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Genotoxicity testing of chromium trioxide - a study using *Vicia* bioassay

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Abstract

In the present study by employing highly sensitive and simple plant bioassay, *Vicia faba* cytogenetic test, genotoxic property of chromium trioxide (CrO_3) was evaluated. Roots of *Vicia faba* were treated with 10, 20. 30, 40 and 50 mg/l of CrO_3 at room temperature in dark. The dosages selected for evaluation were below LD50 dose of the compound. Cytotoxicity and chromotoxicity were expressed in terms of depression in mitotic activity and frequency of chromosomal aberrations in mitotic phases. Treatment of roots caused dose and period of treatment related inhibition of mitotic activity. Dose related increase in the frequencies of cells with chromosomal aberration in metaphase and anaphase stages were observed. Statistical analysis revealed significant effects of dose and periods of treatment. The results indicate genotoxic potential of chromium trioxide in the dose range tested.

Keywords: Genotoxicity; chromium; *Vicia faba*; mitotic inhibition, chromosomal aberrations.

INTRODUCTION

Chromium is well known as human and animal carcinogens [1-2]. Chromium has been listed as one of the 129 priority pollutants and one of the 14 most noxious heavy metals. Chromium is a hazardous substance thought to pose the most significance potential threats to human health. Human exposure to hexavalent chromate containing materials has been reported to occur during the production of chromate pigments [3], chrome plating [4], leather tanning [5] and stainless steel welding [1]. The main sources of chromium pollution are chromate-producing factories, metallurgical industries, chrome plating, and the burning of coal. Chromium trioxide, as an analytical reagent and oxidant, is widely applied in chromate manufacture, electroplating, printing, tanning and fabric mordant dyeing, and many other industries. The environmental contamination of chromium is increasingly serious nowadays. Compounds of hexavalent chromium are irritants and corrosives, possibly due to their capacity as oxidants or to their properties as heavy metal. The harmful effects of chromium compounds (especially hexavalent chromium compounds) on human health had been reported [6-7], and soluble hexavalent chromium compounds are considered as carcinogens to human lung [8].

Previous studies showed that potassium dichromate and sodium dichromate could induce significant increase of micronucleus rate in bone marrow polychromatic erythrocyte (PCE) in rodents [9]. CrO and CrCl decreased the fidelity of DNA synthesis in vitro by increasing the error in deoxynucleotide incorporation [10]. Calcium chromate induced errors in recovery mechanisms in *Salmonella* [11]. On the other hand it was shown that the concentration of metals with carcinogenic and mutagenic activity decreased DNA polyrnerase activity and increased errors that incorporate deoxynucleotides [12]. But dichromate ion represents a special case because high concentration inhibited DNA polymerase without altering deoxynucleotide incorporation and has also been described as carcinogenic and mutagenic [12]. A dose response relationship was found when chromium trioxide and potassium dichromate produced chromosomal aberrations in mice [13]. Potassium dichromate and calcium chromate induced chromosomal alterations in *Vicia faba* [14]. Chromium causes a variety of DNA lesions such as DNA strand breaks, SCEs and mutations [15-18]. The oxidation state is the most important parameter for chromium toxicity. Cr(VI) can easily gain entry into cells via sulphate transport protein, while Cr(III) is poorly taken up by the cells. Furthermore, inside the cells, Cr(VI) is reduced by cellular reductants through reactive intermediates such as Cr(V) and Cr(IV), to the most stable Cr(III) state [19-20]. Hydrogen peroxide (H_2O_2), normally present in cells, reacts with Cr(V) and leads to the formation of an hydroxyl radical via the Fenton reaction [21-22]. Formation of hydroxyl radicals seems to be the major cause of Cr(VI) genotoxicity [23]. In the present study genotoxicity of chromium trioxide was evaluated in the root meristem of *Vicia faba.*

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MATERIALS AND METHODS

Primary roots of *Vicia faba* were used as test material in the present experiment.

Chromium trioxide (CrO_3, CAS Registry Numbers: 1333-82-0, M.W.197.82) was tested for its genotoxic property in the present study. Five different dosages i.e. 10, 20, 30, 40 and 50 mg/l were selected for treatment of roots. Solutions were prepared in glass distilled water.

Roots (1-2 cm long) were treated for 4, 8 and 12 h simply by suspending them in the test solutions containing the chemical in separate glass jars. In each experiment, negative controls (distilled water) and positive controls (0.20% EMS solution) were included. After treatment, roots were washed thoroughly in running tap water and the root tips were cut and fixed in a mixture of ethyl alcohol and glacial acetic acid (3:1 v/v) for 24 h. The fixed roots were then transferred into 70 % ethyl alcohol and stored at 4°C in a refrigerator

for future use. All the treatments were carried out simultaneously and under the same condition.

Before slide preparation the root tips of were hydrolyzed with a solution of 1N HCl (1 part) and 45% acetic acid (9 parts). The root tips were stained with 1.5 % carmine in 45% acetic acid. After removing the root caps from the well stained root tips, 1 mm of the meristematic zones were cut in a drop of 45% acetic acid on a clean slide and squashed under cover-slip by exerting thumb pressure on it. Five root tips from each sample were analyzed under 100 x magnifications. 5000 cells from five root tips were scored for mitotically dividing cells to calculate mitotic index. 500 cells from five root tips of control and treated roots were scored for number of abnormal cells and presence of chromosomal and mitotic abnormalities. All experiments were repeated at least three times. The data shown represent the mean ± SE. The data were statistically analyzed using the analysis of variance (ANOVA) and significant differences between the means were assessed by Dunnet multiple comparisons test at P<0.05.

RESULTS

The genotoxic effects following exposures to different doses of chromium trioxide assessed by *Vicia faba* assay are presented. During this study, ethyl methane sulphonate (EMS), used as positive control chemical, led to significant effects which had validated the sensitivity of the plant materials tested.

Effects of chromium trioxide on the intensity of cell division were measured by evaluating mitotic index (MI). 5000 cells from 5 roots (1000 cells/root**)** were examined and the data are presented. As shown in Table 1, the mitotic indices in the root tip cells of chromium trioxide treated roots were significantly lower (P<0.01 and p<0.001) than that of control. Inhibition of mitotic activity increased concomitantly with the increase in dose of the test compound as well as treatment period (Fig. 1). Analysis of variance (ANOVA) test revealed that there exist significant differences between treated groups and periods of treatment (Table 2).

Table 1. Effect of chromium trioxide treatment on the cell division in the root tip cells of *Vicia faba*

Chemical/Concentration	**Miotic Index (%)**		
	4h	**8h**	**12h**
Control	6.00 ± 2.12	16.40 ± 2.26	16.20 ± 2.44
EMS	8.40 ± 1.55***	6.80 ± 0.98***	5.60 ± 0.83***
10	14.50 ± 2.22**	12.40 ± 2.25**	11.10 ± 1.95**
20	12.10 ± 1.65**	10.90 ± 1.24**	9.40 ± 0.90**
30	11.20 ± 1.22**	8.30 ± 0.76**	7.20 ± 0.68**
40	9.30 ± 0.64**	7.20 ± 0.60**	6.10 ± 0.22**
50	7.70 ± 0.98**	5.60 ± 0.19**	4.80 ± 0.34**

EMS 0.2% used as positive control
1000 Cells per root tip and total 5000 cells have been scored in each case
, * Significantly differ from control at p<0.01 and p<0.001 respectively from the control in Dunnet Multiple comparisons Test

Table 2. Two-way analysis of Varience (ANOVA) of percent abnormal cells showing significant variation between treatment cells as wel as periods of treatment

Source of Variation	**df**	**Mean Square**	**F-Value**
Between periods	2	3.749	9.13**
Between Treatment	5	10.056	24.51***
Residual	10	0.4102	

, * differ significantly from control at p<0.01 and p<0.001 respectively

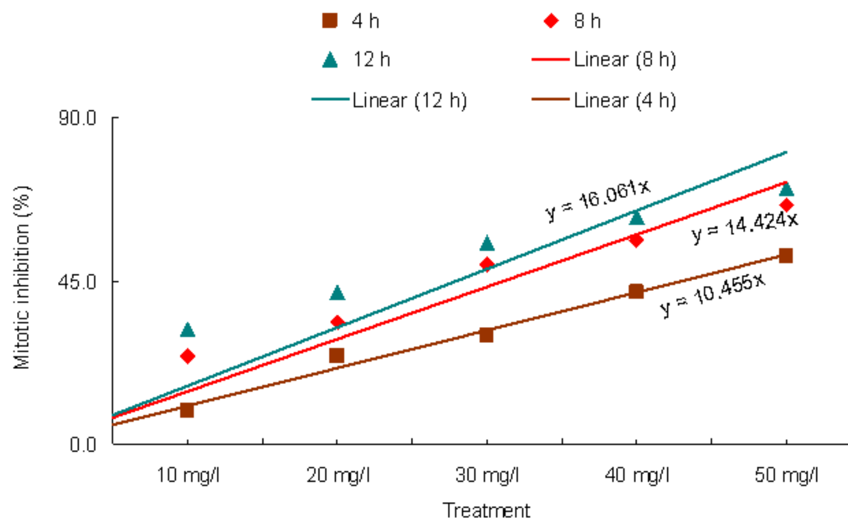

Fig. Showing trends of mitotic inhibition (% that of control) in the root tip cells of *Vicia faba* after treatment with chromium trioxide

Table 3 shows data on frequency of different types of chromosomal aberrations and percentage of abnormal cells induced by different doses of chromium trioxide. Treatment of roots with induced dose and period of treatment related increase in the chromosomal aberration rate. However, statistically significant increases in percent abnormal cells were recorded at the three higher doses i.e. 30, 40 and 50 mg/l of chromium trioxide. Analysis of variance (ANOVA) test revealed that there exist significant difference between treatments as well as periods of sampling (Table 4).

The chromosomal aberrations observed in metaphase and anaphase cells were chromosome and chromatid fragments, lagging chromosome, chromosome and chromatid bridges, stickiness, disturbed metaphase and anaphase viz. arrangement of chromosomes in groups on equator and multipolar arrangements in anaphase and c-mitosis. The occurrence of the different types of chromosomal abnormalities did not show any consistent pattern.

Table 3.Frequency and types of chromosomal aberrations and percent abnormal cells recorded after treatment of roots of Vicia faba with different concentrations of chromium trioxide for different periods

Period of treatment and Dose		Total Cells	Types and Number of Aberrant cells							No. of Abnormal cells	% of Abnotmal cells (a)
			Fg	DM	DA+T	St	Br	Lag	Oth.		
4 h treatment	Control	500	0	2	1	0	0	0	2	5	1.00 ± 0.18
	EMS	500	9	7	6	4	15	8	12	61	12.20 ± 3.56**
	10 mg/l	500	0	1	2	0	0	2	2	7	1.40 ± 0.12
	20 mg/l	500	1	1	2	1	1	3	2	11	2.20 ± 0.55
	30 mg/l	500	2	2	3	2	2	2	2	15	3.00 ± 0.55**
	40 mg/l	500	1	2	4	2	4	4	2	19	3.80 ± 0.86**
	50 mg/l	500	3	2	6	2	4	5	3	25	5.00 ± 0.90**
8 h treatment	Control	500	0	2	1	0	0	0	2	5	1.00 ± 0.18
	EMS	500	17	9	5	7	12	12	9	71	14.20 ± 3.20**
	10 mg/l	500	0	2	3	0	2	2	1	10	2.00 ± 0.58
	20 mg/l	500	0	1	3	2	3	4	1	14	2.80 ± 0.65
	30 mg/l	500	2	3	4	3	4	3	0	19	3.80 ± 0.70**
	40 mg/l	500	1	6	4	5	6	4	1	27	5.40 ± 0.96**
	50 mg/l	500	3	8	8	3	6	4	2	34	6.80 ± 1.30**
12 h treatment	Control	500	0	2	1	0	0	0	2	5	1.00 ± 0.18
	EMS	500	19	12	12	9	12	10	8	82	16.40 ± 3.90**
	10 mg/l	500	0	1	2	3	3	2	0	11	2.20 ± 0.26
	20 mg/l	500	1	3	4	1	3	3	1	16	3.20 ± 0.47
	30 mg/l	500	2	7	4	1	4	2	2	22	4.40 ± 0.68**
	40 mg/l	500	2	6	9	5	5	4	0	31	6.20 ± 0.80**
	50 mg/l	500	4	5	8	7	9	2	1	36	7.20 ± 1.25***

(a) Mean ± SEM (mean of five root tips, 100 cells per root tip were scored)
*, **, *** Significantly differ from control at $p<0.05$, 0.01 and 0.001 respectively in Dunnet multiple comparisons test
EMS 0.2% positive control
Fg. Fragments, DM. Disturbed metaphase, DA+T Disturbed ana+Telophase, St. Sticky metaphase, Br Bridge at anaphase and Telophase
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Table 4. Two-way analysis of variance (ANOVA) of percent abnormal cells showing significant variation between treatments as well as periods of treatment

Sources of Variation	df	Mean Square	F-value
Between periods	2	3.749	9.13**
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Residual	10	0.4102	

** & *** differ significantly from control at $p<0.01$ and $p<0.001$ respectively

DISCUSSION

Mutagenicity testing of chemical pollutants based on plant bioassay has been in existence for many years. Its use for the screening and monitoring of genotoxic properties of environmental contaminants has often been emphasized [23-25]. Heavy metals are extremely toxic elements and they could reduce the mitotic activity and induce many types of chromosomal anomalies [26-28]. Chemical compounds induce chromosomal aberrations by disturbing the process of DNA and protein synthesis or RNA translocation. It may also be induced by preventing the normal repair process of the cells.

Some chromium compounds induced sister chromatid exchanges in cultured human lymphocytes [29 – 30]. Induction of micronuclei and chromosomal aberrations in root tip cells of plants by chromium compounds has been reported [31]. The production of chromosomal aberrations by chromium may be due to its interaction with nucleic acids and nucleoproteins, affecting their physico-chemical properties and by the iron and other metals substituted in essential compounds or in nucleic acids [10-11]. The highly bioactive hexavalent chromium compounds enters into cell through cell membrane, and generate some active oxides, which can combine with the intracellular DNA, and lead to the unreliable intercrossing connection, and duplication in DNA, and ultimately result in chromosomal aberration and tumourigenesis [32]. The hexavalent chromium compound also has cytotoxicity and can even lead to DNA damage [33].

Results of the present investigation revealed that treatment of

roots of *Vicia faba* with chromium trioxide caused dose dependent decrease in mitotic index and increase in percent abnormal cells with chromosomal aberrations. Dose related reduction in the mitotic activity after treatment of roots with chromium trioxide could be due to inhibition of DNA synthesis [34-35] or a blocking in the G_2-phase of the cell cycle thus preventing cells from entering mitosis [36]. The another cause of decrease in mitotic index is the loss of dividing cells due to interference of chromium in the normal sequences of mitosis by disturbing the spindle function. Chromium reacts with proteins, binds with the carboxyl groups of the peptide chains and forms complexes with proteins. Therefore, when chromium bonds to tubulin molecules, it may change their structure leading to spindle dysfunction. Chromium induced various forms of chromosomal aberrations and mitotic abnormalities in root tip cells which indicate that the compound is clastogenic and also act as spindle poison. Chromosome and chromatid aberrations such as breaks, bridge with fragments and mitotic anomalies such as lagging chromosome, c-mitosis, and disturbed meta-anaphase were induced in present study. The similar findings were observed in *Allium cepa* L. [37] and sugarcane [38-39] due to chromium treatment. Chromosomal aberrations such as chromosome stickiness, disturbance, C-metaphase may be due to the effect on the spindle apparatus.

Chemicals that induce chromosome breakage are known as clastogens and their action on chromosomes is generally regarded to involve an action on DNA [40-41]. The formation of acentric fragments might have resulted from different chromosome aberrations, and the lagging of chromosomes caused by disturbances in the mitotic spindle/ centromere or the failure of a chromosome to get attached to spindle fibre. The occurrence of c-mitosis indicates inhibition of spindle formation. Chromosome and chromatid bridges at ana-telophase are formed by breakage and fusion of chromosomes and chromatids. Chromosome bridges are also formed due to the chromosomal stickiness and failure of separation of chromosomes at anaphase. It may also be formed due to unequal translocation or inversion of chromosome segments. In the present investigation any one of the above mentioned causes might be responsible for the formation of chromosome bridges at anatelophase. In addition to the above mentioned types of abnormalities, stickiness was also observed in present study. Chromosome stickiness reflects highly toxic effects, usually of an irreversible type probably leading to cell death.

The significant decrease in mitotic indices and increase in the frequency of chromosomal aberrations following chromium trioxide treatments, as observed in the present study, indicate genotoxic effects of the compound in the dose range tested. The findings suggest that chromium trioxide is a clastogenic as well as aneugenic.

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