Bull. Environ. Contam. Toxicol. (1994) 53:434–441

Environmental Contamination and Toxicology

Developmental Toxicity of $PbCl_2$ in the Echinoid *Paracentrotus lividus* (Echinodermata)

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Received: 15 July 1993/Accepted: 30 January 1994

Heavy metals are known or suspected to be hazardous for the marine environment; 13 of them are listed in the official list drawn up by the U.S. Environmental Protection Agency (Manfredi 1988). In marine waters, lead is generally one of the most concentrated heavy metals (Aubert et al. 1983; Ferrara and Seritti 1989); the main sources of lead input into the marine environment are rivers (domestic and industrial wastes) and atmosphere (mainly tetraethyl lead from automobile exhaust) (Bryan 1984).

Heavy metal toxicity for the marine environment is currently assessed by means of various biological tests based on different marine species (see e.g., Dinnel et al. 1989; Ringwood 1992). Among these, toxicity bioassays using sea urchin gametes and embryos appear to be quite sensitive and informative, offering a wide range of endpoints. Hence, sea urchin bioassays are now widely used in studies involved in the toxicological characterization of xenobiotics and in environmental monitoring (Kobayashi 1984; Pagano et al. 1986; Dinnel et al. 1988; Gray 1989).

Some previous reports focused on different aspects of lead toxicity in sea urchin development (e.g., Congiu et al. 1984; Kobayashi 1984; Lee and Xu 1984; Dinnel et al. 1989; Brunetti et al. 1991); only Congiu et al. (1984) and Brunetti et al. (1991) investigated lead toxicity in *Paracentrotus lividus*. This sea urchin is widely distributed in the Mediterranean and European Atlantic coasts and is currently used in toxicological assessments. Its development was shown to be very sensitive to various heavy metals (e.g., Pagano et al. 1986). In particular, Congiu et al. (1984) and Brunetti et al. (1991) reported that *P. lividus* development was sensitive to a lead concentration of $2.5 \times 10^{-6} M$ (developmental retardation) and of $5 \times 10^{-7} M$ (skeletal anomalies), respectively.

The purpose of the present study was to extend available information on lead toxicity in *P. lividus*, by testing the effects on the fertilizing capacity of sperm and on offspring quality, as well as the effects on developing embryos.

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MATERIALS AND METHODS

Adult *Paracentrotus lividus* (Lamarck) were collected from the Tyrrhenian Sea (Bay of Naples) in May 1992. Gametes were harvested and embryos were reared as described by Pagano et al. (1986).

Sperm were exposed as follows: 20 µL of sperm pellet were diluted in 30 mL of filtered natural sea water (FSW; salinity: 36 ‰) to which various Pb(II) (as $PbCl_2$) concentrations were added (final Pb(II) concentration range: from $3x10^{-5}$ to $3x10^{-4}M$ or from 6 to 60 mg L^{-1}); sperm were either exposed 15 min or 45 min before insemination; a treated sperm suspension (250 µL) was added to 30 mL of FSW containing untreated eggs (approx. 300 eggs/mL) from a single female. Ten minutes after insemination, zygotes were washed thoroughly to dilute the toxicant to non-embryotoxic levels. Fertilization rate (FR or % fertilized eggs) was scored on a sample of 100 eggs 1 hr after insemination. Offspring quality, expressed as frequencies of developmental defects, was assessed on 72 hr-old pluteus larvae according to the morphological criteria defined by Pagano et al. (1982, 1986) (see Table 1). The plutei were immobilized by chromium sulphate, and a sample of 100 larvae (or embryos) was observed under a light microscope (Pagano et al. 1983a).

Table 1. Morphological criteria used to classify larval developmental defects.

Developmental defects	Morphological criteria
P_1 (pathologic 1)	pluteus larvae affected by anomalies of skeleton, digestive tract, and/or pigmentation
P_2 (pathologic 2)	embryos unable to differentiate to the pluteus stage, e.g. blockage at gastrula or blastula stages; frequently observed are mesenchyme-filled blastulae
R (retarded)	larvae showing unaltered pluteus morphology whose size is ≤ 1/2 that of normal larvae (N)
D (dead)	dead embryos and/or larvae

Embryos were exposed as follows: embryos reared in FSW (salinity: 36 ‰) at 19±1°C were exposed to increasing Pb(II) concentrations (final Pb(II) concentration range: from 10^{-7} to $3x10^{-5}M$ or from 0.02 to 6 mg L^{-1}) either from 15 min after fertilization up to the pluteus larval stage (throughout development) or for 8 hr during the following developmental stages: (1) from zygote up to blastula stage (pre-hatching), or (2) from mesenchyme blastula up to pluteus stage (post-hatching). Embryotoxicity was expressed as frequencies of developmental defects according to the morphological criteria shown in Table 1.

For statistical analysis, the significance of fertilization results was determined by analysis of variance (one way ANOVA). The changes in the distribution of proportions of larval classes (N, R, P1, P2, and D) were tested for significance by the chi-squared (χ^2) test (4 degrees of freedom) (Zar 1984).

RESULTS AND DISCUSSION

No significant effect of Pb(II) was observed on fertilization rate (FR) either for short (15 min) or long (45 min) sperm exposure times in the range of Pb(II) concentrations tested (data not shown). However, Pb(II) concentrations equal to or higher than $10^{-4}M$ exerted some morphological effects on fertilized eggs. Indeed, 1 hr after fertilization, cleavage abnormalities were observed in a few embryos such as asymmetric cleavages and a decrease of blastomeric cohesion.

Table 2. Frequencies of developmental defects in the offspring of Pb(II)-exposed *Paracentrotus lividus* sperm according to sperm exposure time *. Means ± Standard Error. Quintuplicate experiment (100 individuals per replicate). P1: % malformed plutei; P2: % "pre-pluteus" embryos.

Pb(II) *(M)*	sperm exposure time (min)	P1	P2	p (χ^2)
	15			
Control		4.6 ± 1.2	2.8 ± 1.3	
$3x10^{-5}$		8.0 ± 2.6	2.6 ± 1.4	< 0.0005
10^{-4}		8.0 ± 2.4	4.8 ± 1.2	< 0.0005
$3x10^{-4}$		11.0 ± 2.8	4.2 ± 1.9	< 0.0005
	45			
Control		8.0 ± 1.3	2.6 ± 0.8	
$3x10^{-5}$		9.8 ± 2.6	1.2 ± 0.6	= 0.4
10^{-4}		7.8 ± 2.9	3.4 ± 1.5	= 0.7
$3x10^{-4}$		11.2 ± 3.3	1.4 ± 0.8	< 0.01

* Although only relevant developmental defects are reported here, χ^2 tests were performed taking into account the data distribution among N, R, P1, P2, and D classes.

Table 2 shows the effects of Pb(II) on offspring quality. The scores of developmental defects of larvae generated by Pb-exposed sperm showed that offspring quality was significantly affected by Pb(II) at all concentrations tested ($p<0.0005$) for a sperm exposure time of 15 min and at $3x10^{-4}M$ Pb(II) for a sperm exposure time of 45 min ($p<0.01$). However, although significant, the differences between control and experimental cultures were relatively low (the maximum decrease of normal development frequency was about 8% for short Pb(II)-exposure time at the concentration of $3x10^{-4}M$; data not shown).

Table 3. Frequencies of developmental defects in *Paracentrotus lividus* larval populations exposed to Pb(II) throughout embryogenesis (72 hr), or for 8 hr before or after hatching*.
Means ± Standard Error. Quintuplicate experiment, except when n is specified (100 individuals per replicate). R: % retarded plutei; P_1: % malformed plutei; P_2: % "pre-pluteus" embryos.

Pb(II) *(M)*		R	P_1	P_2	p (χ^2)
exposure throughout development					
Control		0.2 ± 0.2	5.8 ± 2.5	2.1 ± 0.8	
10^{-7}		0.4 ± 0.2	6.2 ± 1.7	5.0 ± 1.4	< 0.01
10^{-6}		0.0 ± 0.0	5.8 ± 2.0	6.0 ± 1.9	< 0.0005
$3x10^{-6}$	(n = 4)	2.0 ± 1.2	36.3 ± 5.8	33.8 ± 18.5	< 0.0005
10^{-5}		0.0 ± 0.0	16.6 ± 9.7	83.6 ± 9.7	< 0.0005
$3x10^{-5}$	(n = 4)	0.0 ± 0.0	1.3 ± 1.3	98.8 ± 1.3	< 0.0005
pre-hatching exposure					
Control		0.0 ± 0.0	3.6 ± 1.1	5.4 ± 0.9	
10^{-7}		0.0 ± 0.0	3.4 ± 0.7	4.2 ± 0.6	= 0.9
10^{-6}		0.2 ± 0.2	3.4 ± 0.9	6.4 ± 1.3	= 0.9
10^{-5}		0.0 ± 0.0	10.8 ± 2.4	4.4 ± 1.0	< 0.0005
post-hatching exposure					
Control		0.2 ± 0.2	4.0 ± 0.6	11.0 ± 2.3	
10^{-7}		0.2 ± 0.2	6.6 ± 1.1	11.6 ± 2.7	< 0.05
10^{-6}		0.0 ± 0.0	7.2 ± 2.0	11.6 ± 1.5	< 0.01
10^{-5}		26.4 ± 3.8	52.0 ± 5.5	18.8 ± 2.9	< 0.0005

* see note Table 2.

The frequencies of developmental defects in Pb(II)-exposed larvae are reported in Table 3. When embryos were exposed to Pb(II) throughout embryogenesis (72 hr), a highly significant dose-response increase was observed in developmental defects at Pb(II) concentrations ranging from $10^{-7}M$ to $3x10^{-5}M$. This trend was characterized by a progressive shift from larval malformations (P_1) up to the arrest of differentiation at the gastrula stage (P_2), which reached approx. 99% for Pb(II) at $3x10^{-5}M$. Stage-specific toxicity was tested by rearing the cultures for 8 hr in Pb(II) concentrations ranging from $10^{-7}M$ to $10^{-5}M$. When *P. lividus* embryos were exposed to Pb(II) before hatching, only the highest concentration tested ($10^{-5}M$) caused a significant but minor increase of skeletal malformations. Vice versa, a post-hatching exposure led to a dose-dependent increase in developmental defects, including retarded (R) and malformed plutei (P_1), as well as developmental arrest (P_2).

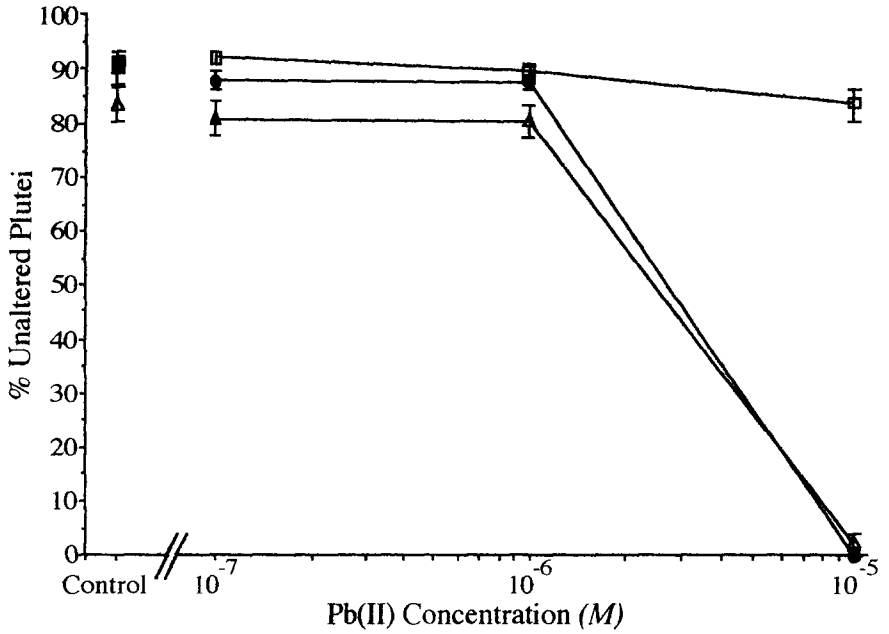

Figure 1. Changes in the percentage of unaltered *Paracentrotus lividus* 72 hr-old plutei following embryo exposure to increasing Pb(II) concentrations (● : throughout development -72 hr-exposure; □ : pre-hatching -8 hr- exposure; △ : post-hatching -8 hr- exposure).
Means ± Standard Error. Quintuplicate experiment (100 individuals per replicate).

As summarized in Figure 1, the frequencies of normal plutei were almost unchanged by a pre-hatching exposure, whereas post-hatching and 72 hr-exposed cultures displayed an overlapping decline in the frequency of normal plutei as a function of Pb(II) concentrations.

The results show that Pb(II) (as $PbCl_2$) does not exert any detectable spermiotoxic effect on *P. lividus* fertilization up to a concentration as high as $3x10^{-4}M$ (60 mg L^{-1}) which is by far out of the range of natural marine Pb(II) levels (Aubert et al. 1983; Bryan 1984), even in dramatically lead-polluted waters (Bryan 1984). The lack of any significant effects of Pb(II) on fertilization rate suggests that Pb(II) does not affect any physiological processes controlling sperm activity during fertilization. However, *P. lividus* sperm seem to have a higher Pb(II)-resistance compared with other echinoid species. Dinnel et al. (1989) showed indeed that fertilization rate in four echinoid species (*Strongylocentrotus droebachiensis*, *Strongylocentrotus purpuratus*, *Strongylocentrotus franciscanus*, and *Dendraster excentricus*) is reduced in the presence of $PbCl_2$. Calculated EC_{50} values for bioassay of $PbCl_2$ with sperm of these four species ranged from 1.3 to 19 mg L^{-1} (Dinnel et al. 1989).

Yet, the quality of the offspring from Pb(II)-treated sperm appeared to be significantly affected following sperm exposure ranging from $3x10^{-5}M$ to $3x10^{-4}M$ Pb(II) for 15 min and to $3x10^{-4}M$ Pb(II) for 45 min. These data possibly suggest a minor transmissible damage to sperm cells induced by high, non-realistic, Pb(II) levels that could turn to larval malformations in the offspring.

Lead-induced embryotoxicity in *P. lividus* appeared to be higher compared with other inorganic toxicants, e.g., Cr(VI), Cd(II) or As(III) and As(V) (Pagano et al. 1982, 1983a, 1983b). As in the case of cadmium, a stage-dependent toxicity was observed, as lead affected sea urchin embryogenesis only following "long-term" (72 hr) or post-hatching exposures, whereas pre-hatch lead-exposed embryos displayed a minor, if any, increase in larval malformations. When comparing the outcomes of "long-term" versus post-hatching exposures (Table 3), a shift was observed from a prevalence of developmental arrest (P_2 embryos) to the prevalence of malformed (P_1) and retarded (R) plutei, respectively. This finding is consistent with a time-dependent severity of lead-induced toxicity, confined to gastrulation and skeletal differentiation periods.

Our results are consistent with other studies of lead embryotoxicity in echinoids (Congiu et al. 1984; Lee and Xu 1984; Brunetti et al. 1991). Congiu et al. (1984) noted a complete blockage at gastrula stage in *P. lividus* embryos exposed to $2.5x10^{-5}M$ $PbNO_3$; for lower Pb(II) concentrations ($2.5x10^{-6}M$), these workers reported a 24-hr delayed effect on larval development. Brunetti et al. 1991 noted an increase of skeletal anomalies in *P. lividus* plutei exposed for 40 hr to $5x10^{-7}M$ Pb(II) as $PbCl_2$. Our data confirm these data of Congiu et al. and Brunetti et al. since we observed a significant increase in larval malformations following embryo exposure to $10^{-7}M$ Pb(II) (for 72 hr-exposure). Lee and Xu (1984) reported that embryos of the echinoid *Temnopleurus toreumaticus* are affected just after gastrula stage by $2.5x10^{-7}M$ lead (chemical form not specified).

Authors generally agree that Pb(II) mainly affects post-hatch gastrulae, though the peculiar sensitivity of this larval stage is unexplained. The two following interpretations could be suggested. (1) During pre-hatch development, fertilization envelope (FE) that surrounds the embryo could act as a "shield" (more or less effective impermeability, adsorption surface) against Pb(II) dissolved in the medium; once hatched, the larva would not be protected any more against Pb(II). The toxicant would then affect, possibly via non-specific mechanism(s), morphogenetic processes occurring after hatching, e.g., skeletal differentiation. (2) FE has little or no protective effect against the metal, and the observed embryotoxicity results from a Pb(II)-specific action towards one or several processes related to skeletal differentiation.

The second interpretation appears to be the more plausible because other divalent toxic cations [e.g., Cu(II) and Hg(II)] affect cleaving echinoid embryos (Pagano et al. 1982, 1986; Walter et al. 1989). Since lead, like cadmium, competes with calcium fixation (Pagano et al. 1982; Pounds et al.

1982), the data are consistent with a specific lead toxicity to skeletal differentiation.

Acknowledgments. Thanks are due to M. Iaccarino and G. Autiero for skillful assistance, and to the staff of the Zoological Station of Naples for providing sea urchins. Authors thank Dr. M. Jangoux, Dr. Ph. Dubois, and A. Temara for criticism of the manuscript. Research was supported by an IRSIA grant to M. Warnau (ref. 910528), by a CNR/FNRS-cooperation grant, and by a CCE STEP Program (ref. STEP-0063-C). Contribution of the "Centre Interuniversitaire de Biologie Marine" (CIBIM).

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