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Effect of pretreatment with chromium picolinate on haematological parameters during dengue virus infection in mice

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***Background & objectives*: Dengue virus (DV) has caused severe epidemics of dengue fever (DF) and dengue haemorrhagic fever (DHF) and is endemic all over India. We have earlier reported that exposure of mice to hexavalent chromium [Cr(VI)] compounds increased the severity of dengue virus infection. Trivalent chromium picolinate (CrP) is used worldwide as micronutrient and nutritional supplement. The present study was therefore, carried out to investigate the effects of CrP on various haematological parameters during DV infection of mice.**

***Methods*: The Swiss Albino smice were inoculated with dengue virus (1000 LD_{50}, intracerebrally) and fed with chromium picolinate (CrP) in drinking water (100 and 250 mg/l) for 24 wk. Peripheral blood leucocytes and other haematological parameters, and spleens were studied on days 4 and 8 after virus inoculations and the findings were compared with those given only CrP and the normal control age matched mice.**

***Results*: CrP in drinking water for 24 wk had no significant effects on peripheral blood cells of mice. On the other hand, there was significant decrease in different haematological parameters following inoculation of normal mice with DV. In CrP fed mice the effects of DV infection were abolished on most of the haematological parameters.**

***Interpretation & conclusions*: The findings of present study showed that the adverse effects of DV infection, specially on platelets and leucocytes, were abrogated by pretreatment of mice with CrP. The therapeutic utility of CrP in viral infections including dengue needs to be studied in depth.**

Key words Blood cells - chromium picolinate - dengue virus - haematological indices - leucopenia - thrombocytopoenia

Dengue virus (DV) produces a benign self-limiting illness, the dengue fever (DF) or a life-threatening serious illness, the dengue haemorrhagic fever (DHF). Dengue virus is endemic in India with frequent epidemics of DF and DHF[1-6]. The dengue disease is characterized by leucopenia, thrombocytopenia, bone marrow suppression, increased haematocrit value and increased capillary permeability, which may result in

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haemorrhage and shock[7-9]. Immunopathological mechanisms appear to be responsible for the pathogenesis of DHF. A rapid increase in the levels of cytokines and chemical mediators apparently induced by a unique cytokine, cytotoxic factor (CF), plays a key role in inducing plasma leakage, shock and haemorrhagic manifestations[10,11].

Pollution of environment by chromium (Cr) is common in industrialized areas as a consequence of effluent discharge from tanneries and other industries, which include metal plating, manufacturing industries and ferrochrome production. This poses serious problem for environmental quality. In aqueous environments chromium has two oxidation states: hexavalent chromium (Cr-VI) and trivalent chromium (Cr-III). Cr (VI) compounds are generally soluble over a wide *p*H range and have been shown to exert toxic and carcinogenic effects in humans and experimental animals, also induce DNA damage such as DNA single-strand breaks and DNA-protein crosslinks *in vivo* and in cultured cells. On the other hand, Cr (III) compounds are mainly non-toxic[12-15] .

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[16,17]. It is, therefore, possible that the chromium toxicity may adversely affect the disease process during DV infection. In our earlier study we have reported that Cr (VI) in the form of potassium dichromate drinking led to reduction in lymphocytes, haemoglobin and the haematocrit values while the granulocyte, monocyte and platelet counts were increased. The most significant finding of these experiments was that the DV-induced reduction of platelet counts was cancelled in Cr (VI)-fed mice[18].

Chromium picolinate monohydrate, (CrP) is a synthetic Cr (III) compound that has widespread use as a nutritional supplement. It promotes a variety of beneficial health effects including weight loss, serum cholesterol reduction, treatment of diabetes, and increased muscle mass[19]. CrP administered to mice in the diet for 3 months up to the maximal possible dose (approximately 20,000-30,000 times that of the average daily human supplement 200 - 400 μg) to show any adverse effects[20-23]. No effects of CrP exposure on clinical chemistry or haematology parameters, gross lesions, or microscopic findings have been observed[24-26]. We, therefore, undertook this study to investigate the effects of dengue virus infection in mice fed with non toxic trivalent chromium in the from of chromium picolinate (CrP).

Material & Methods

Animals: The study was carried out on Swiss Albino mice weighing 25-30 g, aged 6-8 wk obtained from the animal breeding facility of the Industrial Toxicology Research Centre (ITRC), Lucknow. Study protocol was approved by the Animal Ethical Committee of the ITRC, Lucknow. Mice were maintained on pellet diet supplied by local supplier.

Dengue virus: Dengue type 2 virus, strain P23085 (received from National Institute of Virology, Pune) was used in the form of infected infant mouse brain suspension. Mice were given light ether anaesthesia and then inoculated with 1000 LD_{50} of DV intracerebrally (ic) in volume of 30 μl.

CrP treatment: Mice were divided in three groups of 18 animals each. One group was given *ad libitum* drinking water containing 100 mg/l chromium picolinate, second group was given drinking water containing 250 mg/l chromium picolinate and third group (control) was given plain water to drink. After 24 wk of CrP drinking, 12 animals in each group were inoculated i.c. with dengue virus in doses of 1000 LD_{50}. The mice were scarificed by cervical dislocation the 4^{th} (6 animals, clinical signs of illness appear) and 8^{th} (6 animals, paralysis of hind limbs set in) day post virus inoculation (pi).

Haematological study: Animals were sacrificed and 1.5 ml blood was collected from the jugular vein in tubes with 0.1 ml freshly prepared 10 per cent K_3 EDTA solution as anticoagulant. The samples were shaken gently. The following estimations were performed immediately with the help of a fully automatic haematology analyzer (Bayer; Technicon H1*E, Germany): total leucocyte count (TLC), differential leucocyte count (DLC), total RBC count, haematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV) and platelet counts.

Preparation of spleen cell culture: The spleen cells were teased out in cold minimal essential medium (MEM, Hi-Media, Mumbai) and a single cell suspension was prepared. The viable nucleated cell count as ascertained by trypan blue dye exclusion test[27] was more than 95 per cent.

Spleen cell proliferation assay[28]: The cell suspension of normal mice spleen was adjusted to $2x10^6$ cells/ml

in Eagle's MEM supplemented with 10 per cent foetal calf serum. Cell cultures were set up in 96 wells U-bottom microtitre plates in triplicate. Each well contained 0.2 ml cell suspension with or without 5 µg/ml concanavalin A (Con A). The cultures were incubated for 72 h at 37°C in presence of 5 per cent CO_2. Five hours before the termination of the cultures, 20 µl of MTT3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (5mg/ml PBS, phosphate buffer saline) was added to each well. Cells were harvested and supernatant discarded and 200 µl of dimethyl sulfoxide was added; readings were recorded after 10 min at 570 nm using 630 nm as reference OD. All the chemicals used were procured from Sigma, USA.

Preparation of spleen homogenate: Spleens were washed free of blood, homogenized in a tissue homogenizer giving a 10 per cent solution (w/v) in chilled PBS (*p*H 7.2). The homogenate was centrifuged in the cold at 8000 *g* for 10 min and clear supernatant was stored in small aliquots at -20°C. Normal mouse spleen homogenates were prepared similarly and used as control.

Cytotoxicity test: Cytotoxic factor (CF) is present in the homogenates prepared from the DV-infected mouse spleen and has the capacity to kill normal mouse spleen cells in one hour time. The cytotoxic activity of the spleen homogenate isolated from DV infected mice was tested using normal mouse spleen cells as target. The test was carried out in 96 wells plates, using 0.1 ml volume of each preparation. The target single cell suspension of normal mouse spleen was prepared. Then the cell viability was measured by MTT assay. The MTT assay was done in triplicate and the mean value of % of non-viable cell as obtained in repeated experiments are presented[24]. All procedures were carried out at 4°C in ice bath.

Statistical analysis: Two-way analysis of variance (Two-way ANOVA) was done by Systat Software, Inc. (USA), to compare the mean values of the outcome variables 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

Effect CrP feeding on blood cells during DV infection:

Effects on leucocytes: In normal control mice the total leucocyte count was 11.5+4.6x10^3/cubic millimeter (Cu mm) with 87 per cent lymphocytes and about 9 per cent granulocytes. By giving CrP (100mg/l and 250mg/l) in drinking water for 24 wk the total as well as differential leucocyte counts were not affected significantly (Table I). In the normal mice DV infection caused a significant reduction in the total leucocyte at day 4 (P<0.05) and 8 (P<0.01) compared to uninfected mice. The predominant cells in the blood remained lymphocytes in all the groups (Table I).

Effects on red blood cells: Feeding CrP had no effect on the red blood cell count, haemoglobin and MCV, where as haematocrit concentration was significantly increased at 250 mg/l dose of CrP (P<0.05) (Table II). Normal mice inoculated with DV showed reduced total RBC counts (P<0.01), haematocrit (P<0.05) and haemoglobin (P<0.05) at day 8 of DV inoculation (Table II). In CrP fed mice DV inoculation caused no significant change in these indices.

Effect on platelet count: The platelet count in the blood of normal control mice was 406 ± 81×10^6/Cu mm, whereas after 100 mg/l CrP drinking it significantly increased to 484 ± 51 x10^6/Cu mm (P<0.05) but with 250 mg/l CrP feeding count was slightly decreased to 366 ± 52×10^6/µl compared to normal controls. When normal control mice were inoculated with the dengue virus, a significant reduction in the platelet count was seen on days 4 and 8 being 179 ± 57 ×10^6 (P<0.001) and 312 ± 51x10^6 (P=<0.01) respectively. In CrP fed mice DV inoculation caused no significant change (Fig. 1).

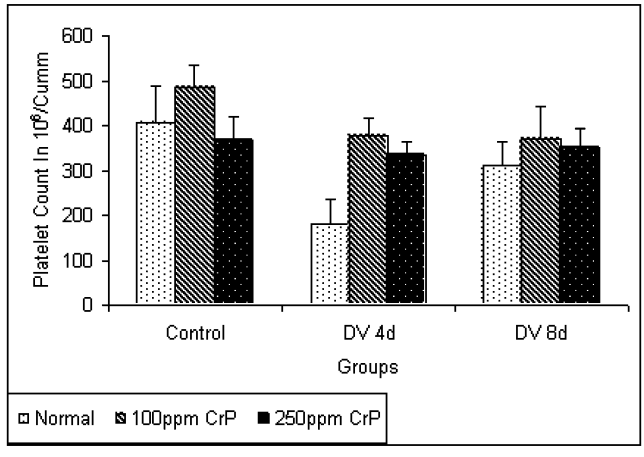

Fig. 1. Effects of CrP feeding (24 wk) on platelet count during dengue virus infection. CrP, chromium picolinate; DV, dengue virus; 4, 8, days after the virus infection compared to controls. (n=6 in each group). Values are mean ± SD.

Table I. Leucocytes count in cromium picolinate fed mice after dengue virus (DV) infection

CrP treated groups	TLC (10^3/Cu mm)			Lymphocytes (%)			Granulocytes (%)			Monocytes (%)		
	Control	DV4d	DV8d	Control	DV4d	DV8d	Control	DV4d	DV8d	Control	DV4d	DV8d
Normal	11.5 ± 4.6	8.0 ± 3.2*	5.8 ± 2.3**	87 ± 1.9	82 ± 6.0	80 ± 3.0	9.5 ± 1.8	14 ± 5.6	15 ± 1.4**	3.3 ± 0.3	4.0 ± 0.8	4.5 ± 0.9
100ppm CrP	10.8 ± 1.5	10.5 ± 2.1	9.7 ± 3.0	90 ± 3.5	88 ± 3.5	82 ± 0.5	8.0 ± 2.9	8.0 ± 2.7	12 ± 1.0*	2.3 ± 0.6	3.9 ± 0.8	6.0 ± 0.6
250ppm CrP	11.2 ± 2.3	10.8 ± 0.3	9.3 ± 1.2	86 ± 6.0	85 ± 1.0	87 ± 9.0	10 ± 3.0	11 ± 1.0	12 ± 1.7*	3.2 ± 1.5	4.1 ± 3.2	3.3 ± 1.1

Control, normal mice; DV4d, 4th day after infection DV; DV8d, 8th day after infection with DV; TLC, total leucocyte count
Values are mean ± SD of 6 animals in each group
*P**<0.05; ** <001 compared to normal

Table II. RBC indices in cromium picolinate fed mice after dengue virus (DV) infection

CrP treated groups	RBC (10^6/Cu mm)			Hb (g/l)			HCt (%)			MCV (fl)		
	Control	DV4d	DV8d	Control	DV4d	DV8d	Control	DV4d	DV8d	Control	DV4d	DV8d
Normal	8.8 ± 0.5	6.2 ± 0.3	5.9 ± 0.5**	14 ± 1.9	11 ± 0.8	9.0 ± 1.2*	29 ± 3.8	21 ± 2.0	23 ± 2.3*	36 ± 1.7	37 ± 2.3	34 ± 1.8
100ppm CrP	8.0 ± 0.5	7.3 ± 1.2	7.7 ± 1.0	14 ± 0.9	15 ± 0.9	13 ± 2.5	29 ± 3.3	26 ± 5.6	31 ± 4.5	36 ± 2.2	36 ± 2.7	37 ± 1.2
250ppm CrP	8.7 ± 0.6	7.7 ± 1.7	7.6 ± 0.5	16 ± 0.9	14 ± 3.4	14 ± 0.9	37 ± 1.0*	31 ± 0.7	30 ± 2.5	39 ± 2.7	35 ± 3.5	37 ± 1.7

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; Hb, haemoglobin
*P** <0.01 **<0.01 compared to control
$^{+}$ *P*<0.05 compared to control of normal unfed group
Value are mean ± SD of 6 animals in each group

Effect on weight of spleen: The weight of the spleen in the control normal mice was 477 ± 59 mg/100 g body weight. Significant reduction in the weight of the spleen with 100 mg/l CrP was observed [388 ± 55 mg/100 g ($P \leq 0.05$) body weight] whereas with 50 mg/l CrP it was increased significantly ($P \leq 0.05$) being (587+79 mg/100g). The weight of the spleen of normal mice inoculated with DV was 439 ± 30 and 340 ± 25mg/100 g body weight on day 4 and 8 post-inoculation, respectively (Figs 2 & 3). When CrP treated mice were inoculated with DV, only with 250 mg/l CrP dose a slight protection was observed against DV induced reduction in spleen weight in comparison to DV alone.

Effects on production of cytotoxic factor (CF): The homogenate from DV-infected mice spleen produced 13 to 25 per cent cytotoxicity (Fig. 4). On the other hand, no cytotoxic activity was seen in the spleen homogenates obtained from CrP fed mice in comparison to normal control mice. A significant decrease ($P < 0.001$) in cytotoxic activity of spleen homogenate of DV infected mice was observed after pretreatment with CrP at both doses.

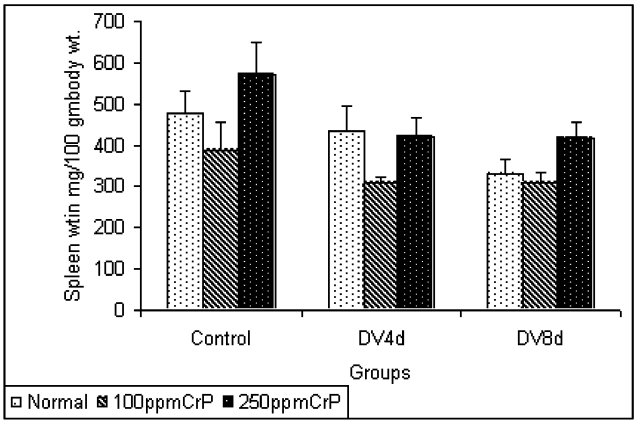

Fig. 2. Effects of CrP on spleen weight (in mg/100g body weight) during dengue virus infection. CrP, chromium picolinate; DV 4, 8 d, dengue virus 4, 8, days after the virus infection compared to controls. Values are mean ± SD of n=6 mice in each group.

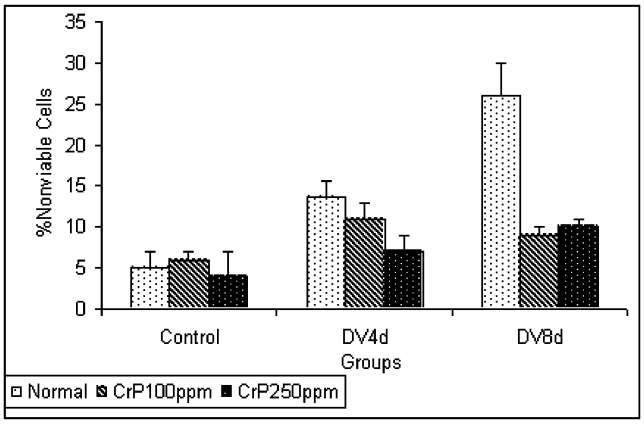

Fig. 3. Cytotoxic activity of the homogenates of spleens obtained from various groups of mice. Values are mean ± SD of n=6 mice in each group.

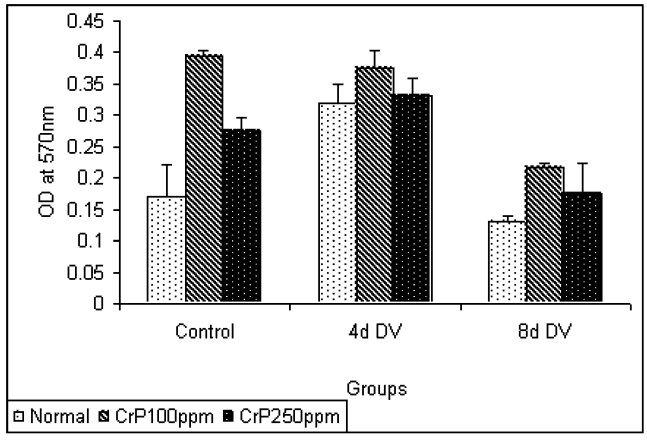

Fig. 4. Effects of CrP feeding on mitogenic stimulation of spleen cells during dengue virus infection. Value are mean ± SD (n=6).

Effect on mitogenic stimulation: When normal mice spleen cells were treated with mitogen the observed stimulation was 0.171 + 0.05. When normal mice were inoculated with DV, maximum proliferation occurred at the fourth day (0.375 ± 0.030) and it was suppressed significantly on day 8 ($P<0.05$). On the other hand, after 24 wk of drinking of 100 mg/l CrP, spleen cell proliferation in mice increased by two-fold of normal mice spleen cells. When CrP (100 mg/l) fed mice were inoculated with DV, on day 8 of infection there was no DV induced suppression in mitogenic stimulation in comparison to the DV alone mice group.

Discussion

The present study showed abrogation of DV-induced adverse effects on different haematological parameters in mice after feeding CrP for 24 wk. The characteristic leucopenia, thrombocytopenia and increased haematocrit are common findings in clinical dengue disease and together with combination of clinical picture are used as markers for early diagnosis of dengue infection[7,9,29,-31]. Constant haematological abnormalities occurring in severe dengue disease include bone marrow suppression[32]. La Russa & Innis[33] have reviewed data from experimental dengue infections of volunteers and bone marrow studies from patients with severe dengue virus infection which showed that marrow suppression evolves rapidly through several phases and recovers. The early blast

cells and the differentiated haematopoietic elements are abortively infected, killed and eliminated by phagocytosis by specialized marrow dendritic cells. Cytokine production by virus-infected stromal cells is also altered[33]. The combined effect of these changes is transient bone marrow suppression resulting in leucopenia and thrombocytopenia.

Our present findings are supported by those on human subjects that the haematocrit, haemoglobin, RBC count, MCV, MCH, MCHC, red blood cell distribution width, platelet count, and mean platelet volume are within normal clinical ranges and are unchanged by chromium picolinate supplementation for 12 wk[28]. Recent studies[35,36] demonstrate that chromium picolinate supplementation significantly improves insulin sensitivity and glucose control in subjects with type 2 diabetes and also in obese subjects with polycystic ovary syndrome.

In this study haemoglobin concentration and the haematocrit value decreased day 8 after DV inoculation in normal mice. Pimpan & Prasert[7] reported that in patients with DHF haematocrit value is increased initially but later on it decreased. On the other hand, DV-induced fall in RBC count, haemoglobin as well as on haematocrit % was abolished in CrP fed mice. During DV infection of mice a significant reduction in the weight of the spleen occured, which was associated with a sharp decline in the proportion of T-lymphocytes and macrophages in the spleen[27]. CrP feeding had no effect on the spleen weight of normal mice which was similar to the findings reported by Rhodes *et al*[26]. When CrP fed mice were inoculated with DV, a significant protection against reduction of spleen weight was observed on day 8 of the virus infection. Further, the production of DV-induced cytokine, CF was significantly reduced in CrP fed mice.

These findings showed that the mitogenic response of splenic lymphocytes to Con A was increased in CrP fed mice similar to the findings reported in cows[37] and in pigs fed supplemental chromium[38]. Organic chromium supplements are known to increase immune function in animals. The exact mechanism by which chromium picolinate enhances the immune system is not known. However, chromium supplementation has been shown to reduce serum cortisol levels. Glucocorticoids, which include cortisol, are known to suppress the immune system[39].

Pollution of environment by Cr (VI) poses serious problem due to its toxicity. On the other hand, Cr (III) compounds are mainly non-toxic[16,17]. We had earlier shown that Cr (VI) drinking led to reduction of most of the haematological parameters (except platelet counts) enhancing the severity of dengue illness in mice[40].

In conclusion, abrogation of DV-induced adverse effects on the haematological parameters by feeding CrP, indicates a need for in depth investigation before it is used as supportive therapy in dengue and other viral infections.

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