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LYSOSOMAL MEMBRANE IMPAIRMENT IN BLOOD CELLS OF *PERNA VIRIDIS* : AN *IN VITRO* MARKER OF CONTAMINANT INDUCED DAMAGE

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ABSTRACT

Lysosomal membrane damage was investigated in mussels (*Perna virdis*) transplanted to a clean reference site at the PMBC jetty (4 days) and to a contaminated site at the Phuket Oil Depot (6 days). The results of a neutral red retention assay on the blood cell lysosomes of the mussels transplanted to the Oil Depot indicated a significant decrease in the dye retention capacity. These results are indicative of a disturbance to the membrane structure resulting from exposure to the contaminants resident in that environment.

In a separate complementary study the capacity of blood cell lysosomal membrane, from the mussel *Myilus edulis*, for repair was investigated following long term chronic to contaminant exposure. The results of this study, which involved depuration for up to 7 days, indicated that lysosomal membrane damage was not a transient phenomenon and, as such, could have serious consequences for the animals concerned.

INTRODUCTION

Lysosomal membrane damage as a consequence of exposure to contaminants both in the form of environmental pollutant "cocktails" or as individual compounds, under experimental conditions, is well documented in the scientific literature (Kohler, 1991; Kohler *et al.*, 1992; Lowe *et al.*, 1992; Lowe & Pipe, 1994; Moore, 1985; Moore & Clarke, 1982; Viarengo *et al.*, 1987). Lysosomes are remarkable in that they have the capacity to accumulate a wide range of toxicants, however, this role can be potentially hazardous for the cells. The presence of elevated levels of toxicants in lysosomes can result in the breakdown of their limiting membrane and leakage of their acid hydrolases and toxic contents into the cytosol resulting in cell injury or death.

The speed at which contaminant exposure will result in lysosomal damage depends on a complex set of factors which relate not only to the nature and concentration of the contaminant but also the physiological state of the animals. For example, when mussels are spawning the test most widely used to assess damage in the lysosomes, the so called, lysosomal latency test which determines the permeability of the lysosomal membrane to substrates, is unreliable due to natural physiological stressors on the membrane system which effect its stability.

The speed with which the lysosomal membrane becomes damaged is important from a water management viewpoint in that if it is known that the membranes of some of the key species are highly sensitive to contaminant impact then remedial measures, following a spill, would be all the more urgent.

A study was therefore undertaken to simulate a petroleum product spill by transplantation of mussels from a clean population to a contaminated environment and to explore lysosomal damage as a determinant of effect.

Another factor which must also be considered when assessing contaminant impact is the capacity of the cells for repair as this to may effect management decisions.

Studies with *Mytilus galloprovincialis* (Regoli, 1992), determined using a reduction in latency as a marker (Moore, 1975) demonstrated that there was no evidence of lysosomal membrane repair following contaminant induced damage after a depuration period of 4 weeks. A complementary investigation to the spill simulation study was, therefore, undertaken using mussels (*Mytilus edulis*) obtained from a population exposed to chronic industrial waste as well as a clean reference population, to examine, using the same methodology, the potential of the blood cell

lysosomal membrane for repair following a period of depuration.

MATERIALS AND METHODS

Mussels (*Perna viridis*) were collected from Phuket fish market and allowed to recover for 2 days prior to being transferred in cages (70 mussels per cage) to a clean reference site at the PMBC jetty and a contaminated site adjacent to the Phuket oil storage depot. Following 6 days (reference site) and 4 days (contaminated site) transplantation the mussels were removed from the cages for the determination of neutral red retention by blood cell lysosomes as follows.

The valves were carefully prised apart with a solid scalpel and a blue Eppendorf pipette tip inserted between the valves in order to hold them apart whilst 0.5 ml of haemolymph withdrawn from the anterior adductor muscle into a 2.5 ml hypodermic syringe, fitted with a 25 gauge needle, containing 0.5 ml of physiological saline (Peek & Gabbot, 1989). The needle was them removed from the syringe and discarded, in order to reduce shearing forces, and the contents of the syringe ejected into a 2 ml siliconised (Sigmacote) Eppendorf tube which was held in water ice until required.

For the investigation into the capacity of blood cell lysosomal membrane for repair mussels (30), *Mytilus edulis*, were collected from the population at Beggar's Island, at the confluence of the rivers Tamar and Lyhner near Plymouth U.K., and from the population at Sharrow Point in Whitsand Bay, Cornwall U.K. On return to the laboratory haemolymph was extracted from each of 10 mussels from both populations as described for *P. viridis* above and a neutral red retention assay performed. The remaining mussels were then placed in a recirculating aquarium system and further samples of haemolymph taken from each of 10 mussels following 24 hrs and 7 days depuration.

Neutral Red Retention Assay

The neutral red retention assay used for these studies was developed from the methods described in Lowe *et al.* (1992) and Lowe & Pipe (1994).

A stock solution of neutral red was prepared by dissolving 20 mgs of dye (C.I.50040) in 1 ml of DMSO and a working solution then prepared by diluting 10 µl of the dye stock solution with 5 ml of physiological saline.

A 40 µl aliquot of the cell suspension was dispensed onto a 76x26 mm microscope slide and suspended on a rack in a humidity chamber consisting of a shallow plastic box containing water ice for 15 mins to allow the cells to attach. The excess solution was then carefully removed and 40µl of the neutral red working solution was added to the area containing the attached cells and a 18x18 mm coverslip applied. After 15 mins incubation the preparations were inspected under a microscope (x400 mags). Following a further 15 mins incubation the preparation was examined again and systematically thereafter at 30 min intervals to determine at what point in time the dye, which had been readily taken up into the lysosomal compartment of the cells, was lost to the remainder of the cytosol. The test for an individual was terminated when dye loss was evident in 50% (assessed qualitatively) of the small granular haemocytes (Pipe, 1990) and the time recorded; the study was truncated to 240 minutes. Following each inspection the preparations were returned to the incubation chamber.

RESULTS

The neutral red retention assay, which determines the efflux of a chromogenic probe (neutral red) from lysosomes into the cytosol, indicated that whereas some of the mussels caged at the PMBC jetty site were able to retain the dye for up to 240 minutes by contrast those caged at the Oil Depot were only able to retain the dye for 60 minutes. However, there were considerable variability in the retention times of the individual mussels at the PMBC site, which was not reflected in mussels caged at the Oil Depot, resulting in a very high sample variance for that particular treatment group. In order to reduce the differences in the sample variances, which would otherwise render a planned t-Test of the data invalid, it was considered desirable to log transform the raw data. The results of the t-Test on the log transformed

data indicated a significant decrease in dye retention in the mussels caged at the Phuket Oil Depot as compared to those transplanted to the PMBC Jetty (Fig. 1, $P<0.02$).

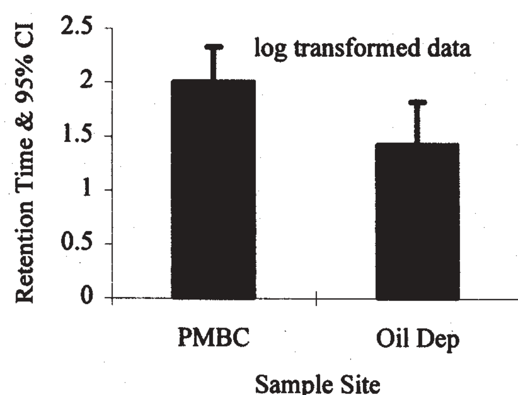

Figure 1. Neutral red retention time (minutes) with associated 95% confidence interval for mussels *Perna viridis* transplanted to the PMBC jetty and Phuket Oil Depot.

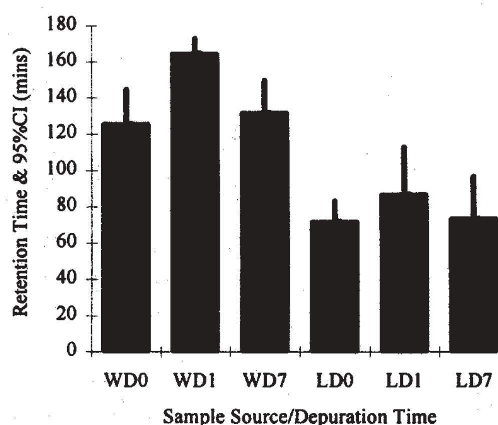

Figure 2. Neutral red retention in mussel (*Mytilus edulis*) blood cell lysosomes from a contaminated site at Beggar's Island (BI) and a clean site at Shallow point (SP) on day 0 (DO) and following depulation at 24 hrs and 7 days (D7).

The membrane repair studies (Fig. 2) indicated no significance difference in the retention time of mussels collected from the contaminated site of Beggar's Island following depuration. There was, however, a significant increase in the retention time of the mussels collected from the clean site at Sharrow Point following 24 hrs depuration ($p<0.001$) although this increase was not maintained up to the 7 day depuration period.

DISCUSSION

The objective of these studies, some of which formed a component of the UNEP (*Perna viridis*) the consequences for the of blood cell lysosomal membranes of short term exposure to a petroleum products spill by means of a site transplantation program. Additionally, a complementary study was undertaken to explore the capacity of the lysosmal membrane for repair following chronic long term contaminant exposure.

The results demonstrated a significant reduction in dye retention capacity in mussels caged at the Oil Depot site as compared to those caged at the clean reference site at PMBC. The failure of the lysosomal membranes, as determined by this assay, is indicative of a disturbance to their structure and in all probability the associated Mg^{2+} ATPase dependent proton pump (Holzman, 1989) by a cytotoxic agent. On the basis of previous field (Lowe *et al.*, 1992) and experimental studies (Lowe & Pipe, 1994; Lowe, *et al.,* 1995)) the cause of this disturbance to lysosomal function is chronic petroleum hydrocarbon seepage/spillage resulting from operations around the Oil Depot.

The failure of the lysosomal membranes to recover following a period of depuration supports the observations of Regoli (1992), for metal contaminants, and indicates that lysosomal damage following contaminant exposure is not transient and as such may have long term consequences for individuals and populations of animals.

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