wk of Cr(VI) drinking

haematocrit and haemoglobin contents were decreased. The decrease in haemoglobin appears to be due to inhibition of its biosynthesis. In the initial stage of pathway, succinyl Co-A combines with glycine to form the first of the intermediates, δ-amino levulinic acid. Chromium may cause an increase in SDH synthesis and therefore decrease the succinyl Co-A pool. It may also interact with serine, which is the precursor of glycine and may thus decrease the serine pool. Chromium causes the DNA damage, producing inhibition of the activities of one or more enzymes involved in heme synthesis[30,31]. Sharma *et al*[32] have reported that in an 18 yr old women who ingested few grams of potassium dichromate showed decreased haemoglobin and haematocrit on day 4 after ingestion. In another study, a 44 yr old man had decreased haemoglobin levels 9 days after ingestion of chromium[33] .

Haemoglobin concentration was decreased by 42 per cent on day 8 in normal mice inoculated with DV. The haematocrit value was also decreased by 40 per cent. Pimpan and Prasert[11] reported that in patients with DHF haematocrit value increased initially but later on it decreased. Gastrointestinal bleeding might initially be occult and usually manifested as a drop in haematocrit without clinical improvement[34,35]. Cr(VI) feeding led to an increase in platelet counts in the present study, while in normal mice inoculated with DV, platelet count was decreased significantly at days 4 and 8 post inoculation. Thrombocytopenia is one of the simple diagnostic criteria proposed by the WHO for clinical diagnosis of DHF[11]. The cause of thrombocytopenia is either impaired megakaryocytes production or increased platelet distruction[10].

Cr(VI) compounds have been declared as a potent occupational carcinogen among workers in chrome plating, stainless steel, and pigment industries[3]. The cells of immune system have capacity to reduce toxic Cr (VI) to non-toxic Cr (III) and in the process some immune functions are disturbed[20,36]. On the contrary, Cr (III) salts such as chromium polynicotinate, chromium chloride and chromium picolinate, are used as micronutrients and nutritional supplements, and have been demonstrated to exhibit significant health benefits in animals and humans[9,37,38].

In conclusion, the reduction in platelet counts following inoculation with DV was markedly less in Cr (VI)-fed mice than that seen in DV-inoculated normal control mice. Further studies need to be done to see whether Cr (III) therapy with chromium polynicotinate, chromium chloride or chromium picolinate will be able to prevent the pathognomonic fall in platelet count in patients with dengue virus infection.

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