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SUB LETHAL MERCURIC CHLORIDE TOXICITY INDUCED STRESS RELATED ALTERATIONS IN THE EPITHELIAL LINING OF FOOT OF THE FRESHWATER MUSSEL *LAMELLIDENS MARGINALIS* (LAMARCK)

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ABSTRACT

Sub lethal (0.2 ppm) mercuric chloride induced stress related histopathological alterations in the epithelial linings of foot (podium) of the edible freshwater mussel *Lamellidens marginalis* (Lamarck) were studied using histochemical techniques up to 60 days of exposure. The histomorphological changes were manifested only slowly and its intensity was somewhat proportional to the duration of exposure. The immediate response of the exposed mussels was the altered mucous secretion. There was a progressive incorporation of sulphated glycoproteins into the secretory contents of the mucous cells especially in the first half of the experiment. Marked histopathological changes including necrosis, appearance of pyknotic nuclei, sloughing of epithelial cells and appearance of non-tissue spaces, etc., started appearing during the later half of the experiment. The fag end of the experiment, which witnessed prominent histomorphological changes, was accompanied by highly decreased mucous secretion.

KEYWORDS*:* heavy metal toxicity, mercuric chloride, *Lamellidens marginalis*, freshwater mussel, histopathology.

INTRODUCTION

Among the myriad of heavy metal pollutants, mercury is considered as a dangerous pollutant which is biologically non-essential, persistent and highly toxic to humans and wild life. The extreme group II b character gives it high affinity towards thiol groups and enhanced covalence when compared to zinc and cadmium leading to increased bio-transport, distribution and toxicity (Venugopal and Lucky, 1978; Kent, 1998). Various man made activities have caused the elevated levels of mercury in different ecosystems and the toxicity caused by excessive mercury exposure is now recognized as a wide spread environmental problem. Even though many of the molluscan forms constitute protein rich food sources for human, they are also common source of mercury in people (Raloff, 1991, USEPA, 2000). In India also, the fresh water mussel *Lamellidens marginalis*, which is having comparatively enough tissue mass, is consumed by native rural folks as a food item. Mussels being primarily benthic filter feeding organisms are continuously exposed to metals that are dissolved in water, associated with suspended particles as well as deposited in the bottom sediments (Naimo, 1995).

In spite of all these facts, exploitation of *L. marginalis*, which is widely distributed throughout the Indian subcontinent, as a model for toxicological studies is, limited (Hameed and Raj, 1990; Sreedevi *et al.*, 1992; Das and Jana, 2003) and reports dealing with mercury toxicity are rare (Sonawane, 2004). In this context, the present investigation has been designed to evaluate the histopathological manifestations of the sub lethal mercuric chloride toxicity on the epithelial lining of foot of the freshwater mussel *L. marginalis*. The selection of the tissue is based

on the fact that it is an edible tissue and at the same time it makes immediate contact with the medium, once the shells opened by the animal.

MATERIALS AND METHODS

Freshwater mussel *L. marginalis* (20 to 23 g weight and 5 to 6 cm length) were acclimated to the laboratory conditions for 20 days (d) in plastic aquaria bearing well water. Feeding was done following Sreedevi *et al.* (1992) and Sonawane (2004) on every day. The medium was renewed after every 24 hours (h). Prior to the commencement of the experiment, 96 h median lethal concentration (96 h LC_{50}) of mercuric

Table 1. Summary of the histochemical alterations in the epithelial covering of the foot of *L. marginalis* at various stages of 0.2 ppm mercuric chloride exposure

Staining Techniques	Cell types	Control	Exposure periods				
			5 d	10 d	20 d	40 d	60 d
Periodic acid Schiff (PAS) for neutral glycoproteins	ECs	0	0	0	1	1	± ~ 1
	MCs	2 ~ 3	1 ~ 2	2	2 ~ 3	2 ~ 3	1 ~ 2
Alcian blue pH 1.0 (AB 1.0) for sulphated mucoproteins	ECs	0	1 ~ 2	±	0	±	0
	MCs	± ~ 1	2 ~ 3	3 ~4	1 ~ 2	± ~ 1	±
Alcian blue pH 2.5 (AB 2.5) for acidic glycoproteins	ECs	0	±	0	0	0	0
	MCs	2 ~ 3	2 ~ 3	1 ~ 2	1	± ~ 1	±
AB 2.5/PAS for acidic and neutral glycoproteins	ECs	0	± b	0	1 pk	± ~ 1 pk	± pk
	MCs	2 ~ 3 P	3 ~ 4b	2 pk	2 ~ 3 pk	2 ~ 3 pk	1 ~ 2 pk
Bismarck brown (BB) for water stable mucoproteins	ECs	1	±	0	±	0	0
	MCs	0	1 ~ 2	± ~ 1	2 ~ 3	1 ~ 2	0

Note: b = Bluish; d = Day(s); ECs = Epithelial cells; MCs = Mucous cells; P = Purple; PK = Pinkish; O = Negative reaction; ± = Very weak reaction; 1 = Weak reaction;2 = Moderate reaction; 3 = Strong reaction; 4 = Very strong reaction; ~ = To

Five groups of 10 mussels each were exposed separately to 50 litres (l) each of 0.2 ppm (sub lethal) mercuric chloride solution prepared in well water having dissolved oxygen 5.6 ± 0.2 ppm, pH 7.2 ± 0.1, water hardness 110 ± 3.0 mg/l and water temperature 28 ± 1°C. Parallel control groups were also kept in separate aquaria containing 50 l of well water without the addition of mercuric chloride. Feeding was allowed in the experimental as well as control groups everyday throughout the tenure of the experiment. No death was observed either in the experimental or in the control groups. After the expiry of 5, 10, 20, 40 and 60d of exposure, 3 freshwater mussels each from the respective experimental as well as control groups were sacrificed. Foot tissue of the sacrificed mussels were excised separately and fixed in 10% neutral formaldehyde and aqueous Bouin's fluid. After dehydration, tissues were cleared in xylene and cedar wood oil and 5 □m sections were stained with Ehrlich's Haematoxylin/Eosin (H/E) for routine histopathological observations. Various glycoprotein moieties (Table 1) were histochemically detected by periodic acid – Schiff's (PAS), Alcian blue pH 1.0 (AB 1.0), Alcian blue pH 2.5 (AB 2.5), AB 2.5/PAS (AB/PAS) and Bismarck Brown (BB) (Pearse, 1985) methods. Thickness of the epithelium was measured using a stage and ocular micrometer. Densities (number/mm^2) of various cell types were calculated using a stage micrometer and camera lucida following Rajan and Banerjee (1992). Data were obtained by random sampling of five different sites of experimental as well as control tissues from each of the three individual mussels at each sampling period. One way analysis of variance (ANOVA) followed by Duncan's multiple range tests was performed to determine whether the morphometric parameters were influenced significantly by the exposure periods. Since there were no significant variations between the measurements taken from various control groups at different exposure periods, their average values were taken into account.

OBSERVATION

Macroscopic (behavioural) alterations

In contrast to the control mussels, the sub lethally exposed mussels in the present study kept their valves (shells) tightly closed for about 45 minutes. Later, even though the mercury exposed mussels slowly opened the valves, they were closing it instantaneously after releasing a few bubbles of air. This process went on for about 5 to 7 hours in the exposed groups. Later the mussels were keeping their valves slightly open for short durations. Desquamation of the mucous coating in the form of streaks into the medium was commonly exhibited by the exposed mussels at various stages the experiment. From 10d onwards, rejected flakes of cells and cell debrises were also seen entangled in the mucus. Another prominent observation especially after 40d of exposure onwards was the appearance of oedematic (swollen) foot in some of the exposed mussels, which were not able to fully withdraw the foot into the mantle cavity even after mechanical stimulation by glass rode. However, no death was noticed either in the experimental or in the control groups through out the tenure of the experiment.

Table 2. One way analysis of variance (ANOVA) showing significant changes in the various morphometric parameters of the epithelium of foot in *L. marginalis* at various stages of 0.2 ppm mercuric chloride exposure

Parameters	Source	ss	df	Ms	F	P
Epithelial thickness	Total	5357.84	17			
	Between Groups	4325.13	05	865.03	10.35	< 0.001
	Within groups	1002.71	12	83.56		
Density of AB 1.0 MCs	Total	267568.89	17			
	Between Groups	265834.21	05	53166.84	367.78	< 0.001
	Within groups	1734.68	12	144.56		
Density of AB 2.5/PAS MCs	Total	173737.74	17			
	Between Groups	207959.99	05	40902.06	142.28	< 0.001
	Within groups	204510.28	12	287.48		
Density of Bismarck brown MCs	Total	3449.71	17			
	Between Groups	8229.06	05	1645.81	30.25	< 0.001
	Within groups	652.89	12	54.41		

Microscopic alterations

Control epithelium

In *L. marginalis*, foot (podium) appeared as a muscular, extensible, hatchet like protrusion of the visceral mass hanging down from the mantle cavity and was fully surrounded by the epithelial covering, which formed ridges and grooves all over the surface (Fig. 1). The epithelium consisted mainly of columnar epithelial cells (ECs), mucous secreting glandular cells (mucous cells or MCs) and blood cells. The ECs were lying on a thin basement membrane, which received rich supply of blood through minute blood vessels and below which the connective tissue was located. Histochemical staining behaviour of MCs is shown in the Table 1 and Figures 2, 3). The outer border of the epithelium was covered with a weak to moderately PAS positive and eosinophilic mucoid lining. AB 1.0 positive MCs were less in number and were exhibiting weak staining. BB positive MCs were generally absent. The various morphometric parameters (Tables 2, 3) of the epithelium in comparison to control exhibited periodic alterations throughout the tenure of the experiment.

Experimental epithelium

The epithelium did not show much histomorphological changes after 5d of sub lethal mercuric chloride exposure. However, the overall thickness of the epithelium along with the mucogenic activity was increased when compared to that of control mussels (Tables 1, 2,3 and Fig. 4). Hyperplasia of MCs was

evident. With AB 2.5/PAS staining, most of the MCs in the upper region of the epithelium showed a bluish colouration and the PAS positive cells were seen only in the basal region. Some of the MCs appeared as fused with each other. The PAS positive and eosinophilic mucous coating seen over the control tissue was absent after 5 d of exposure. In comparison to control, the density and staining intensity of AB 1.0 positive MCs were increased significantly (Tables 1, 2, 3). In contrast to the control tissue, BB positive MCs were seen scattered throughout the epithelium with weak to moderate staining intensity after 5d of exposure (Tables 1, 2, 3).

Mild sloughing of the ECs and development of vacuoles in the epithelium marked the tenth day of exposure. The thickness of the epithelium remained more or less same as that of 5 d (Table 3). The weak to moderately stained AB 2.5 positive MCs in the middle and basal regions of the epithelium mainly maintained the density of AB/PAS positive MCs at the level of 5d. PAS positive MCs were restricted only to the basal region with moderate staining intensity. The epithelium also showed hyperplasia AB 1.0 positive MCs (Tables 1, 2, 3 and Fig. 5). The density of BB positive MCs was reduced when compared to that of 5d, and were with a weak-staining intensity.

After 20d of exposure, the degenerative action of mercuric chloride was evident form the sloughing of necrotic epithelial cells in flakes or in layers (Fig. 6). Prominent non-tissue spaces were also seen in the basal

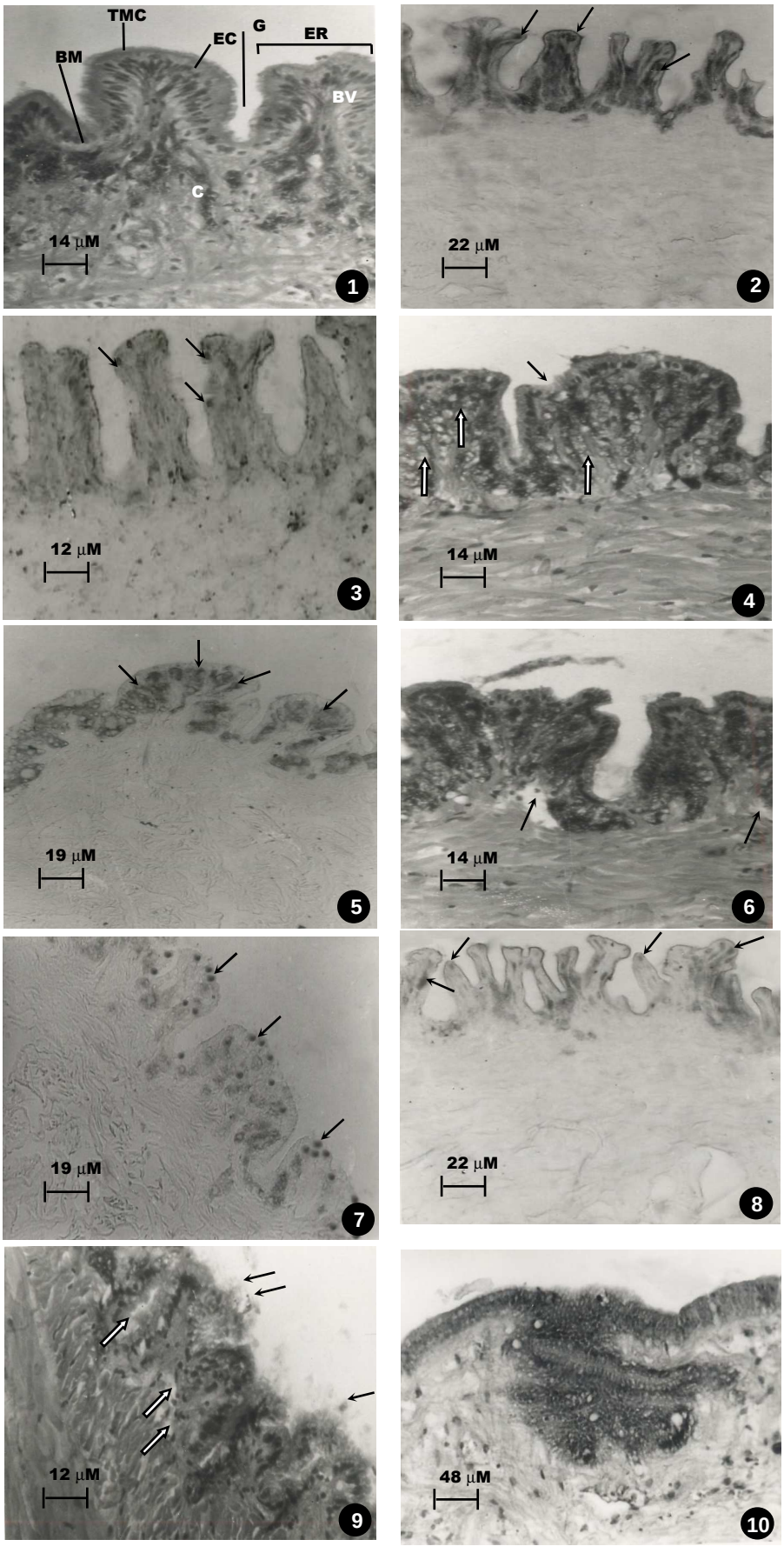
Table 3. Variations in the various morphometric parameters of the epithelium of foot in *L. marginalis* at various stages of 0.2 ppm mercuric chloride exposure

Parameters	Control	5 d	10 d	20 d	40 d	60 d
Foot epithelium thickness	51.58 ± 5.82	66.60 ± 5.81 a^{*}	66.96 ± 3.66 a^{*} b^{NS}	60.77 ± 4.25 a^{NS} b^{NS}	34.86 ± 5.69 a^{*} b^{**}	26.75 ± 6.37 a^{**} b^{NS}
Density of AB 1.0 MCs	107.24 ± 5.23	294.92 ± 9.37 a^{**}	412.57 ± 8.66 a^{**} b^{**}	363.94 ± 4.70 a^{**} b^{**}	188.16 ± 6.81 a^{**} b^{**}	97.72 ± 5.51 a^{NS} b^{**}
Density of AB 2.5/PAS MCs	205.72 ± 8.76	395.31 ± 6.47 a^{**}	371.71 ± 17.59 a^{**} b^{NS}	270.74 ± 8.19 a^{**} b^{**}	174.57 ± 6.89 a^{*} b^{**}	94.48 ± 5.69 a^{**} b^{**}
Density of Bismarck brown MCs	0.0 ± 0.0	23.66 ± 4.09 a^{**}	11.10 ± 3.52 a^{*} b^{*}	57.11 ± 5.91 a^{**} b^{**}	42.54 ± 6.69 a^{**} b^{*}	0.0 ± 0.0 a^{NS} b^{**}

Note: $\overline{X} \pm$ SEM (based on Duncan's multiple range test); a = between the respective experimental group and control group; b = between the respective experimental group and preceding experimental group; d = days; NS = not significant; * = $p < 0.05$; ** = $p < 0.01$.chloride (99% pure, E. Merck, India) to *L. marginalis* was estimated following Finney (1971) through 24 h renewal bioassay system, and was found to be 4 ppm. Feeding was withheld during the bioassay for the estimation of LC_{50}.

region of the epithelium. The overall thickness of the epithelium remained more or less same as that of 10d (Tables 2,3). Even though, there was a progressive increase in the AB 2.5 staining intensity, the developing small MCs showed strong PAS reaction. While the staining intensity as well as the density of AB 1.0 positive MCs decreased significantly than the previous stage, those of the BB positive MCs were increased significantly (Tables 2, 3 and Figs.7).

After 40d of continued sub lethal exposure, the over all thickness of the epithelium reduced significantly (Tables 2, 3). Pyknotic nuclei, sloughing of cells and prominent non-tissue spaces were seen along the epithelium. The density of AB/PAS positive MCs decreased significantly than that of 20d and those present were of smaller size. The densities as well as staining intensity of AB 1.0 positive MCs and BB positive MCs were also reduced from that of the previous stage (Tables 1, 2, 3 and Figs. 8).


TMC
EC
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14 µM
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22 µM
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12 µM
3
14 µM
4
19 µM
5
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6
19 µM
7
22 µM
8
12 µM
9
48 µM
10

Figs. 1-10: Photomicrographs showing transverse sections (T.S.) of the epithelium of foot of control and 0.2 ppm mercuric chloride exposed freshwater mussel *L. marginalis.*

Fig. 1 : Structural organization of control epithelium (H/E).
BM = Basement membrane, BV = Blood vessels, C = Connective tissue, EC = Epithelial cell, ER = Epithelial ridge, G = Groove, TMC = Thick mucoid coating.

Fig. 2 : Distribution of AB 2.5/PAS positive MCs (arrows) and carbohydrate moieties in the control epithelium (AB 2.5/PAS).

Fig. 3 : Distribution of AB 1.0 positive MCs (arrows) and carbohydrate moieties in the control epithelium (AB 1.0).

Fig. 4 : Mild sloughing of the epithelium (arrow) after 5d of exposure. Note the hyperplasia of MCs (open arrows) (H/E).

Fig. 5 : Distribution of AB 1.0 positive MCs (arrows) and carbohydrate moieties in the epithelium after 10d of exposure (AB 1.0).

Fig. 6 : Sloughing of ECs in flakes after 20d of exposure. Note the development of prominent non-tissue spaces in the basal region (arrows) (H/E).

Fig. 7 : Hyperplasia of BB positive MCs (arrows) after 20d of exposure. (BB).

Fig. 8 : Decreased density of AB 2.5/PAS positive MCs after 40d of exposure (AB 2.5/PAS).

Fig. 9 : Extensive necrotic lesions in the epithelium after 60 d of exposure. Note the sloughing of epithelial cells (arrows) and development of non-tissue spaces (open arrows) (H/E).

Fig. 10 : Formation of sub-epithelial granulation tissue after 60d of exposure (H/E).

The sustained exposure unto 60d caused extensive sloughing of ECs and significant reduction in the epithelial thickness (Tables 2, 3 and Figs. 9, 10). The epithelium exhibited necrotic lesions at many places. At some places the sloughing ended up in the loss of the epithelial ridges and in the formation of sub epithelial granulation tissue (Fig. 10). In this undifferentiated granular tissue, the process of epithelialization was discernible. The mucogenic activity of the epithelium was drastically reduced (Tables 2 ,3). The MCs present were more PAS positive than AB 2.5 (Table 1). The density and staining intensity of AB 1.0 positive MCs were also considerably reduced. BB positive MCs were not seen at this stage of exposure.

DISCUSSION

The tight closure of the valves on exposure to the toxic medium by the mussels for a considerable period may be considered as an immediate effort by the organism to ward off the toxicity by preventing the entry of the mercury containing ambient medium into the mantle cavity. The appearance of the oedematic foot with reduced response towards mechanical stimulation in the later stages of the present study may be due to the muscular dystrophy caused by the reduced contractile ability of myofibrils and deterioration of nerve cells because mercury is reported to cause decreased transmembrane potential (Crinnion, 2000) as well as demyelination in the myelin sheaths of nerve fibers (Chang, 1977). Occurrence of such oedematic swollen foots have also been reported by Sreedevi *et al.* (1992) and Balchandra *et al.* (2001) in nickel exposed *L. marginalis* and thereby reducing its bioavailability. The presence of BB positive water stable mucoproteins may be

imparting more stability to the mucous coating as foot faces the additional risk of lose of mucus by friction as it is the organ of movement.

In contrast to the first half, the fag end of the experiment showed extensive histopathological changes. The present investigation reveals that under the sub lethal mercuric chloride toxicity, the cells may exist in the xenobiotic induced altered state exhibiting pathological signs like loss of cell volume regulating capacity, vacuolization, chromosome condensation, etc. and with the increase in exposure period the pathological signs become more acute leading to cell death and sloughing. Trump *et al.* (1975) are of the opinion that the cell response to an injurious chemical stimulus is a biphasic one. That is, they may exist for a time in either recoverable or non-recoverable phase. The cells in the recoverable phase do not lose their ability to recover if the stimulus is removed. But those in the latter state cannot regain its cellular activities even if the stimulus is removed and the effect become lethal to the cell.

Another worth mentioning observation is the significant increase in the thickness of the epithelium in the early stages of exposure and its decreasing trend after 20d. Different reasons may be attributed to the increased epithelial thickness at different stages of exposure. At many stages, it is basically due to the differentially stained mucous cell hyperplasia. Another major factor contributing towards the increased epithelial thickness is the development of non-tissue spaces resulting from extensive vacuolization and/or necrosis. Whatever be the reason for the increased epithelial thickness, it automatically increases the diffusion distance between the ambient medium and the underlying cells, which could reduce the influx of the toxicant. Laurent and Dunel (1980) also consider hyperplasia as an attempt by the organism to maintain homeostasis in lieu of the permeability changes.

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