Ecotoxicology and Environmental Safety 137 (2017) 78–85

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Differential sensitivity to cadmium of immunomarkers measured in hemocyte subpopulations of zebra mussel Dreissena polymorpha

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ARTICLE INFO

Keywords:
Dreissena polymorpha
Hemocytes
Cadmium
Metal
Phagocytosis

ABSTRACT

Increasing discharge of industrial wastes into the environment results in pollution transfer towards hydrosystems. These activities release heavy metals such as cadmium, known as persistent pollutant that is accumulated by molluscs and exercise immunotoxicological effects. Among molluscs, the zebra mussel, *Dreissena polymorpha* constitutes a suitable support for freshwater ecotoxicological studies. In molluscs, homeostasis maintain is ensured in part by hemocytes that are composed of several cell populations involved in multiple physiological processes such as cell-mediated immune response or metal metabolism. Thus, hemocytes constitute a target of concern to study adverse effects of heavy metals.

The objectives of this work were to determine whether immune-related endpoints assessed were of different sensitivity to cadmium and whether hemocyte functionalities were differentially affected depending on hemocyte subpopulation considered. Hemocytes were exposed *ex vivo* to concentrations of cadmium ranging from 10^{-6} M to 10^{-3} M for 21 h prior flow cytometric analysis of cellular markers. Measured parameters (viability, phagocytosis, oxidative activity, lysosomal content) decreased in a dose-dependent manner with sensitivity differences depending on endpoint and cell type considered. Our results indicated that phagocytosis related endpoints were the most sensitive studied mechanisms to cadmium compared to other markers with EC_{50} of $3.71 \pm 0.53 \times 10^{-4}$ M for phagocytic activity and $2.79 \pm 0.19 \times 10^{-4}$ M considering mean number of beads per phagocytic cell. Lysosomal content of granulocytes was less affected compared to other cell types, indicating lower sensitivity to cadmium. This suggests that granulocyte population is greatly involved in metal metabolism. Mitochondrial activity was reduced only in blast-like hemocytes that are considered to be cell precursors. Impairment of these cell functionalities may potentially compromise functions ensured by differentiated cells. We concluded that analysis of hemocyte activities should be performed at sub-population scale for more accurate results in ecotoxicological studies.

1. Introduction

The zebra mussel, *Dreissena polymorpha*, is an invasive freshwater bivalve mollusc distributed in Western Europe and North America. Because of its biological characteristics (abundance, wide geographical distribution, sessile life and high filtration activity), transplanted or natural populations of zebra mussels were used as sentinel species for freshwater pollution monitoring in field studies and under laboratory conditions (de Lafontaine et al., 2000; Minier et al., 2006; Binelli et al., 2009; Bourgeault et al., 2010; Palais et al., 2011; Faria et al., 2014, Parolini et al., 2015; Kerambrun et al., 2016). This species was recently proposed to represent the freshwater counterpart of the blue mussel, *Mytilus edulis*, for ecotoxicological studies (Binelli et al., 2015).

Ecotoxicological risk assessment is partially based on analysis of early physio-pathological responses measured at sub-individual level, providing information on health status of organisms and populations. Among responses evaluated in mussel models, those related to hemocytes are of great interest (Brousseau et al., 1998; Auffret *et al.*, 2005).

Abbreviations: A.U., Arbitrary Unit; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, Dichlorofluorescein; FSC, Forward Scatter; MFI, Mean Fluorescence Intensity; PI, Propidium Iodide; RNS, Reactive Nitrogen Sepcies; ROS, Reactive Oxygen Species; SSC, Side Scatter

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http://dx.doi.org/10.1016/j.ecoenv.2016.11.027
Received 27 September 2016; Received in revised form 22 November 2016; Accepted 29 November 2016
Available online 19 December 2016

Indeed, these cells play a key role in mollusc homeostasis through involvement in multiple physiological functions such as immune defenses. Hemocytes are responsible for humoral and cell-mediated innate responses by ensuring phagocytosis and encapsulation processes. Phagocytosis consists in endocytosis of foreigner particles in phagolysosomes where degradation takes place through action of lysosomal enzymes, reactive oxygen species (ROS) and nitric oxide production (Canesi et al., 2002; Ottaviani, 2011; Soudant et al., 2013; Allam and Raftos, 2015).

Bivalve molluscs have an open circulatory system in which hemocytes may be in direct contact with environmental stressors as chemical contaminants. Consequently, *in vitro* exposures of hemocytes constitute a suitable methodology to simulate interactions between cells and xenobiotics (Renault, 2015). Thereby, measurement of immune-related hemocyte activities after *in vitro* or *ex vivo* exposure to contaminant constitute an interesting support in ecotoxicological risk assessment and a sensitive approach to assess immunotoxicological potential of contaminants (Brousseau et al., 2000; Fournier et al., 2000; Auffret, 2005; Renault, 2015; Ladhar-Chaabouni and Hamza-Chaffai, 2016).

Cadmium is a common and persistent pollutant occurring in the environment from natural processes and anthropogenic activities. Presence of this non-essential metal in aquatic ecosystems is of concern due to its toxicity on human and wildlife (Tchounwou et al., 2012; Sarkar et al., 2013). As filter feeder, zebra mussels are known to accumulate a wide range of metals in their soft tissues and cadmium concentrations ranging from 1 to 90 $\mu g\ g^{-1}$ were measured during biomonitoring surveys (Kraak et al., 1991; Kwan et al., 2003; Richman and Somers, 2005; Anzano et al., 2011). Previous studies performed in multiple bivalve species demonstrated that cadmium exerted immunotoxic effects by disturbing hemocyte functionalities such as phagocytosis, oxidative metabolism, lysosomal activity and induced apoptosis (Brousseau et al., 2000; Olabarrieta et al., 2001; Sokolova et al., 2004; Dailianis, 2009; Latire et al., 2012; Caza et al., 2015). In *Dreissena polymorpha*, Sauvé et al., 2002 demonstrated that hemocyte phagocytosis was reduced in a dose dependent manner in response to an *ex vivo* cadmium exposure. Immunological responses are mechanisms of concern according to their central importance to maintain organism integrity. Moreover, the immunophysiological process is sensitive to contaminants and its alteration may lead to increase susceptibility to pathogens (Pipe and Coles, 1995; Fournier et al., 2000).

Immunotoxicological studies are performed considering hemocytes as a single cell type while numerous mollusc species possess several hemocyte subpopulations expressing different functional capacities suggesting different implications in physiological processes (Cheng, 1984; Auffret, 1988; Hine, 1999; Donaghy et al., 2009a, 2009b; Le Foll et al., 2010). In zebra mussel hemolymph, three hemocyte types were described as hyalinocytes and blast-like hemocyte for agranular cells and one granulocyte population that possess different functional capacities (Giamberini et al., 1996; Evariste et al., 2016). In the present study, we compared sensitivity of cellular activities in hemocytes exposed *ex vivo* to cadmium in a dose-dependent manner. Functional responses were evaluated at cellular sub-population level using a multi-biomarker approach analyzed by flow cytometry. The aim of the study is to analyze how cell functionalities may be differentially affected by cadmium in relation to the hemocyte subtype. Results may lead to a better knowledge of sensitivity levels of the different hemocyte functional markers in an ecotoxicological risk assessment context.

2. Materials and methods

2.1. Animals and hemolymph collection

Zebra mussels were collected by divers at Lac du Der (Marne, France) in September 2015, The mussels were sampled outside their breeding period, minimizing confounding effects related to biotic and abiotic stresses on the different biological responses measured. Prior to experiment, mussels were maintained 2 weeks at laboratory in aerated tanks filled with natural spring water and fed with lyophilized *Chlorella vulgaris* (10^6 cells day^{-1} $mussel^{-1}$). Mussels were maintained at 12 °C in accordance with field water temperatures. Large individuals (N = 14; 28.57 ± 2 mm; 1.76 ± 0.46 g) were selected in order to ensure withdrawn of sufficient hemocyte amount to perform assay at individual scale. Hemolymph was withdrawn from adductor muscle using 0.5 mL insulin syringes fitted with 29 G needles.

2.2. Ex vivo *exposure of hemocytes to cadmium*

After sampling, hemocyte concentration was determined using BD Accuri™ C6 flow cytometer (BD Biosciences). Cells were then transferred in sterile tubes at 2×10^5 cells/mL, diluted with Leibovitz's L-15 medium modified for zebra mussels according to Quinn et al. (2009) with some modifications (L-15 medium (15% v/v), distilled water (85% v/v), HEPES 10 mM, L-Glutamine 2 mM, Penicillin 100 IU/mL, Streptomycin 100 µg/mL, pH 7.5, 0.2 µm filtered).

For exposure, stock and working solution of cadmium chloride hemi-(pentahydrate) (Sigma-Aldrich®) were prepared in hemocyte culture media. Cadmium working solutions was added to cell suspension to reach final cadmium concentration of 10^{-6}, 10^{-5}, 10^{-4}, 5×10^{-4} and 10^{-3} M. Controls were performed by adding cadmium-free culture medium to cell suspension.

2.3. Hemocyte viability and phagocytosis

Hemocyte viability and phagocytic activity were assessed on the same sample after 21 h of incubation with cadmium at 16 °C. This exposure duration corresponds to the time required to experimentally obtain optimal phagocytosis activity in the test described below (Brousseau et al., 1998; Sauvé et al., 2002).

After 21 h incubation with cadmium, cell viability was evaluated using propidium iodide (PI; Sigma-Aldrich) at 10 $\mu g\ mL^{-1}$ final concentration and analyzed by flow cytometry using 488 nm excitation laser and measuring fluorescence in associated channel through 585/15 nm filter. Dead cells with injured membrane are positive to the red fluorescence-emitting probe bound to DNA. Results are expressed as viability percentage (100% – percentage of PI-positive cells).

The phagocytic activity was measured using 2 µm diameter yellow-green fluorescent latex microspheres (Brousseau et al., 1998) (Fluoresbrite®, PolyScience). Latex beads were added to hemocyte suspension 3 h after the beginning of cell incubation with cadmium. Cells were then incubated with beads and xenobiotic for 18 h in dark at 16 °C with a 1:100 hemocyte-beads ratio. Flow cytometric analysis was performed by measuring fluorescence emitted by cells in FL-1 channel (530 nm) associated to 488 nm excitation laser. Percentage of phagocytic hemocytes corresponds to cells that engulfed at least three fluorescent beads and the mean number of engulfed beads per phagocytic cell was calculated as follow: mean fluorescence related to three beads and more divided by mean fluorescence of one bead. However, disturbances of scattering parameters generated by phagocytosis of latex beads did not allowed us to perform analysis of these endpoints at hemocyte subtype scale. Results are expressed considering viability of overall cells and global phagocytic activity of hemocytes.

2.4. Basal oxidative activity

Basal reactive oxygen species (ROS) production by hemocytes was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich) as probe. The nonfluorescent probe freely diffuses into cells where it is de-esterified by intracellular esterase and oxidized by ROS leading to green-fluorescent molecule (DCF). Hemocytes were incubated with 10 µM DCFH-DA for 120 min at 16 °C in dark before processing flow cytometry analysis (Lambert et al., 2003). Relative