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Evaluation of phytotoxicity and genotoxicity of uranyl nitrate in *Allium* assay system

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Uranyl nitrate inhibited root growth of *Allium cepa* at ≥ 25 μ*M* concentration. Fluorimetric analysis of metal uptake indicated the entry and accumulation of uranium into the root cell. Uranyl nitrate was neither clastogenic nor aneugenic as it failed to induce micronuclei significantly, but between 25 and 100 μ*M* concentration, it increased significantly the frequency of sister chromatid exchange over that of control, implying its genotoxicity that possibly interfered with DNA replication and / or repair process.

Uranium occurs naturally in the earth's crust and is present at high concentrations, along with thorium and other rare-earth elements in the areas where monazite sand occurs[1]. Uranium is regarded as the heaviest trace element found in nature. Natural sources of uranium are pitchblende or uranite (U_3O_8, UO_2 or UO_3), carnolite (K_2O_2 UO_3 V_2O_5. $3H_2O$), atunite (KUO_2 PO_4. $8H_2O$) and monazite sand. In oxidizing oceans uranium exists predominantly in the form of uranyl carbonate anions[2] $[UO_2 (CO_3)_2]^{2-}$ and $[UO_2 (CO_3)_3]^{4-}$. Uranium is a weak radioactive element and is treated almost like a non-radioactive metal. Uranium and uranyl compounds, however, are highly toxic to biological systems even in trace[3]. Plants accumulate uranium[4,5]. Ratio of uranium in plants in relation to soil is 4 x 10 (Ref. 3). Intake of uranium is mainly through consumption of vegetables, cereals and table salt[6]. No chemotoxic and radiotoxic effects on microorganisms and plants are known from exposure to environmental radioactivity from natural or industrial sources of uranium, thorium and decay products. Little information is available on toxicity of uranium or uranyl compounds in plants and animals[3].

The present study was undertaken to evaluate the phytotoxicity and genotoxicity of uranium using uranyl nitate $[UO_2 (NO_3)_2]$. It is a stable compound, emits no radiation and is readily soluble in water, ether and alcohol. Uranyl nitrate is tested for toxicity employing *Allium cepa* as the assay system[7,8]. Uranium uptake by the root cell was evaluated by laser fluorimetric analysis. Phytotoxicity and genotoxicity of uranyl nitrate have been evaluated on the basis of estimation of several endpoints that include root growth, mitotic index, induction of micronuclei (MNC)[9,10] and sister chromatid exchanges (SCE)[11] in root meristem cells of *A. cepa*.

Materials and Methods

Test chemical - Uranyl nitrate (Fluka, Switzerland) was used as the test chemical. Stock solution of the chemical was made fresh and diluted with tap water (*p*H 7) in order to prepare the experimental solutions of desired concentrations.

Assay system - Healthy bulbs of *Allium cepa* L. (2n = 16) were peeled of dry scales and the bases scraped to expose the root primordial and set for germination. The growing roots (1-2 cm) were used in experiment. The experiment was conducted at room temperature 24°± 1°C under continuous cool fluorescent light (approximately 100μE m^{-2} s^{-1}).

Chemical treatments were conducted by placing the bulbs with growing roots as said above on glass test tubes (120 × 15 mm, Borosil®, India) filled with experimental solution. After specific periods of treatment, the roots were washed in running tap water

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to terminate the treatments. The investigation was carried out in three experiments.

Experiment I - To assess the phytotoxicity of uranyl nitrate, bulbs of *A. cepa* were treated with different concentrations (0, 5, 10, 15, 20 and 25 μ*M*) of experimental solution and kept for germination. Per treatment, 5 bulbs were used. The test solutions were replaced daily. On day 5, the root lengths were measured and the root tips were excised and fixed in acetic acid : ethanol (1:3) for cytological analysis[9].

Experiment II - To determine the genotoxicity for uranyl nitrate, growing root meristems (2-3 cm) of *A. cepa* were treated with uranyl nitrate (0, 5, 10, 20, 25, 50, 75 and 100 μ*M*) for 1hr followed by recovery in tap water. During recovery, the root tips were excised and fixed in acetic acid : ethanol (1:3) at 6hr intervals from 6 to 48hr for MNC assay [10]. At least 6 bulbs per concentration were used.

Experiment III - The growing root meristems of *A. cepa* (2-3 cm) were treated with 100 μ*M* of 5-bromodeoxy uridine (BrdUrd), 0.1 μ*M* of 5 fluorodeoxy uridine (FdUrd) and 5 μ*M* of uridine (Urd) for 20hr (approximately one cell cycle) followed by treatment with uranyl nitrate at concentrations of 0, 25, 50 and 100 μ*M* for 1hr in the presence of 100 μ*M* of deoxy thymidine (dT; Loba Chemie, Mumbai, India) and 5 μ*M* of Urd. After a brief wash the roots were allowed to grow for another round of treatment with dT and Urd for 19hr. Washing the roots under running tap water and treating them with 0.05% of colchicine (Loba Chemie, Mumbai) for 2.5hr terminated the treatments. All the above treatments were performed in plastic receptacles containing 250 mL of experimental solution under continuous supply of air. Roots were washed, excised and fixed in acetic acid:methanol, (1:3), for 7hr and preserved in methanol (70%) at 4°C for SCE analysis[11]. Per concentration, 5 bulbs were used. At least 30 metaphase spreads from 5 or more root meristems for each concentration were analyzed. Following squashing and tapping of root meristem cells under cover slip, since it was not always possible to obtain metaphase spreads with all the sixteen chromosomes separated, only those metaphase spreads having at least twelve or more chromosomes separated were scored for SCE analysis[12].

Analysis of uranium uptake and cellular accumulation - Two experiments were performed to determine uptake and accumulation of uranium in *A. cepa* roots and distribution of uranium into cell wall and soluble fractions. In the first experiment, 4 bulbs of Allium were kept in 20 μM of uranyl nitrate solution (25 mL in each tube) for 7 days with daily change of solution. Uranyl solution was adjusted to *p*H 5.5. Roots were harvested and pooled and then washed thoroughly and divided into two lots. One lot was dried and kept for determination of uranium. The other lot (2g fresh wt) was fractionated into cell wall and soluble fraction following the method of Chao and Dashek [13]. Cell wall fraction yielded 117mg dry weight while the soluble fraction was 12mg dry weight. Each fraction was dried and used for determination of uranium.

Dried root tissue and fractions were grounded in small glass mortar with a glass pestle to fine powder. The samples were digested in 5mL mixture of HNO_3 and $HClO_4$ (5:1) at 75°C. After complete digestion,; excess acid was evaporated to dryness. The content of the tubes was dissolved in 25 mL of distilled water. Uranium was estimated after proper dilution with water and addition of fluran by Laser Fluorimeter [14] at the laboratory of Centre for Advanced Technology, Indore, India, using the Laser Fluorimeter developed at the centre.

Statistical analysis - Cytological scoring of slides were made from coded slides and data were pooled together after decoding. The data on root length, MNC and SCE were analyzed statistically using Student's t test or the tables of Kastenbaum and Bowman[15].

Results

Data with regard to phytotoxicity of uranyl nitrate were evaluated on the basis of root growth, MI, and induction of cells with MNC following a 5 day exposure. Uranyl nitrate (25 μ*M*) inhibited significantly root growth and cell division of *A. cepa* (Table 1). Uranyl nitrate at sub-toxic concentrations (5-20 μ*M*) allowed cell division to progress and induced cells with MNC at significant frequencies as compared to control. Mitotic aberrations induced were c-metaphase and c-anaphase, chromosome aberrations such as bridges or breaks were noted but their frequencies were insignificant.

Fluorimetric analysis revealed that the root of *A. cepa* after 5 days of growth in uranyl nitrate (20 μ*M*), accumulated 596 μg uranium/g dry weight (bioconcentration factor 127). Further, the cell wall fraction and soluble fraction registered 117 and 999 μg uranium/g dry weight, respectively.

Table 1—Effect of uranyl nitrate on root growth, mitotic index (MI) and frequency of cells with micronuceil (MNC) in root meristems of *A. cepa*.

Concentration (μM)	Root length(cm)*	Total cells analyzed	Cells inmitosis	MI	Cells with MNC	MNC/1000cells
0 (control)	3.32 ± 0.03	2049	120	5.80	3	1.55
5	3.47 ± 0.06	1409	80	5.67	5	3.76 [a]
10	3.34 ± 0.05	3220	173	5.57	11	3.61 [a]
15	3.03 ± 0.08	1989	150	7.54	10	5.44 [a]
20	2.72 ± 0.09	3009	185	6.15	13	4.60 [a]
25	No root growth	(Toxic)				

[a] Significantly increased compared to control ($p \leq 0.05$).
* Sample of 25 roots per treatment was measured.

Table 2—Effect of uranyl nitrate on the frequency of cells with chromosome aberrations and MNC in root meristem cells of *A. cepa*.

Concentration (μM)	Recovery hr.	MI ± SEM	No. of ana -telophases analyzed	Cells with chromosome aberrations	Cells with chromosome aberrations ± SEM	Interphases analyzed	Cells with MNC	Cells with MNC/1000 ±SEM
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
0 (Control)	6	8.98 ± 0.2	288	3	1.1 ± 0.48	7235	0	0
	12	10.82 ± 0.36	365	0	0	7923	1	0.14 ± 0.14
	18	9.97 ± 0.23	326	0	0	7805	5	0.67 ± 0.67
	24	9.59 ± 0.44	300	1	0.27 ± 0.27	7199	2	0.28 ± 0.28
	30	9.02 ± 0.33	258	2	0.87 ± 0.87	5837	2	0.38 ± 0.38
	36	10.86 ± 0.36	345	1	0.23 ± 0.23	7962	3	0.49 ± 0.37
	42	8.00 ± 0.69	240	2	0.34 ± 0.34	7283	2	0.34 ± 0.21
	48	8.49 ± 0.45	269	3	1.25 ± 0.59	7979	2	0.27 ± 0.17
5	6	7.23 ± 0.39	238	1	0.52 ± 0.52	7502	3	0.44 ± 0.21
	12	10.87 ± 0.26	348	0	0	7482	1	0.16 ± 0.16
	18	10.29 ± 0.35	335	5	1.36 ± 0.66	7909	3	0.48 ± 0.32
	24	9.20 ± 0.41	366	4	1.12 ± 0.54	8630	5	0.58 ± 0.27
	30	8.45 ± 0.35	288	1	0.35 ± 0.35	8043	0	0
	36	7.62 ± 0.55	297	3	1.17 ± 0.58	8770	5	0.82 ± 0.46
	42	9.61 ± 0.21	370	4	1.3 ± 0.48	9399	4	0.46 ± 0.22
	48	9.86 ± 0.36	382	2	0.54 ± 0.34	9247	5	0.47± 0.24
10	6	8.22 ± 0.46	351	7	1.84 ± 0.46	8398	0	0
	12	8.70 ± 0.48	344	1	0.29 ± 0.29	8963	0	0
	18	8.31 ± 0.38	294	8	2.58 ± 1.0[a]	9013	5	0.59 ± 0.35
	24	8.19 ± 0.48	283	5	1.5 ± 0.77	8338	2	0.25 ± 0.25
	30	6.26 ± 0.77	240	3	0.95 ± 0.43	8753	8	0.95 ± 0.43
	36	6.54 ± 0.42	244	1	0.37 ± 0.37	8348	1	0.12 ± 0.12
	42	10.08 ± 0.37	445	5	1.12 ± 0.38	9614	7	0.82 ± 0.38

Contd.

Table 2—Effect of uranyl nitrate on the frequency of cells with chromosome aberrations and MNC in root meristem cells of *A. cepa*.

Concentration (μM)	Recovery hr.	MI ± SEM	No. of ana -telophases analyzed	Cells with chromosome aberrations	Cells with chromosome aberrations ± SEM	Interphases analyzed	Cells with MNC	Cells with MNC/1000 ± SEM
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	48	7.75 ± 0.36	260	4	1.44 ± 0.71	8636	7	0.88 ± 0.22
20	6	7.04 ± 0.25	280	10	4.55 ± 1.94[a]	9079	4	0.50 ± 0.16
	12	9.27 ± 0.28	443	5	1.37 ± 0.55	10350	2	0.10 ± 0.10
	18	7.79 ± 0.72	281	5	2.87 ± 0.72[a]	8181	5	0.7 ± 0.28
	24	8.16 ± 0.13	376	5	1.24 ± 0.6	9674	4	0.42 ± 0.21
	30	6.29 ± 0.43	219	3	1.31 ± 0.58	7760	3	0.24 ± 0.15
	36	8.9 ± 0.46	235	2	1.1 ± 0.63	6248	1	0.18 ± 0.18
	42	NCD	--	--	--	--	--	--
	48	8.1 ± 0.28	338	2	0.93 ± 0.64	9139	5	0.56 ± 0.3
25	6	6.74 ± 0.77	282	3	2.23 ± 1.4	8170	2	0.47 ± 0.47
	12	7.11 ± 0.3	306	0	2.0 ± 0.8	8226	6	0.69 ± 0.3
	18	6.72 ± 0.51	312	0	3.93 ± 0.97	7949	4	0.69 ± 0.31
	24	6.78 ± 0.41	304	1	2.2 ± 0.56	6759	6	0.92 ± 0.45
	30	8.73 ± 0.45	307	2	2.45 ± 0.38	8105	11	1.36 ± 0.27
	36	7.93 ± 0.9	267	1	1.19 ± 0.29	7505	8	1.3 ± 0.67
	42	7.16 ± 0.57	294	2	1.09 ± 0.2	8009	5	0.66 ± 0.24
	48	7.06 ± 0.6	275	3	0.79 ± 0.79	7597	5	0.79 ± 0.23
50	6	NCD	--	--	--	--	--	--
	12	7.95 ± 0.31	251	18	6.95 ± 1.27[a]	7054	5	0.76 ± 0.35
	18	7.34 ± 0.31	258	2	1.18 ± 0.69	8002	3	0.8 ± 0.3
	24	6.83 ± 0.51	233	8	4.23 ± 2.01	8386	12	1.68 ± 0.16
	30	7.9 ± 0.18	282	3	1.11 ± 1.11	8183	6	1.11± 0.2
	36	6.94 ± 0.43	207	9	5.25 ± 1.43	7211	5	1.63 ± 0.51
	42	7.73 ± 0.68	223	17	7.51 ± 2.13	7458	13	1.94 ± 0.43
	48	7.79 ± 0.42	253	9	2.91 ± 1.2	7382	7	1.18 ± 0.21
75	6	5.49 ± 0.17	214	3	0.48 ± 0.44	8431	0	0
	12	5.16 ± 0.34	200	10	7.82 ± 4.01[a]	8022	2	0.26 ± 0.26
	18	6.3 ± 0.21	215	6	3.8 ± 0.82	8275	1	0.27 ± 0.27
	24	7.58 ± 0.33	260	16	6.77 ± 0.78 a	7380	6	0.61 ± 0.31
	30	8.59 ± 0.32	288	16	5.98 ± 2.94	7564	10	1.45 ± 0.73
	36	8.73 ± 0.28	214	9	6.87 ± 3.53	8223	6	0.71 ± 0.36
	42	9.93 ± 0.38	339	15	3.74 ± 0.95	7947	9	1.24 ± 0.39
	48	10.85 ± 0.44	386	12	3.5 ± 0.14[a]	7935	10	1.34 ± 0.51
100	6	6.48 ± 0.3	249	4	1.76 ± 1.11	8010	4	0.67 ± 0.67
	12	6.98 ± 0.6	260	9	3.71 ± 1.74	7403	8	1.07 ± 0.34
	18	6.48 ± 0.27	239	6	2.3 ± 0.7	6450	2	0.28 ± 0.18
	24	8.71 ± 0.63	297	16	6.56 ± 1.65[a]	7687	12	1.95 ± 0.62
	30	7.3 ± 0.35	288	18	7.09 ± 1.02[a]	7992	14	1.99 ± 0.4
	36	7.7 ± 0.7	302	16	6.9 ± 1.21[a]	7952	10	1.2 ± 0.22
	42	7.35 ± 0.26	310	8	2.7 ± 1.5	8212	10	1.16 ± 0.42
	48	7.33 ± 0.8	302	1	0.5 ± 0.5	8004	2	0.27 ± 0.17

Chromosome aberrations include ana-telophase with chromosome fragment or bridge.
NCD: No cell division
[a]Significantly increased compared to control ($P \leq 0.05$)

Table 3—Dose-response induction of SCE in root meristem cells of *A. cepa* by uranyl nitrate.

Concentration (μM)	No. of metaphases analyzed (no. of roots)	No. of chromosomes analyzed	Total no. of SCEs	SCE/cell* $\pm$ SD	SCE/chromosome* $\pm$ SD
0 (Control)	30 (6)	392	930	38.06 $\pm$ 5.44	2.37 $\pm$ 0.34
25	39 (7)	524	1485	45.12 $\pm$ 6.89[a]	2.80 $\pm$ 0.42[a]
50	49 (8)	665	1999	48.05 $\pm$ 4.80[a]	2.99 $\pm$ 0.33[a]
100	57 (11)	756	2181	46.15 $\pm$ 5.55[a]	2.88 $\pm$ 0.33[a]

[a]Significantly increased compared to control ($p \leq 0.05$)
* Values are mean $\pm$ SD of number of metaphase cells or chromosomes analyzed

Data on the effect of uranyl nitrate on the frequencies of cells with chromosome aberration and MNC in root meristem cells of *A. cepa* have been presented in Table 2. Growing root meristems were treated with uranyl nitrate at different concentrations (5-100 μM) for 1hr followed by recovery in tap water. MI values remained approximately same in root meristems treated with uranyl nitrate (5-10μM) and control. A gradual fall in MI was evident with the increase of concentration of uranyl nitrate (20μM or more). Cells with chromosome aberrations such as chromosome bridge and fragments were recorded at low frequencies, exceptionally significant in some cases. Frequencies of cells with MNC determined for uranyl nitrate at various concentrations and recovery hours were insignificant as compared to that of control

Uranyl nitrate was tested for induction of SCE at 25, 50 and 100μM. Frequency of SCE was calculated either per cell or per chromosome (Table 3). In both the cases induction of SCEs were higher than controls. Increase of SCE induced by uranyl nitrate followed a dose response, best fitted to a polynomial curve($r^2 = 1$). Frequency of SCE calculated either per cell or per chromosome was significant ($p \leq 0.05$).

Discussion

Conflicting reports are there on phytotoxicity of uranium in soil[16]. However, present study clearly indicated toxic effect of uranyl nitrate on root growth of *A. cepa.*

Effect of uranyl nitrate on viability, cell kinetics, MNC, chromosome aberrations and SCE in Chinese hamster ovary cells has been investigated[17]. Inhibition of cell viability (50%) has been observed at 49μM, while decrease in cell kinetics and increase in frequencies of chromosome aberration, MNC and SCE has been reported between 10 to 300 μM concentration of uranyl nitrate[17]. Thus uranyl nitrate has been shown to be genotoxic. Likewise, uranyl flouride has been found positive for induction of chromosome aberration in male mouse germ cells[18]. Although uranyl ion (UO_2^{2+}) by itself is not radioactive, its decay products such as thorium, radium and particularly radon are alpha radiation emitters, and are both cytotoxic and genotoxic[19]. According to estimation, in order to produce chromosomal damage, a dose rate of 18 cGy of radon exposure for more than 8 hr is needed[20]. Genotoxicity of UO_2^{2+} in the range of 10-300 μM with no detectable radioactivity has therefore been assumed to be due to chemical toxicity of UO_2^{2+} rather than to its radioactivity. Chemical toxicity of UO_2^{2+} has been attributed to its binding to phosphate groups of DNA. High affinity of UO_2^{2+} to phosphate groups of membrane phospholipids has been reported[21,22].

Phytotoxicity of uranyl nitrate has been well established in the present study. Analysis of uranyl uptake further provided evidence that UO_2^{2+} entered the root cells, crossing the cell wall and membrane. Although uranyl nitrate induced significantly MNC in root meristem cells of *A. cepa,* from the point of genetic toxicology importance was not attached to the findings owing to the severity of the treatment conditions (chronic treatment). Chronic treatments might result in chromosome stickiness, accompanied by a high frequency of cells with MNC, a cytotoxic manifestation, rather than due to genotoxicity. Experiment II was thus designed to eliminate cytotoxicity by treating the growing root meristems only for 1hr followed by recovery when the cells receiving the genotoxic insult would show aberrations at a later period of time as the cell-cycle progressed[23]. Since the frequencies of chromosome aberrations or MNC, induced by uranyl nitrate was either inconsistent or insignificant, the data could not implicate uranyl nitrate to be either clastogenic or aneugenic[10].

SCE assay has been shown to be one of the sensitive short-term genotoxicity assay owing to its ability to

detect genotoxins at very low doses[24]. Uranyl nitrate (25-100μ*M*) enhanced SCE significantly over the control that followed a dose-response in the present study. Although the exact mechanisms underlying the induction of SCE is still a matter of discussion[12], SCE are generally believed to represent the interchanges of DNA replication products at apparently homologous loci, involve DNA breakage and reunion[25]. SCE assay thus provided evidence that uranyl nitrate could be genotoxic, possibly through interacting with DNA and/or interfering with DNA replication-repair process. UO_2^{2+} might interfere with Ca fluxes and functions, including membrane and macromolecular integrity leading to some of the lesions that warrant further investigation.

Efforts made in the present study in standardization, calibration and validation of the *Allium* assay in Indian conditions would greatly benefit those Departments, which are concerned with the risk assessment of solid wastes and fly ash. This test based on *Allium* assay could be the first step for polluted soils (nuclear wastes, metals, pesticides, hydrocarbons etc.) also. Since it is simple not requiring too much expertise or equipment and is fairly quick, it could be ideal for inclusion in protocols for risk assessment of contaminated water, soil or solid waste, and therefore best suited to the conditions prevailing in developing countries.

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