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Responses of Antioxidants and Lipid Peroxidation in *Villorita Cyprinoides* to Mercury Exposure

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Abstract:
Oxidative damage and antioxidant properties have been studied in Villorita cyprinoides subjected to short-term exposure to mercury (Hg) as pollution biomarkers. Villorita cyprinoides is a black water clam that belongs to a group of the genus Villorita; species cyprinoides (Fam:Corbiculidae) were found in the backwaters of Kerala, mainly in Vembanad backwaters. Clams are considered to be nutritious and delicious and are fished in considerable quantities in some coastal places, known to scavenge and deactivate free radicals All aerobic organisms generate free radicals such as reactive oxygen species (ROS) in the process of their oxidative metabolism. The assessment of oxidative stress using superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) and levels of malondialdehyde (MDA) performed on the hemolymph were estimated as biomarkers in clams exposed to mercury. The decreased MDA levels observed in hemolymph under exposure to Hg, showed that the organisms have effectively regulate the exposure stress. Increased activities of antioxidants such as superoxide dismutase catalase, glutathione peroxidise, glutathione reductase and glutathione-S-transferase in hemolymph under short term exposures to Hg suggesting activation of physiological mechanism to scavenge the ROS produced during stress. The results suggest that Hg stress does alter the active oxygen metabolism by modulating antioxidant enzyme activities, which can be used as biomarker to detect sublethal effects of pollution.

Keywords: *Villorita cyprinoides (black water Clam), free radicals scavenger, lipid peroxidation, antioxidant enzymes and hemolymph*

1. Introduction

Heavy metals have a great ecological consideration due to their toxicity and accumulation. Mussels may accumulate significant concentration of metals in water in which those metals are below the limit of detection in routine water samples. Thermal and nuclear power plants often generate heated effluents, which find their way in coastal waters. Similarly sewage and industrial out falls may give rise to metal contaminants of which Hg is considered to be most toxic. The impact of heavy metals on aquatic environment affects directly or indirectly human health.

Antioxidant systems are efficient protective mechanisms against reactive oxygen species (ROS) produced by endogeneous metabolism or by the biotransformation of xenobiotics.The activity of these systems may be induced or inhibited after chemical stress. An induction can be considered an adaptation, allowing the biological systems to partially or totally overcome stress resulting from exposure to an unsafe environment. In contrast, a deficiency of the antioxidant system will induce a precarious state, making biological species more susceptible to toxic agents and preluding toxicity. Indeed, such a deficiency will impair the ability to neutralize ROS and to prevent cell damage. Thus, the parameters of antioxidant systems could be useful biomarkers reflecting not only exposure to contaminants, but also toxicity. Biomarkers that could measure exposure and provide a reliable indication of toxic effect can be very useful for assessing the environmental impact of pollutants (Walker, 1995). Antioxidant systems have been studied for some years in fish and bivalves exposed experimentally to chemicals (Di Giulio *et al.*,1989; Winston and Di Giulio, 1991; Stegeman *et al.*, 1992; Lemaire and Livingstone, 1993).

Bivalve molluscs have proved to be suitable test species for biological monitoring of contaminants in marine ecosystems. Molluscs are dependent, at least in part, on circulating blood cells (hemocytes) for natural internal defense against pathogens. Thus, analogous to mammals, molluscan hemolymph contain hemocytes which are phagocytic and appear to attack invading pathogens via release of ROS (Bachere *et al.*, 1991; Le Gall *et al.*, 1991; Pipe, 1992). To defend themselves against ROS generated by hemocytes during active phagocytosis hemocytes contain the enzymatic antioxidants superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidise (GPx;

EC 1.11.1.9), glutathione-S-transferase (GST; EC 2.5.1.18) and catalase (CAT; EC 1.11.1.6) (Pipe *et al*.,1993).Hemocytes appear highly granulated owing to the fact that they are rich in lysosomes (Moore, 1988; Pipe *et al*.,1993).

The immune system is a sensitive target to the toxic action of chemicals in several organisms (George,1983; Wong *et al*., 1992; Brousseau *et al*.,1998; Fournier *et al*., 2002). SOD, has been reported to correlate well with immune competence of molluscs (Liu et al., 2004). As a free radical elimination enzyme, SOD is essential to minimize the oxidative damage to host cells in the immune defense (Zhang *et al.,* 2005). Superoxide dismutase and Se-dependent glutathione peroxidase are two main antioxidants in organisms (Orbea *et al.,* 2000; Dautremepuits *et al.,* 2004; Chandran *et al.,* 2005). Catalase (CAT) is a commonly studied antioxidant enzyme involved in the initial anti-oxidative mechanism and widely used as a biomarker in mussel (Cajaraville *et al.,* 2000; Khessiba *et al.,* 2001; Nasci *et al.,* 2002; Lau and Wong, 2003; Romeo *et al.,* 2003). Malondialdehyde (MDA) is one of the final products of the membrane fatty acid degradation, mainly total thiobarbituric acid-reactive substances (TBARS), and is considered as a good biomarker of lipid peroxidation and consequently of oxidative stress (Pellerin-Massicotte 1997; Cheung *et al.,* 2003, 2004). Variations in SOD, CAT, GPx and MDA activities suggest their potential use as biomarkers of effects, such as oxidative stress, resulting from Cd contamination in the mollusc *R. decussates* (Geret, *et. al.,* 2002)

This work is a contribution to a crucial need to understand the effects of exposure to Hg on antioxidant and lipid peroxidation in *Villorita cyprinoides.* It represents the first level of contamination via the trophic way, and may be of real importance in complement to other trophic level contamination studies to better understand the bioaccumulation process.

2. Materials and Methods

Specimens of *Villorita cyprinoides* size group, 25–30 mm) were collected from pristine locations, in Cochin backwaters (9° 55'N; 76° 17′E). The animals were laboratory conditioned in aerated, biofiltered sea water (salinity—18×10^{-3}, pH 7·6 ± 0·2, temperature 28 ± 0·l°C). Acute toxicity was performed following the standard methodology of EPA/ROC (1998) to determine the lethal concentration (LC50) and the sub- lethal level of mercury. The LC50 values were calculated using Probit Analysis (PASW 18). The three sub- lethal concentration selected for the metals were 6 µg/L, 12 µg/L and 30 µg/L. Hemolumph was collected with a sterile syringe from the posterior adductor muscle of both control and treated animals. The extracted haemolymph was centrifuged at 700rpm, and the cell-free haemolymph was used for the analysis.

SOD activity was measured by following the oxidation of NADH at 340 nm as described by Paoletti *et al.* (1986). Catalase activity was determined following the method of Maehly and Chance (1955). The activity was estimated spectrophotometrically following a decrease in absorbance at 230 nm due to H_2O_2 consumption.

The activity of GPX was determined by the method of Pagilia and Valentine (1967). GPX catalyses the oxidation of GSH to GSSG in the presence of H_2O_2. This reaction was coupled to NADPH oxidation in presence of exogenous GR to maintain GSH concentration. Total GPX activity was measured taking cumene-hydroperoxide as the substrate. The absorbance was recorded at 340 nm. Enzyme activity was expressed as nmol NADPH oxidized / min/ mg proteins using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

GST activity was measured according to Habig *et al*., (1974) using CDNB as a substrate. The change in absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed / min / mg protein using a molar extinction coefficient of 9.6 mM^{-1}
cm^{-1}.

LPX level was assayed by measurement of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids. Hydroperoxides were determined by the TBA reaction
as described by Ohkawa *et al.,* (1979). The absorbance was read at 532 nm after removal of any flocculated material by centrifugation. The amount of TBARS formed was calculated by using an extinction coefficient of 1.56 X 105 M^{-1} cm^{-1} and expressed as nmol TBARS formed per mg protein. Protein content was estimated by Folin-Phenol method of Lowry *et al.,* (1951) using bovine serum albumin as a standard.

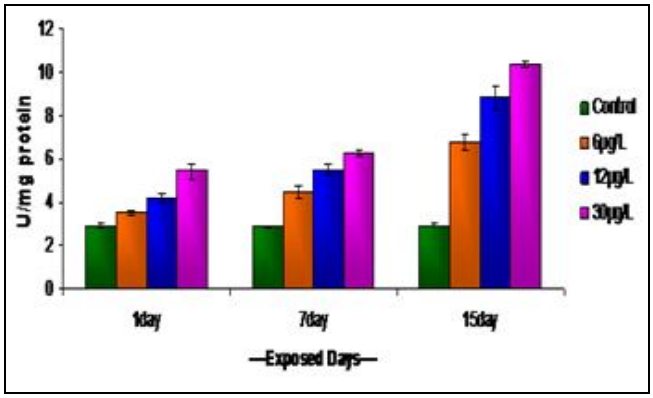
3. Statistical Procedures

All values quoted are mean values ± SD. Statistical analyses were carried out with the aid of the Sigmastat 3.5 statistical package. Data were analyzed by two way analysis of variance and significance was concluded at $P < 0.01$ probability level. The statistical difference between the control and exposed bivalves was tested with Dunnett's test. Tests for data normality and homogeneity of variance were carried out previously.

4. Results

SOD showed a significant increased activity with increasing concentrations of mercury. Increase in activity was proportional to days of exposure as well, with highest SOD activity on the 15th day. (Table1and Fig 1).

Mean catalase activity was highest in animals from treatments exposed to the higher Hg concentrations. However, the lowest concentration 6 and 12 µg/L of mercury did not appear to elicit any induction of the enzyme above control levels (Fig. 2). On the 7th and 15th days of observation, CAT activity showed dose and time dependent elevation.



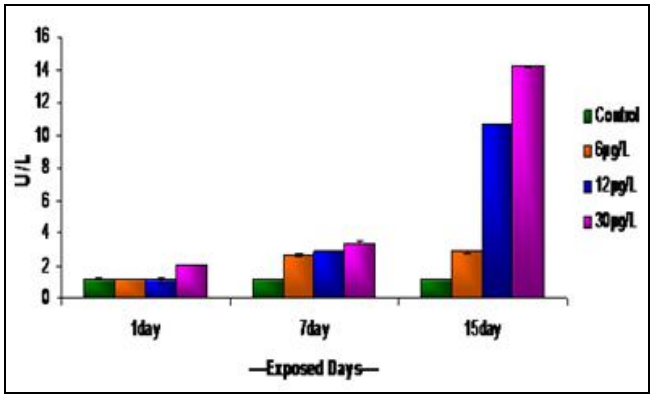
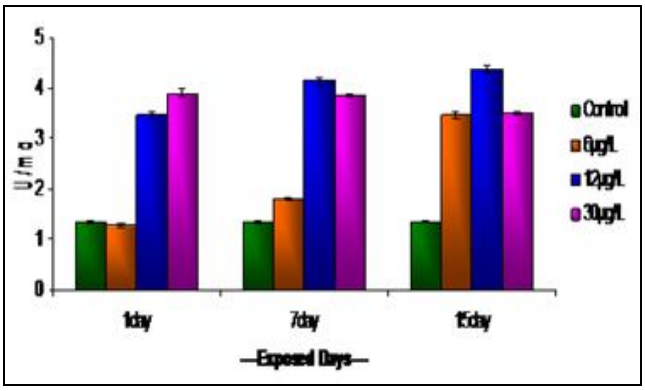


Figure 1: Superoxide dismutase activity in mercury exposed V.cyprinoides
Figure 2: Catalase activity in mercury exposed V.cyprinoides



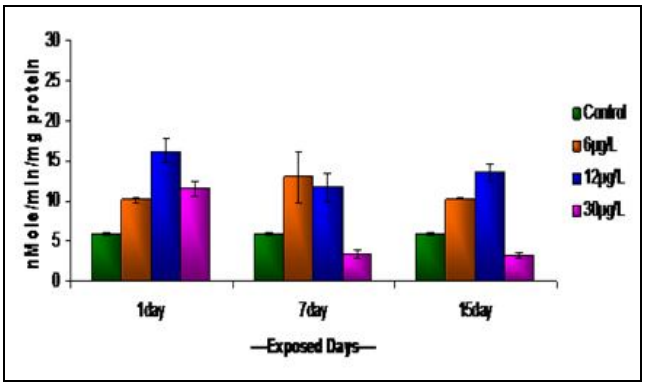


Figure 3: Glutathione peroxidase activity in mercury exposed V.cyprinoides
Figure 4: Glutathione S Transferase activity in mercury exposed V.cyprinoides

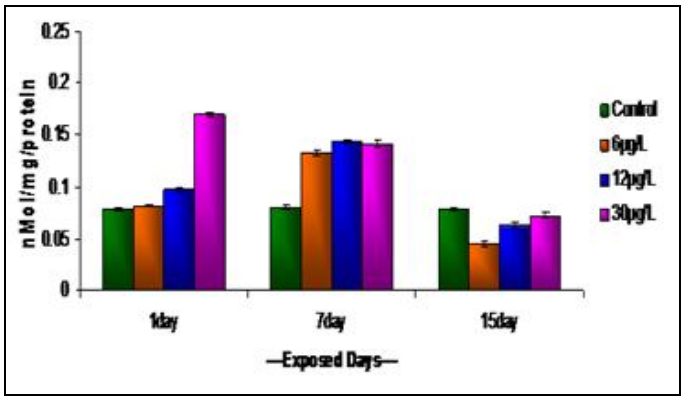


Figure 5: Lipid Peroxidation in mercury exposed V.cyprinoides

Source of variation	df	Sum of Squares	Mean Square	F	P-value
Day	**2**	135.870	67.935	1051.265	<0.001
Concentration	**3**	194.949	64.983	1005.582	<0.001
Day X Concentration	**6**	51.425	8.571	132.630	<0.001
Residual	**60**	3.877	.065		
Total	**72**	2443.091			

Table 1: Two way ANOVA Table for Superoxide dismutase activity in mercury exposed V.cyprinoides

Source of variation	df	Sum of Squares	Mean Square	F	P-value
Day	**2**	445.124	222.562	14075.558	<0.001
Concentration	**3**	325.838	108.613	6869.028	<0.001
Day X Concentration	**6**	386.188	64.365	4070.630	<0.001
Residual	**60**	.949	.016		
Total	**72**	2125.630			

Table 2: Two way ANOVA Table for Catalase activity in mercury exposed V.cyprinoides

Source of variation	df	Sum of Squares	Mean Square	F	P-value
Day	**2**	5.458	2.729	1030.866	<0.001
Concentration	**3**	86.900	28.967	10941.087	<0.001
Day X Concentration	**6**	13.425	2.237	845.133	<0.001
Residual	**60**	.159	.003		
Total	**72**	677.444			

Table 3: Two way ANOVA Table for glutathione peroxidase activity in mercury exposed V.cyprinoides

Source of variation	df	Sum of Squares	Mean Square	F	P-value
Day	**2**	110.583	55.291	79.326	<0.001
Concentration	**3**	607.300	202.433	290.431	<0.001
Day X Concentration	**6**	199.313	33.219	47.659	<0.001
Residual	**60**	41.821	.697		
Total	**72**	7226.127			

Table 4: Two way ANOVA Table for glutathione S transferase activity in mercury exposed V.cyprinoides

Source of variation	df	Sum of Squares	Mean Square	F	P-value
Day	**2**	.045	.023	3541.200	<0.001
Concentration	**3**	.025	.008	1294.093	<0.001
Day X Concentration	**6**	.028	.005	727.087	<0.001
Residual	**60**	.000	6.41E-006		
Total	**72**	.803			

Table 5: Two way ANOVA Table for Lipid Peroxidation in mercury exposed V.cyprinoides

Except for a marginal decrease at 6 µg/L Hg on the first day, GPx activity increased with concentration and period of exposure in organisms treated with mercury. (Table. 3, Fig.3). There were significant differences in GPx activities related to the treatments applied, after 15th day of exposure . ANOVA revealed significant differences in GPx activities among treatments after Hg exposure .
In mercury treated *V.cyprinoides* GST activity was found to be higher for those mussels exposed to 6 and 12 µg/L mercury (Table. 4 and Fig. 4).
V.cyprinoides exposed to mercury showed interesting result for lipid peroxidation. The increased lipid peroxidation activities that occurred in the animals on the 1st and 7th day were seen to decrease. Decreased Lipid peroxidation show Hg exposed mussel could effectively regulate mercury stress(Table,5 and Fig.5).
Dunnet result shows that the changes in activity for the toxicants were found to be statistically significant at $P<0.01$ level in terms of days and concentration .

5. Discussion

Increased oxidative stress has been suggested to enhance the antioxidant enzymes activities in animals, as a protective action towards oxidative stress. Superoxide dismutase (SOD) is one of the main antioxidant defence enzymes generated in response to oxidative stress. It converts the highly toxic superoxide anions into hydrogen peroxide. In the present study, the activity of SOD was significantly increased in the haemolymph. On the15th day, *V.cyprinoides* exposed to the highest concentration of mercury had a 3.5 fold increase in SOD activity . It is interesting to note that the production of superoxide anion in Hg stressed clams was higher in all

concentrations and time periods. Obviously, Hg stressed ones, higher production of superoxide anions is countered by higher production of SOD to neutralize the adverse effect. This explains the higher antioxidant activity in mussels exposed to heavy metals. Increased SOD activity on 15th day of 0.45mg/L Hg exposure was reported by Verlecar *et al.,* (2008) indicating protective behaviour of the cell against super oxide radicals. Increase in SOD activity has been reported by Prakash and Rao (1995) in mussels exposed to Al, Pb and Cd.

Hydrogen peroxide is toxic to cells. CAT and GPX are the major primary antioxidant defense component that catalyses the decomposition of H_2O_2 which is produced by the action of superoxide dismutase to H_2O.

Glutathione peroxidase catalyses the reduction of hydroperoxides, with the conversion of reduced glutathione (GSH) to glutathione disulfide (GSSG). At high H_2O_2 concentrations, organic peroxides are metabolized by Catalase. Geret *et al.,* (2002) observed Hg to have a significant inhibitory effect on the activity of CAT and glutathione peroxidase for the first day at concentration 25 µg/L. In the present study slightly reduced CAT and GPx activities were noted on the first day for lower mercury concentrations. According to Geret (2002) this inhibition is metal dependent. Mercury is known to be involved in redox reactions (Fenton reactions), which result in the production of oxyradicals.

GPx is inactivated with increased levels of hydrogen peroxide, which in turn is due to the lower activity of catalase . GPx activity was elevated as a response to chronic toxicity for mercury exposed *V.cyprinoides.* Other studies in which increased CAT (Prakash and Rao, 1995) and GPX activities were reported are in the digestive gland of *P. viridis* in response to metals, seasonal changes in environmental parameters and pollutants (Verlecar *et al.,* 2008). Besides participating as a hydrogen donor in the glutathione peroxidation reaction, reduced glutathione has a direct antioxidant function by reaction with superoxide, hydroxyl radical and singlet oxygen, leading to the formation of oxidized glutathione, and in the present study, the activity of glutathione-*S*-transferase was also significantly increased when compared with the activity in the controls except for a few observation. Significant increase in GST activity as observed on the 1st day in mercury exposed specimen suggests the protective action against reactive oxygen radicals. An effective antioxidant control will end up with low MDA level and vice versa (Lau and Wong, 2003). CAT activity was well evident in Hg exposed mussel on chronic exposure of 15 days, and hence MDA produced was almost similar to that of control mussels or below.

This shows animals can adapt themselves to Hg stress, by mussels on prolonged exposure. Elevated SOD and CAT activities along with decreased lipid peroxidation prove mussels' efforts to curtail oxidative damage caused by mercury. An enhanced level of thiobarbituric acid reactive substances (TBARS) was observed in the digestive gland of the mussel, *P.viridis* in response to mercury (Geret *et al*., 2002; Almeida *et al.,* 2004; Verlecar 2008).

All marine organisms are known to contain high levels of polyunsaturated fatty acids, which are the substrates for lipid peroxidation, and the presence of antioxidants elevates the resistance of their cell membranes to oxidative stress. In present study LPO was high on 1st and 7th day of mercury exposure, which decreased by the 15th day well below that of the control value. This result is similar to those obtained in gills and digestive gland of *Mytilus edulis* on mercury exposure by Geret *et al.,* (2002). Mercury have high affinities for glutathione (GSH), which is the primary intracellular antioxidant agent, and can bind and cause the irreversible excretion of GSH leading to depletion of GSH. Present study showed decreased MDA production by 15 days when exposed to mercury. Similar decrease in MDA level was obtained in gills of *Mytilus edulis* on exposure to 40 µg/L-1 Cu for 21 days (Geret *et al.,* 2002). The probable reason may be due to the pairing of mercury with metallothionein resulting in an intensification of antioxidant systems.

6. Conclusion

In the present study, significant differences have been recorded in the activities of antioxidant enzymes in the haemolymph of *V.cyprinoides* exposed to mercury as compared with the control mussels. This indicates that there is an increased level of oxidative stress due to the presence of heavy metals, and that an imbalance is generated between pro-oxidants and antioxidants. The study made in *V.cyprinoides* haemolymph can help to understand mechanism through which metals exert their toxicity in organisms and hence the results can be used to explain the impact of heavy metal toxicity on organisms .Hg exposed mussels are likely to adapt themselves even to the highest concentration.

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