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Comparative Toxicities of Benzene, Chlorobenzene, and Dichlorobenzenes to Sea Urchin Embryos and Sperm

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The leukemogenicity and myelotoxicity of benzene are well-known and the major cause of benzene's banning from most industrial applications (International Agency for Research on Cancer 1974a; Gad-el-Karim et al. 1984). Various benzene derivatives such as alkylbenzenes and chlorobenzenes, however, continue to be used as chemical intermediates, solvents, pesticides, etc. in spite of incomplete knowledge of their chronic toxicity (Colson 1979). Several of the chlorinated benzenes are known to be porphyrogenic in animals and humans (Rimington and Ziegler 1963). There are two case reports of human carcinogenicity from long-term exposure to dichlorobenzenes (International Agency for Research on Cancer 1974b; Girard and Revol 1970), but animal studies for carcinogenicity, mutagenicity, and developmental toxicity of dichlorobenzenes (Hayes et al. 1985; National Research Council 1983; Loeser and Litchfield 1983) and of monochlorobenzene (John et al. 1984) are all essentially negative.

This study was designed to obtain comparative data on developmental, genetic and reproductive toxicities of benzene (B), chlorobenzene (CB) and dichlorobenzenes (o-, m-, and p-DCB) in the sea urchin bioassay. This test system, as reported previously (Pagano et al. 1986) permits observation of a number of biological endpoints on mitotic activity, embryogenesis and fertilization, and thus provides multiparametric toxicological data on xenobiotics.

MATERIALS AND METHODS

Mediterranean sea urchins (Paracentrotus lividus) were caught in the Bay of Naples by the staff of the Zoological Station, Naples. Gametes were obtained and embryos were cultured as described previously (Pagano et al. 1983; 1986). The experiments were run in natural filtered (Ø = 0.45 μm) seawater (fsw), at 20°C and pH 8.2 to 8.4; salin-

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ity was 37.6 to 37.8 g/l and density 1.03. The developmental toxicity of agents was tested by rearing the embryos in their presence from zygote to pluteus larval stage, occurring 48 h after fertilization. Sperm inactivation experiments (Pagano et al. 1986) were run by suspending a fixed aliquot of sperm in fsw to which were added increasing levels of test agents; sperm suspensions then were used to inseminate untreated eggs after subsequent time intervals. In every experiment, the following treatment schedules were performed: a) untreated control (blank); b) standard control (solvent: 0.01% DMSO); c) B; d) CB; e) o-DCB; f) m-DCB; g) p-DCB. The concentrations of the agents were all expressed as nominal levels. All chemicals were ≥99% pure and were purchased from Merck, Darmstadt, W. Germany (DMSO and B) or from Aldrich, Milwaukee, Wis. (CB and DCBs). The endpoints of embryo exposure consisted of observing the frequencies of: (1) developmental defects, involving skeletal and/or gut malformations; (2) mitotic abnormalities, including total mitotic aberrations (TMA), mean number mitoses per embryo (MPE), % interphase embryos (IE), and metaphase/anaphase ratio (M/A). In this study, an additional cytogenetic endpoint consisted of % anaphase aberrations (AA), referring only to anaphase figures. Following sperm exposure, the outcomes were scored for fertilization success (% fertilized eggs), and quantitated for offspring quality (developmental defects and mitotic abnormalities) (Pagano et al. 1986). The results were analyzed for statistical significance by Yates' corrected χ^2 test (2xk tables), and by Student's |t| test (Armitage 1971).

RESULTS AND DISCUSSION

The sea urchin bioassay was used to test B, CB and o-, m-, and p-DCB on embryos from P. lividus for 48 h of exposure. The results are shown in Table 1 on embryo exposure to the test agents at levels ranging from 10^{-6} to 10^{-4}M with the exception of B, which was only tested at the nominal level of 10^{-6}M. Benzene showed a significant increase in developmental defects. The metaphase/anaphase (M/A) ratio was elevated indicating a partial blockage of metaphase; however, there was no increase in mitotic aberrations.

For CB, developmental defects were only increased when its level was raised to 10^{-4}M; regarding mitotic abnormalities, CB increased the M/A ratio; there was no dose-related increase in TMA; however, the % AA was increased significantly for 10^{-5} and 10^{-4}M CB. In the case of the DCBs, o-DCB demonstrated a dose-related response (r^2=0.82) in developmental defects, mainly consisting of highly reduced skeletal size; however, no effect on the mitotic process was evident except for an increase in M/A ratio. For m-DCB there was a strong increase in developmental defects, as well as mitotic abnormalities. The latter

consisted both of mitotoxicity (decreased MPE, r^2=0.83; increased IE and M/A) and aberrations for 10^{-4}M m-DCB (mainly, multiple breaks and scattered chromosomes). The apparent lack of a dose-related trend in developmental defects is due to the disappearance of a fraction of the pathologic embryos from evident cytolysis which, however, was not quantified. Ortho- and p-DCB showed lesser developmental and mitotic toxicity, the latter primarily consisting of increased M/A ratio.

When the chemicals were tested on P. lividus sperm, an entirely different set of results was obtained. First, regarding sperm fertilization success, only o-DCB and, to a lesser extent, p-DCB caused spermiotoxicity (data not shown). Developmental defects in the offspring of pretreated sperm, as shown in Table 2 were found to be significant for all compounds but primarily for B and CB. On the other hand, regarding mitotic effects (Table 3) clear inhibition of mitosis (reduced MPE and increased IE and M/A) resulted for CB and o-DCB. Increased TMA occurred for B, as well as m-DCB and p-DCB; by considering AA, however, the greatest effect was observed for CB with lesser effects for m-DCB, o-DCB, and B. It should be emphasized that CB and o-DCB caused a sharp decrease in active mitotic figures (decreased MPE) and even more so in anaphase figures (increased M/A) representing the main source of aberrations, as detected in our cytogenetic analysis. Thus, the lack of an increase in TMA did not necessarily reflect the absence of any effect, as displayed by AA data.

As summarized in Table 4, toxicity orders were different depending on whether the embryos or sperm were treated, which suggests different action mechanisms in the two targets. According to whether embryos or sperm are considered, a different ranking of toxicity for these compounds is obtained, even more so if a single endpoint is used, such as developmental defects, mitotoxicity, spermiotoxicity or mitotic aberrations.

The results suggest that B is active on P. lividus sperm in causing developmental and mitotic abnormalities in offspring. B is a known human leukemogen (International Agency for Research on Cancer 1974a) and recently it has been demonstrated (Gad-el-Karim et al. 1984) that in rats the myeloclastogenic effect of B is greatly enhanced by pretreatment of the rats with 3-methylcholanthrene (3-MC), a known inducer of the mixed function oxygenase system. Therefore, it can be suggested that P. lividus sperm can biotransform B to genetically active derivatives; the mechanisms of this action are presently under investigation in our laboratory.

Regarding the activities of the chlorinated benzenes tested, it has been reported that liver glutathione (GSH) is decreased after feeding halogenated benzenes which are metabolized in rodents to mercap-

Table 1. Developmental and cytogenetic effects on P. lividus embryos exposed 48 h to benzene (B), chlorobenzene (CB), and dichlorobenzene (DCB) isomers. Triplicate experiment; data are shown as Mean ± Standard Error.

	% Developmental Defects	No. Mitoses per Embryo	% Interphase Embryos	Metaphase/ Anaphase	% Total Mitotic Aberrations	% Anaphase Aberrations
Blank	11.8± 6.7	8.8±0.5	33.3±10.7	2.2±0.8	1.4±0.5	4.3±1.7
0.01% DMSO	9.1± 1.6	8.7±0.5	34.7±12.0	2.1±0.3	1.7±0.8	4.6±2.6
10^{-6}M B	29.5±14.3[c]	8.7±0.5	38.7±16.6	3.5±0.5[c]	1.3±0.2	4.5±1.6
10^{-6}M CB	15.1± 8.8	8.1±0.5	39.0±11.5	3.2±1.1[c]	0.9±0.4	3.8±1.9
10^{-5}M	15.7±12.0	9.6±0.6	33.0±14.7	2.9±0.6[c]	3.0±0.6[c]	8.1±2.8[c]
10^{-4}M	42.2±25.4[c]	9.6±0.5	34.0±14.5	3.0±0.9[c]	1.9±0.6	7.5±3.3[a]
10^{-6}M o-DCB	20.0± 7.1	9.8±0.5	31.0±11.8	3.2±0.8[c]	2.7±1.1	8.0±3.3[b]
10^{-5}M	28.3± 5.6[c]	10.6±0.5	29.3±15.5	4.1±1.3[c]	1.3±0.7	5.5±2.9
10^{-4}M	46.8±28.9	7.9±0.5	39.3±13.6	3.3±0.4[c]	1.0±0.6	4.1±0.5
10^{-6}M m-DCB	60.8±21.0[c]	9.2±0.5	38.7±16.5	1.8±0.7	0.6±0.3	3.3±2.5
10^{-5}M	48.0±28.0[c]	6.4±0.4	43.3± 4.8[a]	3.3±0.6[c]	0.9±0.6	2.9±2.0
10^{-4}M	37.1±16.3[c]	3.3±0.4[b]	62.0± 5.5[c]	17.2±9.7[c]	5.6±4.8[c]	2.6±2.6
10^{-6}M p-DCB	20.0± 8.1	6.8±0.4	39.3± 9.8	7.1±4.7[c]	0.7±0.07	3.2±1.2
10^{-5}M	21.3±12.4	9.5±0.5	34.3±10.4	5.6±1.8[c]	1.1±0.3	5.9±0.9
10^{-4}M	19.7± 8.1	7.8±0.5	37.0±11.1	5.2±1.9[c]	1.4±0.4	4.2±2.4

a= $p<0.05$; b= $p<0.01$; c= $p<0.001$

Table 2. Sperm inactivation experiment, P. lividus. 4♂(pooled)x 1♀. Frequencies of developmental abnormalities in the offspring of exposed sperm. Exposure times: 5 to 90 min. Nominal agent level: 10^{-5}M.

	% Developmental Defects ($\bar{x}$±SE)	$\Sigma\chi^2$
Blank	13.8± 2.9	
0.01% DMSO	15.9± 3.0	
B	65.9±15.3	639.32
CB	41.6±15.0	329.94
o-DCB	30.2±13.9	75.91
m-DCB	21.8± 7.7	71.01
p-DCB	22.0± 5.0	41.48

Table 3. Sperm inactivation experiment, P. lividus. 4♂(pooled)x 1♀. Cytogenetic analysis on the offspring of 30-min exposed sperm. Nominal agent level: 10^{-5}M.

	MPE ($\bar{x}$±SE)	IE	M/A	TMA	AA
Blank	13.1±0.4	2	2.2	4.1	11.2
0.01% DMSO	11.9±0.4	4	7.1	0.7	4.8
B	11.5±0.3	5	7.0	2.4[b]	13.3[a]
CB	9.0±0.6[b]	21[c]	95.8[c]	1.3	55.6[c]
o-DCB	6.2±0.5[c]	37[c]	27.2[c]	1.9	22.7[b]
m-DCB	11.2±0.3	4	8.3	2.6[c]	24.2[c]
p-DCB	10.2±0.6	16[b]	3.3	2.7[c]	9.7

a= $p<0.05$; b= $p<0.01$; c= $p<0.001$

Table 4. Summary of comparative toxicity records for B, CB and DCBs according to the different treatment schedules and outcomes in the sea urchin bioassay.

Testing Object	Agents	End points			
		Developmental Defects	Mitotic		Spermiotoxicity
			Delay or Blockage	Aberrations	
Embryos	m-DCB	+ +	+ +	+	
	B	+	+/-	-	
	CB	+/-	+	-	
	o-DCB	+/-	+/-	+/-	
	p-DCB	-	+/-	-	
Sperm	o-DCB	+	+ +	+	+ +
	B	+ +	-	+ +	-
	CB	+/-	+ +	+	-
	p-DCB	+/-	+/-	+	+
	m-DCB	+/-	-	+ +	-

turic acids (Barnes et al. 1959). Interestingly, it was reported by Koch-Weser et al. (1953) that benzene halogenated in the meta position is most effective in forming mercapturic acids, thus producing liver damage in rats.

As to possible comparisons between the active levels of agents in this study, and those occurring in polluted water bodies (US Environmental Protection Agency 1980), we may note that the latter are one to two orders of magnitude below our effective levels. Although our data may not reflect realistic concentrations (except for a few instances), even so they might contribute to further quantitative structure-activity relationship (QSAR) studies of halogenated organics. Moreover, bioconcentration of these pollutants may increase their levels in biota up to those investigated in the present study.

In conclusion, our data show some distinct toxicity patterns for the different agents. Thus, the present data, as well as our similar unpublished data on biphenyl hydroxy derivatives, point towards the need to reconsider criteria for ranking toxicities by utilizing multiple toxic endpoints even when a single test species is considered. This supports the suggestion of Legator and Harper (1982) to utilize a multiple test protocol for ranking genetic toxicity, and implies that a number of established toxicity ranking schemes could be revised by introducing an adequate number of suitable toxicological parameters, so that toxicity manifestations could be better delineated.

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