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BIOMARKER STUDIES FOR THE ASSESSMENT OF CADMIUM TOXICITY IN GREEN MUSSEL *PERNA VIRIDIS* LINNAEUS

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Abstract: Mussels are widely used in both, field and laboratory experiments as sensitive markers of trace metal or organic substances contamination. For the assessment of degree of contamination, the use of biomarkers is a promising approach and allows detection of early biological changes, which may result in long term biological disturbances. Among them lysosomal membrane stability is considered as a very reliable biomarker of general stress in animals exposed to contaminants. In the present study the sublethal effect of cadmium chloride ($CdCl_2$) on the haemocytelysosomal membrane stability of the most common edible bivalve *Pernaviridis* is evaluated by neutral red retention (NRR) assay. The mussels were exposed to various concentrations (0.01, 0.025, 0.05, 0.1 and 0.4 ppm) of $CdCl_2$ for a period of 21 days. Haemocytes were collected from the mussels and destabilization of lysosomes was quantified for 5th, 15th and 21st day of exposure. Similarly the frequency of micronuclei formation was also noted in the dosed mussels. Exposure of mussel to Cadmium Chloride resulted in reduction in NR retention time, indicating lysosomal membrane destabilization in the haemocytes. Lysosomes showed reduced capability to retain neutral red compared to controls and the observed effects were found be dose dependent with concentration of the heavy metal. On exposure of $CdCl_2$ the frequency of micronuclei (MN) found to increase with the increase in concentrations of heavy metal for an exposure period of 5th, 15th and 21st day. From the results it can be concluded that the NRR assay and MN test could be used as effective bio-monitoring tool to evaluate aquatic heavy metal pollution especially in mussels considering their value as an edible commodity.

Key words: biomarkers, *Perna viridis*, cadmium, lysosomal membrane stability, NRR assay, micronuclei

INTRODUCTION

The use of biomarkers is a promising approach in the assessment of ecosystem health. It might allow the detection of early biological changes, which may result in long terms physiological disturbances. It has been reported that biomarkers are influenced rather by the fluctuations in the levels of organic or inorganic contaminants than by fluctuations in natural or physiochemical parameters (Moore, 1991). Cellular biomarkers are used as measure of the sublethal effects of pollutants that can indicate progressive cell damage with physiological consequences (Hebel *et al.,* 1997). Thus measuring at the cellular level may provide a fast and sensitive indication of environmental pollution (Moore, 1985; Viarengo and Canesi, 1991).

Lysosomal membrane stability is considered as a very reliable biomarker of general stress in biomonitoring studies (Krishnakumar *et al.,* 1994; Regoli, 1992). Lysosomes are able to accumulate high amounts of heavy metals in a non toxic form, there by representing an important cellular compartment for metal detoxification (Viarengo and Nott, 1993). Alternation of the integrity of lysosomal membranes may cause undesired release of hydrolases into the cytosol, with consequent damage for cells themselves (Lowe *et al.*, 1995). It has been demonstrated that the exposure of marine organisms to nano-micromolar concentrations of essential heavymetals (Cu, Fe) or of metals without a biological function (Hg, Cd, Cr, Pbetc) causes lysosomal membrane destabilization(Moore, 1990; Viarengo *et al.,* 1981).

Another important cellular level biomarker is

micronuclei. They are small DNA containing bodies which can be present near the cell nucleus during interphase as a consequence of both chromosome breakage and spindle disfunction. The micronucleus assay (MN) has been considered as a cytogenetic test to assess the effects of clastogenic and aneugenic agents in various cell types and organisms (Das and Nanda, 1986; Majone *et al.,* 1987).

Mussels are widely used in both, field and laborataory experiments as sensitive markers of trace metal or organic substance contamination (Krishanakumar *et al.,* 1994; Regoli, 1998; Viarengo *et al.,* 1997). The study organism, *Pernaviridis* commonly known as green mussel is an economically important mussel, a bivalve belonging to the family Mytilidae. They are filter feeders and have the ability to accumulate a wide range of contaminants. Because of its capacity to magnify the heavy metals in its tissues this organism is considered as a sentinel organism and widely used in pollution studies. So the present study was aimed to evaluate the sublethal effect of Cadmium Chloride on lysosomal membrane stability and frequency of micronuclei formation in mussel haemocytes.

MATERIALS AND METHODS

Mussel collection and handling

The green mussel, *Perna viridis* (3 to 4 cm) were collected from rocky shores of Chellanam, Eranakulam district, Kerala. The collected male and female mussels were transported in plastic containers with sea water of ambient salinity. Inside the lab they were cleaned to remove the algae, mud or other fowlers and then washed in a jet of water. Then they were acclimatized in the laboratory conditions for 48 hours in sea water. The sea water was filtered prior to use. Salinity of sea water ranged between 30-35‰ and P^H between 8.15 and 8.30.

Determination of LC_{50}

For LC_{50} studies, ten mussels were randomly selected and exposed to Cadmium Chloride ($CdCl_2$) at a dose range of 0.05, 0.1, 0.5, 1 and 4 ppm for a period of 96 hours. Sea water was changed every day and algal suspension as feed was given throughout the experiment. The number of mussels dead and alive was scored on each day for each concentration. The experiment was done in triplicate and the average mortality for each concentration was taken and the LC_{50} value was estimated.

Heavy metal exposure to sublethal concentrations

The sublethal concentrations were selected on the basis of lethal toxicity studies of $CdCl_2$ on mussels. Ten mussels of shell length 3 to 4 cm were used for each sublethal concentration which was in the range of 0.01, 0.025, 0.05, 0.1, and 0.4 ppm. Bivalves were exposed to metals of different sublethal concentration and were inspected every 12 hours for 21 days. During the course of experiment, sea water was changed daily and the animals were given algal diet in the required quantities.

Collection of haemolymph

Haemolymph was withdrawn from the posterior adductor muscle of mussels with a syringe. The mussel valves should be carefully raised apart along the ventral surface, using a solid scalpel, which should remain in position in order to keep the valves apart. Allow any water retained within the shell cavity to drain out before attempting to withdraw haemolymph. Haemolymph was collected with a syringe (2ml) containing physiological saline so as to obtain a 50/50 of cell/ physiological saline suspension. The needle was then withdrawn and syringe content was poured into a vial placed in ice cubes.

NRR assay for haemolymph

Neutral red retention procedure was performed according to Low and Pipe (1994) with slight modifications. For NRR assay, 40µl haemolymph suspension were spread on microscope slides, transferred to a light proof humidity chamber for 10 minutes and allowed to attach. Then 40µl of the neutral red solution was added to the cell monolayer and incubated for 15 minutes to allow the neutral red to penetrate into cells. After incubation, slides were examined systematically under a light microscope every 15 minutes interval for 120 minutes. The lysosomal destabilization

was expressed as the percentage of haemocytes showing dye loss from lysosomes into cytosol, which consequently appeared reddish pink. The cells were scored using 40X lens, as dye present in cytosol, and the percentage lysosomal destabilization was calculated as follows,

Percentage lysosomal destabilization = (Number of dead cells / Total number of cells) x 100

MN test in the haemolymph

The MN test was conducted according to the procedure proposedby UNEP (1999). A 50/50 of cell/physiological saline suspensions were spread on slides, transferred to a light proof humidity chamber and allowed to attach. Cells were then fixed with methanol: acetic acid (3:1) stained with 5% Giemsa stain for 10 minutes. The cells were scored for each sample using a light microscope under oil immersion at 100X magnification and the results were recorded as micronucleus frequency (MN frequency).

Statistical analysis

LC_{50} value for $CdCl_2$ toxicity in mussels was calculated by Probit analysis using Biostat software.

RESULTS AND DISCUSSION

The NRR assay is based on the principle that only lysosomes in healthy cells take up and retain the vital dye neutral red. In clams and mussles NRR times were depressedfollowing exposure to heavy metals (Matozzo *et al.*, 2001; Viarengo *et al.,* 2000). Exposure of mussel *Perna viridis*to $CdCl_2$ resulted in the lysosomal membrane destabilization. It showed reduced retention capability of lysosomes to neutral red when compared to controls. The observed effects varied with concentration of the heavy metal. After staining with neutral red, the control cells showed a reddish orange granular appearance due to intense staining of lysosomes and prolonged retention of dye within the lysosomes, while mussels exposed to $CdCl_2$ showed gradual destaining of lysosomes. The lysosomal membrane stability was found to reduce with increasing concentration and time.

The percentage lysosomal destabilization on 5th day, 15th day and 21st day were shown in figure 1, 2 and 3 respectively. As compared with control, haemocytes in higher concentrations showed high lysososmal destabilization. After 21 days of exposure all haemocytes in 0.4ppm were destabilized within 60 minutes. These results showed a dose dependent increase in neutral red inf iltration into the cytosol.

Lysosomal membrane alterations are probably due to the high levels of metals that may accumulate in lysosome (Moore, 1990; Regoli, 1992), leading to the inhibition of Mg^{2+}- ATPase, a proton pump of lysosomal membrane that maintains the internal environment of lysosomes acids (Low *et al.*, 1992). Dysfunction of this ATP-ase allows free passage of lysosomal contents, including NR into the cytosol (Lowe *et al*., 1995).

Figure 4 (a) and (b) represents the neutral red activity in control and sublethal concentration of $CdCl_2$ exposed hemocytes. The lysosomes of the control cell take up and retain the dye, while in $CdCl_2$ exposed cells showed lysosomal membrane destabilization and efflux of lysosomal contents into the cytososl.

Micronuclei are formed from the entire chromosome or from a fragment of it. Such micronuclei were induced by genomic stress of heavy metal. It involves the induction of either chromosome fragments that lag behind the separating chromosomes or a chromatin bridge between chromosomes at the anaphase of mitosis. MN test constitute the fast and sensitive test to detect genomic damage due to both clastogenic effects and alterations of mitotic spindle (Bolognesi *et al*., 1999). Due its simplicity micronuclei test is one of the most applicable techniques to identify genomic alternations in the animals.

The results for MN assay on haemolymph were shown in Table 1, 2 and 3. On exposure of $CdCl_2$ the micronuclei frequency found to increase with increase in concentrations of heavy metal. After the 21 days of exposure, there were more than 50% of the cells which possessed micronuclei at higher concentrations. The increase in micronuclei denotes increased genomic damage. As compared to the lower concentration of $CdCl_2$, a three time increase in micronuclei frequency

was observed in higher concentrations. Time dependant increase in MN frequency was also observed with 5 to 6 fold increase over control values after continuous exposure.

In conclusion, the neutral red retention assay and MN test applied in the haemolymph of mussels exposed to sublethal concentrations of $CdCl_2$ proved sensitive enough to be used as a biomonitoring tool to detect early warning for heavy metal toxicity

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