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Short communication

Comparison of cytochemical procedures to estimate lysosomal biomarkers in mussel digestive cells

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Abstract

Enlargement and membrane destabilisation in digestive cell lysosomes of mussels are biomarkers of pollution effect. Cytochemical methods are currently applied to determine lysosomal membrane stability (LMS) and lysosomal structural changes (LSC). LMS, determined after grading *N*-acetyl-β-hexosaminidase activity on cryotome sections of digestive gland, is measured as labilisation period (LP). LSC, determined after image analysis of cryotome sections where β-glucuronidase activity is revealed, are measured as lysosomal volume (Vv), surface (Sv), numerical (Nv) densities and surface-to-volume ratio (S/V). Both methods have now been compared in a field study. Mussels were collected from Biscay Bay (Plentzia, reference; Muskiz, moderately polluted) and North Aegean Sea (Olympiada, reference; Limani, heavily polluted). Higher Vv and Sv and lower S/V and LP were recorded in polluted sites than in reference sites. Significant correlations with LP were found for Vv and S/V. The cost/effectiveness and environmental significance of both methods are discussed.

Keywords: Lysosomal membrane stability; Lysosomal structural changes; Biomarkers; Mussels; Marine pollution

Lysosomal responses to pollutants in molluscan digestive cells include alterations in the latency of hydrolases (Moore, 1976; Viarengo et al., 1981, 1987; Harrison and Berger, 1982; Regoli, 1992; Cajaraville et al., 1995; Tremblay and Pellerin-Massicotte, 1997), the size of target lysosomal compartments (Lowe et al., 1981; Cajaraville et al., 1989; Marigómez et al., 1989; Etxeberria et al., 1994; Marigómez and Baybay-Villacorta, 2003), the lysosomal matrix pH and the membrane permeability (Moore, 1982; Moore and Viarengo, 1987; Lowe and Pipe, 1994; Grundy et al., 1996).

Altered membrane permeability can be measured by examining the osmotic fragility of lysosomes in tissue homogenates (Sellinger et al., 1960; Lin and Steichen, 1994) or by examining lysosomal membrane preparations at the molecular level (UNEP/RAMOGE, 1999), which require extensive work and delicate sample manipulation. More easily, membrane alterations can be assessed by examining whether its normal physiol-

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ogy has been affected or disrupted following exposure to pollutants (UNEP/RAMOGE, 1999). The neutral red retention (NRR) assay reflects on the efflux of lysosomal contents into the cytosol after membrane damage, so any impairment of this latter system will result in a reduction of the dye retention time (UNEP/RAMOGE, 1999). A protocol specifically adapted to be used in mussels has been applied to investigate lysosomal responses in both haemocytes and digestive gland cells (UNEP/RAMOGE, 1999). This assay has proven particularly useful in haemocytes (Grundy et al., 1996) whereas its application to digestive cells has been more limited (Ringwood et al., 1998). One important drawback for this test is the need to be performed immediately after collection of hemolymph samples from living animals, which can be a serious problem in certain field conditions.

Cytochemistry has been successfully applied to assess lysosomal integrity in the lysosomal membrane stability (LMS) test (Bitensky et al., 1973), which is based on the demonstration of the latent activity of lysosomal hydrolases, most commonly *N*-acetyl-β-hexosaminidase, measured as the increased permeability of the substrate into lysosomes (UNEP/RAMOGE, 1999). For this purpose, tissue sections are pre-treated at 37 °C or with a weak acid at room temperature for different time intervals before visualising hexosaminidase activity. The time (min) corresponding to the maximum enzyme activity observed is the labilisation period (LP), which diminishes as membrane stability decreases (UNEP/RAMOGE, 1999). LP can be more accurately determined after microdensitometry or cytophotometry (Krishnakumar et al., 1994). Significant decreases in LP values have been reported in response to exposure to organic chemicals (Moore, 1988; Krishnakumar et al., 1994), metals (Harrison and Berger, 1982; Viarengo et al., 1981, 1987; Regoli, 1992) and other sources of environmental stress (Moore, 1976; Bayne et al., 1981; Tremblay and Pellerin-Massicotte, 1997).

Reduction in LP and the increase in osmotic fragility are associated with a change in the size distribution of the lysosomes (Deter and De Duve, 1967; Cajaraville et al., 1995). The lysosomal enlargement, which presumably follows the merging of steady-state lysosomes with autophagosomes, seems to be primarily due to the enhanced sequestration in lysosomes of intracellular proteins of diverse ethiology (Deter and De Duve, 1967). In molluscan digestive cells, exposure to pollutants increases the protein turnover (Moore and Viarengo, 1987) and provokes lysosomal enlargement (Lowe et al., 1981; Moore, 1988; Cajaraville et al., 1989, 1995; Marigómez et al., 1989, 1996; Etxeberria et al., 1994). The prevalence of lysosomal enlargement has been determined by subjective grading (Moore, 1988; Regoli, 1992). Changes in lysosomal size have also been quantified by image analysis after using *N*-acetyl-β-hexosaminidase and β-glucuronidase as lysosomal enzyme markers (Cajaraville et al., 1991b, 1995) to which we will refer to as lysosomal structural changes (LSC) test. The application procedures vary from direct visual reading of stereological grids (Lowe et al., 1981; Cajaraville et al., 1989; Marigómez et al., 1989) to the use of automatic image analysis systems (Cajaraville et al., 1995), final calculations being in most cases based on the equations published by Lowe et al. (1981). Lysosomal structural changes have been also quantified at the ultrastructural level (Domouthsidou and Dimitriadis, 2001).

Several studies compare different analytical approaches to measure lysosomal responses in molluscan digestive cells (Moore, 1976, 1988; Regoli, 1992; Lowe and Fossato, 2000). Domouthsidou and Dimitriadis (2001) found a significant correlation between the volume density of residual bodies and LP values. However, to our knowledge, conventional cytochemical LMS and LSC tests (Table 1) have never been compared.

The purpose of the present study was to compare LMS and LSC tests in order to determine their virtues and limitations and to provide help to those who have to select a procedure to estimate lysosomal biomarkers or have to compare data obtained by these two different approaches. Two different scenarios at European scale (North Aegean Sea (NAS) and Bay of Biscay (BB)) were considered for comparison in order to contribute to the standardisation of lysosomal biomarkers in environmental monitoring studies. Mussels, *Mytilus galloprovincialis*, from NSA are not subjected to severe tidal changes, which may limit the plasticity of digestive cell lysosomes, whereas those collected in BB endure marked tidal variations, which confers a high plasticity to digestive cell lysosomes.

Mussels of 30–40 mm shell length were collected from intertidal populations in Plentzia (reference site) in BB (≈43°24′N, 2°56′W) and subtidal populations