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Comparative toxicities of selected rare earth elements: Sea urchin embryogenesis and fertilization damage with redox and cytogenetic effects

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ABSTRACT

Background: Broad-ranging adverse effects are known for rare earth elements (REE), yet only a few studies tested the toxicity of several REE, prompting studies focusing on multi-parameter REE toxicity.
Methods: Trichloride salts of Y, La, Ce, Nd, Sm, Eu and Gd were tested in *Paracentrotus lividus* sea urchin embryos and sperm for: (1) developmental defects in either REE-exposed larvae or in the offspring of REE-exposed sperm; (2) fertilization success; (3) mitotic anomalies in REE-exposed embryos and in the offspring of REE-exposed sperm, and (4) reactive oxygen species (ROS) formation, and malondialdehyde (MDA) and nitric oxide (NO) levels.
Results: REEs affected *P. lividus* larvae with concentration-related increase in developmental defects, 10^{-6} to 10^{-4} M, ranking as: Gd(III) > Y(III) > La(III) > Nd(III)≅Eu(III) > Ce(III)≅Sm(III). Nominal concentrations of REE salts were confirmed by inductively coupled plasma mass spectrometry (ICP-MS). Significant increases in MDA levels, ROS formation, and NO levels were found in REE-exposed embryos. Sperm exposure to REEs (10^{-5} to 10^{-4} M) resulted in concentration-related decrease in fertilization success along with increase in offspring damage. Decreased mitotic activity and increased aberration rates were detected in REE-exposed embryos and in the offspring of REE-exposed sperm.
Conclusion: REE-associated toxicity affecting embryogenesis, fertilization, cytogenetic and redox endpoints showed different activities of tested REEs. Damage to early life stages, along with redox and cytogenetic anomalies should be the focus of future REE toxicity studies.

1. Introduction

Current literature on REE-associated toxicity is mostly confined to three REEs (Ce, La and Gd). Consequently, comparative information for several REEs remains relatively scarce in spite of their widespread industrial utilization and as emerging environmental contaminants (US Environmental Protection Agency, 2012; EU-OSHA, 2013; Gambogi and Cordier, 2013; Snow et al., 2014; Pagano et al., 2015a,b; González et al., 2015).

The present study aimed at providing comprehensive data on multiple toxicity endpoints after exposure to selected REEs in sea urchin early life stages. The sea urchin assay system has been utilized extensively in the past -up to present-day studies- to address questions concerning the effects of a number of agents, including inorganics, organics, and complex mixtures, e.g. whole sediment or industrial effluents. Sea urchins have provided valuable insights on the toxicity mechanisms of many xenobiotics. This extensive body of literature is beyond the scope of this experimental report and will be reviewed in a paper currently in preparation. Multiple toxicity endpoints can be tested in sea urchin early life stages such as effects on fertilization success, embryogenesis, mitotic activity, redox balance, and other endpoints such as gene expression (Stumpp et al., 2011a,b; Evans and Watson-Wynn, 2014; Migliaccio et al., 2014). Thus, sea urchin assays can

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provide multi-parameter information on the ability of a xenobiotic to interfere with key-events in early life stages such as cell division and differentiation, fertilization and oxidative/nitrosative stress (ONS) (Korkina et al., 2000; Pagano et al., 2001a; Oral et al., 2010; Romano et al., 2011; Migliaccio et al., 2014).

With this long-established background on the use of sea urchin assays in toxicity testing, the present study's main objective included toxicity testing of selected REE trichloride salts, including Y(III), La(III), Ce(III), Nd(III), Sm(III), Eu(III) and Gd(III). Toxicity endpoints investigated included developmental defects in *Paracentrotus lividus* pluteus larvae either following exposures during embryogenesis or in the offspring of REE-exposed sperm (whose fertilization success was also tested). Furthermore, cleaving embryos, either reared in a REE medium or generated by REE-exposed sperm, were tested for REE-induced cytogenetic anomalies, including changes in mitotic activity and induction of mitotic aberrations. Finally, three ONS endpoints were tested in REE-exposed embryos or larvae, including ROS formation and MDA levels related to oxidative stress, and NO levels related to nitrosative activities. ONS is indeed known both to induce damages in biomolecules and to play key-roles in embryogenesis.

2. Methods

2.1. Sea urchins

Sea urchins (*P. lividus*) were collected in the Bay of Naples by the staff of the Stazione Zoologica Anton Dohrn. Gametes were obtained and embryos were reared as reported previously (Pagano et al., 2001b). Controls consisted of embryos reared in natural filtered seawater (FSW) run as triplicate blanks. The embryos were reared in trichloride salt solutions of Y(III), La(III), Ce(III), Nd(III), Sm(III), Eu(III) and Gd(III) that were diluted from a 10^{-1} M stock solution stored refrigerated at pH 3 (by HCl addition). The correspondence of nominal *vs*. analytical concentrations was determined by a set of ICP-MS analyses using an Aurora M90 Brucker apparatus. Embryo exposures to REE trichloride salts at concentrations in the order of 10^{-8} to 10^{-4} M were performed throughout embryogenesis, starting from zygote (10 min post-fertilization) up to the pluteus larval stage (72 h post-fertilization). Embryos were incubated in FSW at 18 ± 1 °C in Falcon™ Tissue Culture Plates (6 wells, 10 ml/well). Experiments were run with a total of 4–12 replicates. A series of experiments was performed on *P. lividus* sperm, by suspending a 50-µl sperm pellet for 1 h in 30 ml FSW containing REE salts, 10^{-5} to 10^{-4} M; thereafter, 50-µl of sperm suspension were used to inseminate 10 ml of untreated eggs (~50 eggs/ml).

2.2. Embryological analysis

Embryological analysis was performed on living plutei immobilized in 10^{-4} M chromium sulfate 10 min prior to observation, approx. 72 h after fertilization. In each treatment schedule, the first 100 plutei were scored for the percentages of: (1) normal larvae (N); (2) retarded larvae (R, size < 1/2N); (3) malformed larvae (P1), mostly observed through damaged skeletal differentiation; (4) embryos/larvae unable to attain the pluteus stage-i.e. abnormal blastulae or gastrulae (P2), and (5) dead (D) embryos or larvae. Total developmental defects (DD) were scored as (P1 + P2).

2.3. Cytogenetic analysis

Cytogenetic analysis was carried out on 30 cleaving embryos from four cultures in each treatment schedule (either embryo exposure or following sperm exposure), and triplicate controls (each in quadruplicate cultures) amounted to a total of 12 control cultures. The embryos were fixed in Carnoy's fluid (60% ethanol, 30% chloroform and 10% glacial acetic acid) 5 h after fertilization, and stained by acetic carmine (Pagano et al., 2001b). The cytogenetic endpoints both allowed for measurements of quantitative and morphological abnormalities. Quantitative parameters included: (a) mean number of mitoses per embryo (MPE), and (b) percent interphase embryos (IE). The frequencies of morphologic abnormalities were scored as: (a) anaphase bridges; (b) lagging chromosomes; (c) acentric fragments; (d) scattered chromosomes; (e) multipolar spindles; (f) total mitotic aberrations per embryo and (g) percent embryos having ≥ 1 mitotic aberrations [E(Ab+)].

2.4. Sperm bioassays

Following a 1-h sperm pretreatment, fertilization success was measured as percent fertilized eggs also expressed as fertilization rate (FR) on live cleaving embryos 1–3 h post-fertilization. Thereafter, the embryos were cultured up to pluteus stage and scored for developmental defects as described above in order to evaluate the effects, if any, of sperm exposure on offspring health status. Each observation was carried out blind on randomly-tagged specimens.

2.5. Redox endpoints – ROS measurement

ROS production was determined by measuring the oxidation of 2′,7′-dichlorohydro-fluorescein diacetate (DCFH-DA, D6883, Sigma-Aldrich). Sea urchin embryos (about 5000) were incubated in 5 µM DCFH-DA in phosphate buffer saline (PBS) for 1 h under darkness. After incubation, embryos were collected by centrifugation at 1800×*g* for 10 min at +4 °C, briefly rinsed in PBS without DCFH-DA and frozen in liquid nitrogen. Frozen embryos were resuspended in 0.2 ml Tris–HCl buffer 40 mM, pH 7.0, vortexed for 1 min, and finally centrifuged for 10 min at 13,500 × *g* at +4 °C. The supernatant was collected and examined for protein concentration using a Bradford assay. Fluorescence was measured using spectrofluorometer (Shimadzu RF-5301 PC) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Fluorescence values were corrected by subtracting the autofluorescence of unlabeled extracts. A standard curve was prepared using dichlorofluorescein (DCF, 35848, Sigma-Aldrich), diluted in the same Tris–HCl buffer. Fluorescence from DCF was linear in the range of 0–50 nM.

2.6. Lipid peroxidation determination

Lipid peroxidation was measured by the thiobarbituric acid method assay (TBA test), which is based on the reactivity of the end product of lipid peroxidation, malondialdehyde (MDA) with TBA to produce a red adduct. Sea urchin developing embryos (about 15,000) were collected at the pluteus stage by centrifugation at 1800 × *g* for 10 min at +4 °C. The pellet was homogenized in Tris–HCl containing 0.1 mM EDTA and 0.2% triton X-100 (1:2 W/V) and centrifuged (at 14,000 × *g* for 30 min, +4 °C). The supernatant (200 µl) was added to 1 ml of the reaction buffer containing 15% trichloroacetic acid (TCA), 0.375% TBA and 0.1% butylated hydroxytoluene (BHT). The buffer was prepared by adding TBA (previously dissolved in 1–2 ml of 12 M HCl) to the TCA solution while stirring on a hot plate. After incubation at +95 °C for 15 min, the reaction mixture was centrifuged and its absorbance was read at 532 nm. A standard curve for MDA was prepared using 1,1,3,3-tetraethoxypropane as reported by Esterbauer and Cheeseman (1990).