Ecotoxicology and Environmental Safety 123 (2016) 72–80

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Detection of malformations in sea urchin plutei exposed to mercuric chloride using different fluorescent techniques

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ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form
22 July 2015
Accepted 23 July 2015
Available online 5 August 2015

Keywords:
Harmonic generation microscopy
Two-photon microscopy
Confocal microscopy
Skeletal rod
Apoptosis

ABSTRACT

Embryos of Mediterranean sea urchin *Paracentrotus lividus* and subtropical *Echinometra mathaei* were exposed to 5,10, 15 and 20 μg L^{-1} and to 1, 2, 3 and 4 μg L^{-1} mercuric chloride ($HgCl_2$), respectively. The effective concentration (EC_{50}) inducing malformation in 50% of 4-arm pluteus stage (P4) was 16.14 μg L^{-1} for *P. lividus* and 2.41 μg L^{-1} for *E. mathaei*. Two-photon (TP), second (SHG) and third harmonic generation (THG) microscopy techniques, TUNEL staining, propidium iodide (PI) and Hoechst 33342 probes were used to detect light signals or to stain apoptotic and necrotic cells in fixed and alive plutei. Signals were detected differently in the two species: TP fluorescence, commonly associated with apoptotic cells, did not increase with increasing $HgCl_2$ concentrations in *P. lividus* and in fact, the TUNEL did not reveal induction of apoptosis. PI fluorescence increased in *P. lividus* in a dose-dependent manner, suggesting a loss of cell permeability. In *E. mathaei* plutei TP fluorescence increased at increasing $HgCl_2$ concentrations. THG microscopy revealed skeletal rods in both species. Different fluorescent techniques, used in this study, are proposed as early-warning systems to visualize malformations and physiological responses in sea urchin plutei.

1. Introduction

Mercury (Hg) is one of the most toxic and persistent elements in the environment, deriving both from natural and anthropogenic sources. This metal can enter the ecosystem through the breakdown of minerals, although significant amounts originate as a result of human activities and discharges from metal smelting, coal-burning power plants, municipal waste incineration, coal and other fossil fuel combustion (Satoh, 2000). In aquatic ecosystems, mercury is present mainly in inorganic elemental (Hg°, Hg^{2+}) or organic methylated form and its bioavailability is influenced by physical-chemical factors such as pH, dissolved organic carbon or temperature of the water (Driscoll et al., 1994). Between the two ionized states, the bivalent form is more stable and more frequently found, and can bond with chloride ($HgCl_2$) in salt water. Mercury concentrations in aquatic environments are highly variable, ranging from very low concentrations in open ocean to concentrations as high as 16 μg L^{-1} in very polluted areas close to industrial discharges (Plaschke et al., 1997; De Riso et al., 2000).

Toxic effects of mercury have been widely studied on fish (Driscoll et al., 1994; Devil, 2006; Sinaie et al., 2010), due to their relevance in human consumption. On the other hand, the impact of mercury on marine environment has been investigated using animal models, belonging to different trophic levels, and taking into consideration various end-points such as behavior, reproduction, embryo and larval mortality or morbidity (Fernandez and Beiras, 2001). In ecotoxicology studies, echinoderms are considered excellent bioindicators due to their dual role in pelagic and benthic compartments (Bellas et al., 2005; Salamanca et al., 2009). Furthermore, echinoderm gametes are easy to collect and fertilization and larval development are well known (Pagano et al., 1986). Studies on the toxicity of mercury on sea urchin species have mainly been focused on the evaluation of the effective concentration (EC_{50}) inducing 50% embryo and larval mortality, as well as deformities, or sperm fertilization inhibition (Fernandez and Beiras, 2001; Warnau et al., 1996).

In this study we analyzed the effect of increasing

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http://dx.doi.org/10.1016/j.ecoenv.2015.07.027
0147-6513/

concentrations of mercuric chloride ($HgCl_2$) on the larval development of two different sea urchin species: *Echinometra mathaei*, the most ubiquitous and abundant shallow-water sea urchin in tropical and subtropical regions, and *Paracentrotus lividus. E. mathaei* is commonly found at depth up to 130 mt and in a temperature range between 24 and 27 °C. It has a flexible behavior and diet, high reproduction and recruitment rates and low resource requirements (McClanahan and Muthiga, 2007). Although genetic, morphological, biochemical, ecological and reproductive studies have been carried out on *Echinometra* species, few data are available on its sensitivity to organic and inorganic contaminants (Kominami and Takata, 2003; Mahdavi et al., 2008; Sadripour et al., 2013). *Paracentrotus lividus* is common in the temperate Mediterranean areas, with winter water temperatures of around 11 to 12 °C and summer temperatures ranging from 18 to 25 °C (Boudouresque and Verlaque, 2007). This species has largely been used as a model animal to study the impact of toxicants (Bellas et al., 2008; Pinsino et al., 2010; Gaion et al., 2013) or natural toxins (Romano et al., 2011, 2003, 2010).

Here we evaluated the effective concentration of $HgCl_2$ inducing malformations in the 4-arm plutei stage (P4) in both temperate and subtropical species. Furthermore, harmonic generation (HGM) and two-photon (TP) microscopy techniques were used to verify whether fluorescence signals could be associated with chemical stress. Harmonic generation and TP microscope are the least invasive laser scanning techniques enabling visualization of autofluorescent and endogenous harmonic generation signals from the whole-mount samples, with submicron spatial resolution and without the use of fluorescent probes. Optical sections along the depth of the sample can detect different cell morphology and physiological activity (Chu et al., 2003). Furthermore, we used TUNEL staining to detect apoptotic cells in *P. lividus* P4 stage exposed to $HgCl_2$, propidium iodide (PI) to visualize necrotic cells in live plutei and Hoechst 33342 to stain cell nuclei. Apoptosis or programmed cell death is a form of cell suicide showing characteristic morphological and biochemical alterations such as cell shrinkage, blebbing and activation of specific caspases that lead to enzymatic breakdown of DNA (Lockshin et al., 1998). Apoptosis is a physiological process occurring during embryo development and metamorphosis (Roccheri et al., 2002; Thurber and Epel, 2007; Agnello and Roccheri, 2010), but can also be activated by external stimuli such as the presence of bioactive molecules and pollutants (Romano et al., 2003; Agnello et al., 2007). Different fluorescent techniques applied in this study, could be used as early stress indicators in sea urchin bioassay tests.

2. Materials and methods

2.1. Gamete collection

P. lividus and *E. mathaei* adults were collected from an intertidal rocky site along the coast of Livorno (Italy) [43° 25′ 31.79″ N, 10° 23′ 37.51″ E] and Keelung (Taiwan) [25° 8′ 30.79″ N, 121° 48′ 11.79″ E] respectively, and immediately transported in an insulated box to the laboratory. Animals were injected with 1 ml of 0.5 M KCl solution into the coelom, through the peristome, to obtain gametes, soon after their arrival. Sperm obtained from at least three males was collected dry from each male using a Pasteur pipette, pooled and conserved in an Eppendorf tube at 4 °C until fertilization within 2 h. Sperm concentration was determined diluting 50 µl of semen in 25 ml tap water to enlarge sperm head, through osmotic shock, which was then measured with a hemocytometer (Thoma chamber) under the Olympus inverted-microscope (Milan, Italy) using a 40*x* objective. Oocytes obtained from at least three females were pooled into 1 L beaker filled with 0.22 µm filtered seawater (FSW) collected at the corresponding sampling site (36 ± 1 psu salinity, $pH = 8.0 \pm 0.2$). The final concentration of 1000 eggs mL^{-1} was prepared by counting subsamples of a known volume with the inverted Olympus microscope at 4*x* objective. Fertilization occurred diluting sperm and eggs in 1 L FSW beaker at 15,000:1 and 10,000:1 sperm:egg ratio, for *P. lividus* and *E. mathaei*, respectively (Rahman et al., 2000; Lera and Pellegrini, 2006). Few minutes after fertilization, an aliquot of embryos was observed under the inverted microscope to verify the presence of the fertilization membrane. The acceptability of the sample was fixed at a fertilization rate above 90%, as also suggested by other authors (from 70% to 95%) (Warnau et al., 1996; Arizzi Novelli et al., 2002; Lera and Pellegrini, 2006).

2.2. Incubation experiments

Each solution was prepared dissolving 135.75 g of $HgCl_2$ in 500 mL bi-distilled water (BDW) to obtain a final concentration of 1M $HgCl_2$ solution. Nominal concentrations of 10, 20, 30, 40, 50, 80, 100, 150, 200 and 250 µg L^{-1} $HgCl_2$ (corresponding approximatively to 3.6–92 µM $HgCl_2$) were then obtained diluting 1M solution in FSW and stirring at the test temperature (1 µg L^{-1} $HgCl_2$ corresponds to 0.74 µg L^{-1} Hg^{2+}). One milliliter of each solution was then added to 9 ml FSW in each well plate containing about 1000 *P. lividus* and *E. mathei* embryos, to obtain final concentrations of 5, 10, 15, 20 and 25 µg L^{-1} for *P. lividus* and 1, 2, 3, 4 and 8 µg L^{-1} for *E. mathaei. Paracentrotus lividus* embryos were maintained at 18 ± 2 °C (Cakal Arslan et al., 2007) while *E. mathaei* where maintained at 24 ± 1 °C (Kominami and Takata, 2003). An untreated control for each species was incubated in FSW alone; six replicates for each condition were applied.

Larval growth followed until the controls reached P4 stage for more than 80%. For *P. lividus* this stage occurred 72 h after fertilization, 48 h for *E. mathaei*. The acceptability of the results was fixed when the percentage of normal plutei was higher than 80% in the controls. Normal and abnormal P4 were identified according to Pagano et al. (1986); the fully developed P4 larvae were considered normal, whereas retarded gastrulae, preastrulae, prism stages and those malformed (showing defects in the skeleton and/or digestive apparatus) were considered abnormal (De Nicola et al., 2007). Median effective concentration inducing 50% of abnormal P4 stages (EC_{50}) was determined for each species, considering the percentage of abnormal P4 stage in each concentration.

2.3. Harmonic generation microscopy analyses

To verify HGM or TP fluorescent signals in living plutei, P4 embyos from both sea urchin species were observed with the second (SHG) and third harmonic generation (THG) microscopy techniques and TP contrast using an adapted upright microscope Olympus BX51 microscope (Taipei, Taiwan) (Hsieh et al., 2008). The scanned SHG and THG images were obtained with a 60x water immersion objectives at numerical aperture of 1.2. The HGM microscopy system is based on a femto-second Cr:forsterite laser operating at wavelengths (λ) of 1230 nm, which can achieve deepest penetration and causes less damage compared with the most commonly used Ti:sapphire laser (700–1000 nm λ) (Chen et al., 2001; Sun et al., 2004). Harmonic generation microscopy is related to the interaction of intense light with matter; in particular the SHG intensity generated depends on the square of the incident light intensity (Chu et al., 2002) and signals are generated at dense, non-centro symmetric structures, such as collagen fibers and striated muscle myosin (Rehberg et al., 2011). The THG fluorescence generated depends on the cubic of the incident light intensity and associated signals were found to be associated with