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LOW DOSE GENOTOXICITY OF *4–BROMO–N,N–DIETHYL–5,5–DIMETHYL–2,5–DIHYDRO–1,2–OXAPHOSPHOL–2–AMINE 2–OXIDE* IN MICE BONE MARROW CELLS AND *ALLIUM CEPA* L. ROOT TIP CELLS

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The chemistry of organophosphorus compounds is a subject of increasing interest and different new compounds have been synthesized. There are data that some known organophosphates are mutagens. Oxaphosphole derivatives possess biological activity and might influence proliferating cells. Bioassays are currently used in ecotoxicology to investigate the effects and mechanisms of action of new chemicals. To provide a broad coverage of the mutagenic potential of a chemical, information on different experimental test-systems is required. Genotoxicity assays are usually performed at high doses, but humans are exposed to most environmental chemicals at low doses. The objective of this study was to determine the genotoxicity of low doses (2.82×10^{-6} µg/kg and 2.82×10^{-9} µg/kg, corresponding to concentrations 10^{-12}M and 10^{-15}M) of *4–bromo–N,N–diethyl–5,5–dimethyl–2,5–dihydro–1,2–oxaphosphol–2–amine 2–oxide* (Br–oxph) in ICR

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mice bone marrow cells and *Allium cepa* L. root tip cells. Treatment with Br–oxph for 3 h produced alterations in the mitotic index *in Allium cepa* cells and induced chromosome aberrations in both test systems. These effects remained 48 h after the treatment. The data from the study showed the existence of cytotoxic and genotoxic effects of Br–oxph at tested doses.

Key words: *Allium cepa* L. root tip cells, Br–containing oxaphosphole derivative, genotoxicity, ICR mice bone marrow cells.

INTRODUCTION

Organophosphorus compounds are widely used and different new compounds have been synthesized (BREL, 2008; LEBLOND *et al.*, 2002). Some known organophosphates are mutagens (MATHEW *et al*., 1990**;** LIEBERMAN *et al*., 1998). Oxaphosphole derivatives possess biological activity (ENCHEV *et al*., 1986) and might influence proliferating cells. Experimental bioassays are currently used in ecotoxicology to provide information for risk assessment evaluation of new chemicals and to investigate their effects and mechanisms of action (REPETTO *et al*., 2001). Cytotoxicity and genotoxicity can be monitored through cytological parameters (EGITO *et al*., 2007). To establish the mutagenicity of any chemical, the damaging effects should be evaluated by different tests (SEETHARAMA RAO and NARAYANA, 2005). Rodent animal bioassays are valuable tools for investigating toxicity of chemicals (ROLDAN-ARJONA *et al*., 1991). The *Allium cepa* assay is an efficient test for chemical screening (FERETTI *et al*., 2007) and also provides comparable results to a number of other test systems (FISKESJÖ, 1985).

The objective of this study was to determine the genotoxicity of low doses ($2.82x10^{-6}$ µg/kg and $2.82x10^{-9}$ µg/kg, corresponding to concentrations 10^{-12}M and 10^{-15}M) of *4–bromo–N,N–diethyl–5,5–dimethyl–2,5–dihydro–1,2–oxaphosphol–2–amine 2–oxide* (Br–oxph) in ICR mice bone marrow cells and *Allium cepa* L. root tip cells.

MATERIALS AND METHODS

Compound Tested

The compound tested (Br–oxph) was synthesised in the Laboratory of Organic Chemistry of the University of Shumen (Bulgaria) (ANGELOV and ENCHEV, 1987). Our preliminary study (KALCHEVA *et al*., 2009) revealed genotoxicity of Br-oxph (10^{-9} M, 10^{-6} M and 10^{-3} M). Some chemicals exerted physiological effects at range 10^{-8}–10^{-14}M (RAYCHOUDHURY *et al*., 1999; SERGEEVA *et al*., 1996) and in present study we tested Br–oxph at similar concentrations – 10^{-12}M and 10^{-15}M. The solutions were prepared just before each treatment. We choose 3 h-treatment period according to papers that reported the first appearance of DNA damage after a certain treatment period (MIYAMAE *et al*., 1997; WILLIAMS and OMOH, 1996).

Animal test system.

ICR mice (2n = 40) were used. The animals were kept at temperature of 24°±2°C; 12/12 hours of light/dark cycle. Water and food were supplied *ad*

libitum. Eight experimental groups were organized, which included 10 animals (5 males and 5 females) each. Mice were treated i.p. (1ml per 100 g/bw) with Br–oxph solutions ($2.82x10^{-6}$ μg/kg and $2.82x10^{-9}$ μg/kg, corresponding to concentrations 10^{-12}M and 10^{-15}M). As a negative control served mice, injected with 0.9% NaCl. Methyl methanesulfonate (MMS) ($1.10x10^{2}$ μg/kg bw, CAS 66–27–3) was used as a positive control. The experimental animals were sacrificed 3 h, 24 h and 48 h after administration of Br–oxph, and the controls – 24h after administration of MMS or 0.9% NaCl.

Bone marrow preparations were produced (PRESTON *et al*., 1987). The animals were injected i.p. with colchicine (4mg/kg) 90 min prior to sacrifice. Bone marrow cells were flushed from femur and hypotonised in 0.075M KCl at 37ºC for 25 min. The cells were fixed in methanol–acetic acid (3:1), air dried and stained with 5% Giemsa stain. The mitotic index was calculated by counting the number of mitotic cells in 1000 cells per animal. 50 well–spread metaphases per animal were analyzed and chromosome aberrations were scored: chromatid and isochromatid breaks, centromeric and telomeric fusions, and fragments; number of tetraploid metaphases (as a result of spindle abnormalities), chromosome gaps (defined as achromatic lesions (ITO and ITO, 2001) and apoptotic cells (identificated by typical fragmented condensed nuclei (GORNEVA *et al*., 2005)).

Plant test system.

Allium cepa L. cv. Shtuttgarter Riesen seeds (2n = 16) were used. Thirty seeds were placed on filter paper in Petri dishes (containing 5 ml of distilled water), the dishes were sealed and incubated at 25±1°C for 72 h. Germinated seeds with equal length of roots (~ 1 cm) were used. Three experiments were made: 1) 5 ml of tested solutions (10^{-12}M and 10^{-15}M) were added to the dishes, and incubated at 25±1°C for 3 h; 2) and 3) in order to establish whether any recovery from the possible damage takes place, the first experiment was repeated, but followed by two recovery periods: treated seedlings were placed in Petri dishes, containing 5 ml of distilled water, and incubated at 25±1°C for 24 h and for 48 h. Distilled water was used as a negative control. Methyl methanesulfonate (MMS) (10^{-4}M for 24 h, CAS 66–27–3) was used as a positive control.

Microscope preparations were produced (RANK, 2003). The roots were fixed in Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 minutes, hydrolyzed in 3N HCl for 8 min and in 45% acetic acid for 30 min at room temperature and stained for 30 min in 1% aceto-orcein. The terminal root tips (1–2 mm) were cut off and squashed in 45% acetic acid. The microscopic analysis included the mitotic index and scoring of aberrant cells. Each sample consisted of six root meristems. At least 600 cells of each root meristem were analyzed. The mitotic index was calculated by counting the number of mitotic cells in 100 cells/root. The index of each phase of mitotic division was calculated as a ratio between the cell number in the respective period and the number of dividing cells, in percents. The following categories of aberrations were scored: chromosome bridges and fragments, vagrant chromosomes, aberrant metaphases and anaphases in dividing cells; micronuclei in interphase cells and binucleated cells.

Statistical analysis

The mean +/– standard deviation for each group was calculated, and the data were statistically analyzed for their significance by Student's t–test.

RESULTS AND DISCUSSION

Effects on animal test-system

In Table 1 we presented the results of the influence of Br–oxph on mitotic index and chromosome abnormalities. We scored the chromosome aberrations only when the frequency of mitotic cells was enough for determination of at least 50 well spread metaphases per animal. Br-oxph triggered apoptosis in bone marrow cells and this apoptogenic effect remained during two recovery periods. When apoptosis was too high, it was impossible to score the mitotic index and chromosome aberrations. Both Br–oxph solutions decreased the mitotic activity, but differences with the control were not significant. The treatment for 3 h significantly increased the level of chromosome aberrations in mitotic cells – about fourfold. 24 h and 48 h after the treatment the frequency of chromosome aberrations decreased, but still remained significantly higher in comparison with the control. Chromatid breaks were the most frequent kinds of aberrations. We found also isochromatid breaks, centromeric and telomeric fusions, but at a lesser frequency. The percent of cells with gaps and tetraploid cells was not affected by the treatment.

Table 1a. Cytogenetic analysis of bone marrow cells of mice 3 h, 24 h and 48 h after treatment with Br-oxph at doses of $2.82x10^{-6}$ μg/kg and $2.82x10^{-9}$ μg/kg

Dose	Time after	MI, %	Metaphase	Type of aberrations				
(μg/kg)	treatment	(mean± SD)	scored (n)	ICB (n)	CB (n)	c/c (n)	t/t (n)	Fr (n)
NC	24h	1.97 ± 1.03	500	1	21	1	1	0
$2.82x10^{-9}$	3h	1.73 ± 0.54	300	12	48	2	3	0
	48h	1.78 ± 0.60	250	12	32	4	0	0
$2.82x10^{-6}$	3h	1.82 ± 0.44	250	8	50	0	0	0
	24h	1.02 ± 0.83	250	5	45	0	1	0
	48h	1.39 ± 0.14	250	6	27	0	3	0
PC	24h	0.67 ±0.29**	400	28	106	1	2	0

Table 1b. Cytogenetic analysis of bone marrow cells of mice 3 h, 24 h and 48 h after treatment with Br-oxph at doses of $2.82x10^{-6}$ μg/kg and $2.82x10^{-9}$ μg/kg

Dose (μg/kg)	Time after treatment	Cells with aberations ‡, % (mean ±SD)	Cells with gaps, % (mean ±SD)	Tetraploid metaphases,% (mean ± SD)
NC	24h	4.4 ± 3.10	3.0 ± 2.71	1.20 ± 1.69
$2.82x10^{-9}$	3h	18.67 ± 7.23 ***	2.33 ± 2.34	1.00 ± 1.10
	48h	16.4 ± 6.54 **	4.80 ± 2.28	0.80 ± 1.10
$2.82x10^{-6}$	3h	18.8 ± 3.35 ***	4.00 ± 2.82	0.80 ± 1.10
	24h	16.8 ± 8.07 **	6.00 ± 5.66	0.40 ± 0.89
	48h	10.8 ± 2.28 ***	6.80 ± 4.38	0.80 ± 1.10
PC	24h	20.44 ± 8.99 ***	2.25 ± 1.98	0.44 ± 1.33

Data are expressed as means ± standard deviation (mean ± SD); *P≤0.05, **P≤0.01, *** P≤0.001; n – number; MI – Mitotic index; NC – Negative control (0.9% NaCl); PC – Positive control (Methyl methanesulfonate, 0.11 mg/kg bw); CB – Chromatid breaks; ICB – Isochromatid breaks chromosoms; c/c – Centromeric fusions; t/t – Telomeric fusion; Fr – Fragments; ‡ – some of metaphases were with more than one aberration

Effects on plant test-system

Table 2 shows the results of the influence of Br–oxph on the mitotic index and on the frequency of mitotic phases. The mitotic index value upon treatment with 10^{-12}M for 3 h was slight decreased in comparison with those observed in the control. Br–oxph at the concentration of 10^{-15}M caused significant decrease in the mitotic activity of about 40%. During the recovery period of 24 h the cell division in root tips treated with both Br–oxph solutions were inhibited compared with the control, but differences were not significant. After recovery of 48 h both Br-oxph solutions significantly inhibited the cell division in comparison with the control. Br–oxph changed the mitotic phase distribution.

The treatment with Br–oxph for 3 h significantly increased the level of chromosome aberrations in mitotic cells (Table 3). During the recovery for 24 h the level of chromosome aberrations was about fivefold higher than the control. The frequency of chromosome aberrations increased during recovery for 48 h in comparison with those observed after 24 h. Br–oxph induced a variety of different chromosome aberrations in mitotic cells. Anaphases with spindle abnormalities and anaphases and telophases with vagrant chromosomes were the most frequent kinds of aberrations. We found anaphase/telophase fragments and bridges, but at a lesser frequency. C–mitoses were observed only in one sample. There were also some abnormalities in interphase cells – micronuclei and binucleated cells but differences with the control were not significant.

Table 2. Effect of treatment with Br-oxph (10^{-15}M and 10^{-12}M) for 3 h and during 24 h and 48 h recovery on mitotic index and phase indices in root tip meristems of Allium cepa L.

Time after treatment	Sample	Number of cells analysed	MI, % (mean ± SD)	Prophase, PhI% (mean ± SD)	Metaphase, PhI% (mean ± SD)	Anaphase, PhI% (mean ± SD)	Telophase, PhI% (mean ± SD)
0h	NC	3993	6.79 ± 2.31	43.01 ± 7.22	20.19 ± 4.93	11.25 ± 4.09	25.55 ± 10.33
	10^{-15}M	3811	4.09 ± 1.58*	40.84 ± 16.77	26.91 ± 10.20	12.88 ± 9.37	19.37 ± 17.35
	10^{-12}M	3742	5.14 ± 0.61	24.67 ± 9.51**	26.17 ± 4.28*	19.73 ± 6.22*	29.43 ± 7.05
24h	NC	3866	7.83 ± 2.60	33.44 ± 10.08	27.46 ± 4.43	16.34 ± 5.75	22.76 ± 9.38
	10^{-15}M	3809	5.01 ± 2.33	31.76 ± 7.52	38.98 ± 9.20*	14.61 ± 9.29	14.64 ± 10.31
	10^{-12}M	3835	5.15 ± 1.98	26.82 ± 7.83	36.95 ± 10.66	12.47 ± 8.39	23.76 ± 5.30
48h	NC	3698	6.61 ± 1.08	28.18 ± 4.90	27.73 ± 5.05	16.89 ± 3.02	27.20 ± 4.30
	10^{-15}M	3711	3.94 ± 1.14**	34.94 ± 16.29	28.90 ± 9.95	16.38 ± 12.95	19.78 ± 10.35
	10^{-12}M	3772	4.55 ± 1.20*	34.27 ± 4.06*	27.67 ± 8.88	18.46 ± 9.35	19.60 ± 5.32*
24h	PC	4012	3.86 ± 1.49**	27.93 ± 8.17	29.97 ± 11.77	19.98 ± 6.78	23.05 ± 9.56

Data are expressed as means ± standard deviation (mean ± SD); *P≤0.05, **P≤0.01; MI – Mitotic index; PhI – Phase index; NC – Negative control (distilled water); PC – Positive control (Methyl methanesulfonate, 11mg/l)

Table 3. Effect of treatment with Br-oxph (10^{-15}M and 10^{-12}M) for 3 h and during 24 h and 48 h recovery on abnormalities in mitotic and interphase cells in root tip meristems of Allium cepa L

		Abnormalities in mitotic cells							Abnormalities in interphase cells			
Time after treatment	Sample	Cells analysed (n)	SA (n)	V (n)	CM (n)	Br (n)	Fr (n)	Total % (mean ± SD)	Cells analysed (n)	Bn (n)	Mn (n)	Total % (mean ± SD)
0 h	NC	269	2	2	0	1	0	1.94 ± 1.13	3724	2	4	0.16 ± 0.26
	10^{-15}M	156	4	6	2	0	0	7.56 ± 4.50 *	3655	2	3	0.14 ± 0.07
	10^{-12}M	192	11	11	0	1	1	12.25 ± 6.83 **	3550	5	2	0.20 ± 0.13
24 h	NC	303	0	3	0	2	1	2.00 ± 2.60	3563	1	2	0.09 ± 0.09
	10^{-15}M	191	8	8	0	0	0	10.47 ± 8.92 *	3618	3	2	0.14 ± 0.07
	10^{-12}M	199	5	16	0	1	0	10.03 ± 8.02 *	3636	9	3	0.33 ± 0.58
48 h	NC	244	1	2	0	1	0	1.46 ± 1.74	3454	6	2	0.22 ± 0.29
	10^{-15}M	146	8	7	0	2	0	11.79 ± 4.72***	3565	12	2	0.39 ± 0.53
	10^{-12}M	171	4	16	0	2	0	14.27 ± 11.49 *	3601	1	3	0.11 ± 0.14
24 h	PC	156	10	16	0	6	1	20.30 ± 11.27 **	3563	18	21	1.11 ± 0.73**

Data are expressed as means ± standard deviation (mean ± SD); *P≤0.05, **P≤0.01, *** P≤0.001; n – number; NC – Negative control (distilled water); PC – Positive control (Methyl methanesulfonate, 10^{-4}M); SA – Spindle abnormalities; V – Vagrant chromosomes; CM – C–mitoses; Br – Bridges; Fr – Fragments; Bn – Binucleated cells; Mn – Micronuclei

DISCUSSION

The continued production and release of different chemicals into the environment has resulted in the need for evaluation of their potential genotoxic

effect. Although humans are chronically exposed to most environmental chemicals at low doses, genotoxicity assays are usually performed at high doses with short treatment period (MASAMURA *et al.*, 2003). Distinct biological assay was not always able to present all aspects of the influence of chemicals and to answer the requirements for high sensitivity (KLESZCZYNSKA *et al*., 2003). Taking this into account, we tested the genotoxicity of low doses of new synthesized oxaphosphole (Br–oxph) in animal and plant test systems.

Br–oxph induced chromosome aberrations in bone marrow cells of ICR mice and *Allium cepa* root tips after treatment for 3 h. Our results are in accordance with data of MIYAMAE *et al*. (1997) and WILLIAMS and OMOH (1996) that described the onset of DNA damage after 3h-treatment period. This effect decreased, but remained during 24 h and 48 h recovery in bone marrow cells. Interestingly, in plant test system the level of chromosome aberrations was higher 48 h after the treatment in comparison with the level of aberrations immediately after the treatment for 3 h. A number of factors can influence the time of appearance of chemically induced aberrations such as compound solubility, rate and distribution of biotransport, availability at the target site as influenced by time and cell permeability (MCFEE and TICE, 1990).

Genotoxic stresses might activate intracellular signaling molecules, which lead to growth arrest, DNA repair, and/or apoptosis (GRISHIN *et al*., 2001). Induction of apoptosis in mice bone marrow cells was a sign for toxicity of Br–oxph. In animal test-system chromatid breaks were more frequent kinds of aberrations. The observed fusions between chromatids can be initiated by simultaneous breakage of the two chromatids or by the loss of telomere capping (MURNANE and SABATIER, 2004). The occurrence of abnormal anaphases and vagrant chromosomes indicated that solutions caused disturbances of spindle formation in plant test-system (RANK, 2003). Relatively low percent of bridges and fragments in *Allium cepa* root tips was in accordance with the relatively low percent of cells with micronuclei (ALBERTINI *et al*., 2000).

The induction of chromosome aberrations demonstrates that Br–oxph has a genotoxic effect. However, compared with the positive control with MMS, Br–oxph had less genotoxic effect. The abnormalities were not peculiar to any concentration or time after the treatment. We noticed also a great variability in the individual response to treatment in the experimental groups. Obviously, some individuals had a higher potential to cope with the compound tested than others. Because of the high variations between the single experiments some data were not significant. We observed genotoxic potential of Br–oxph at low doses – 2.82×10^{-6} μg/kg and 2.82×10^{-9} μg/kg. However, these doses are within the range reported by physiologists to be capable of altering important cell signaling events (KONG *et al.*, 1997; PALMINA *et al.*, 1997).

The results demonstrated that Br–oxph exerted mitodepressive effect in *Allium cepa* cells. The suppression of mitotic activity was often used for tracing cytotoxicity (SMAKA–KINCL *et al*., 1996). Interestingly, 10^{-15}M caused stronger inhibition of cell division than 10^{-12}M. According to MUÑOZ-DE-TORO *et al.* (2005)

for many of the measurements, the lower dose had no detectable effect, but for several it did, and in a few cases the scientists noted that the lower dose actually caused a larger effect than the higher dose. The interference in the cell cycle kinetics may also indicate cytotoxic influence (AMIN, 2002).

Most of the procedures used in toxicology are carried out on mammals, but public pressure to minimize the use of vertebrates in ecotoxicity testing (WALKER *et al.*, 1998). In present study, we verified that *Allium cepa* is a sensitive biosensor for the genotoxic potential of oxaphospholes.

CONCLUSION

The data obtained with ICR bone marrow cells and *Allium cepa* root tip cells showed the existence of cytotoxic and genotoxic effects of low doses Br–oxph ($2.82x10^{-6}$ μg/kg and $2.82x10^{-9}$ μg/kg, corresponding to concentrations 10^{-12}M and 10^{-15}M) for 3 h. These effects remained 48 h after the treatment.

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NISKA DOZA GENOTOKSIČNOSTI *4–BROMO–N,N–DIETIL–5,5–DIMETIL–2,5–DIHIDRO–1,2–OKSAFOSFOL–2–AMINO 2–OKSIDA* U ĆELIJAMA KOŠTANE SRŽI MIŠA I ĆELIJAMA KORENA *ALLIUM CEPA* L.

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I z v o d

Hemija organofosfornih componenti je subjekat povećanog interesa a različite nove komponente su sintetisane. Oksafosfol derivati su biološki aktivni i mogu da utiču na proliferaciju ćelija. Objekat ovih istraživanja je određivanje genotoksičnosti u niskim dozama ($2.82x10^{-6}$ μg/kg i $2.82x10^{-9}$ μg/kg, odgovarajućih koncetrancija 10^{-12}M I 10^{-15}M) *4–bromo–N,N–diethol–5,5–dimetil–2,5–dihidro–1,2–okasfofhol–2–amino 2–oksida* (Br–oxph) u ICR ćelijama koštane srži miša i *Allium cepa* L. ćelijama korena. Tretman sa Br–oxph 3 h dovodi do promena u indeksu mitoze *kod Allium cepa* ćelija i indukuje hromozomske aberacije u oba sistema. Ovi efekti se zadržavaju 48 h nakon tretmana. Podaci iz ispitivanja pokazuju postojanje citotoksičnog i genotoksičnog efekta Br–oxph pri testiranim dozama.

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