

TOXICOPATHOLOGICAL IMPACT OF CADMIUM CHLORIDE ON THE ACCESSORY RESPIRATORY ORGAN OF THE AIR-BREATHING CATFISH *HETEROPNEUSTES FOSSILIS*

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ABSTRACT

Sublethal cadmium chloride (0.3 ppm) toxicity induced stress related morphopathological alterations in the accessory respiratory organ of the air-breathing catfish *Heteropneustes fossilis* (Siluriformes; Heteropneustidae) have been investigated at various intervals of exposure. The histopathological manifestation of the cadmium toxicity includes bulging of the hyperemic secondary lamellae into the lumen of the accessory respiratory organ, necrosis and sloughing of the respiratory epithelium leading to haemorrhage and fusion of SL at various stages of the exposure. Periodic alterations in the densities of epithelial cells and mucous cells along with the development of non-tissue spaces have also been noticed at different exposure periods leading to alterations in the thickness of the respiratory epithelia. The heavy metal salt exposure has affected the mucogenic activity of the respiratory epithelium not only quantitatively but qualitatively also, indicating the probable ameliorative role fish mucus in cadmium toxicity.

Key words: *Heteropneustes fossilis*, histopathology, cadmium chloride toxicity, accessory respiratory organ, catfish

INTRODUCTION

The air-breathing catfish *Heteropneustes fossilis* (Order: Siluriformes; Family: Heteropneustidae) is a cherished table fish in India and is distributed throughout the Indian sub continent in various fresh water ecosystems including muddy, marshy and derelicts ponds having low levels of water and dissolved oxygen. They are seen even in contaminated water bodies also. In fact, the presence the accessory respiratory organ (ARO) keeps the fish fit enough to survive in these oxygen deficient water bodies by means of aerial mode of respiration. The presence of ARO also enables the fish to temporarily stay out of water for hours together through aerial mode of respiration. This ability is being exploited in marketing the fish in live condition. ARO has a common embryological origin with gills and also has histological similarity with the latter but it essentially differs from gills in having no direct contact with the aquatic medium (Munshi, 1962). Through ARO, *H. fossilis* respire aerially by gulping in air above the water surface.

Cadmium is a non-essential, non-biodegradable element with no known biological function and is reported to be a major contaminant of aquatic ecosystems causing adverse effects on aquatic organisms (Hollis *et al.*, 1999). It is released from diverse sources such as electroplating, paper, PVC plastic, pigments and ceramic industries, battery, mining and smoldering units and many other modern industries (Gupta *et al.*, 2003). Many workers have reported the manifestation of toxic effects of cadmium on the gills of fishes (Randi *et al.*, 1996; Hollis *et al.*, 1999). Even though the ARO plays a pivotal role in the survival of the species, almost no data is available on the toxicity of cadmium on this vital organ system of *H. fossilis*. In this context, the present study is an attempt to elucidate the toxicopathological effects of the heavy metal salt cadmium chloride on the respiratory epithelium of the ARO of *H. fossilis*.

MATERIALS AND METHODS

Maintenance of fish and toxicant exposure

Healthy specimens of *H. fossilis* having 15 to 17 cm

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length and 50 to 55 g weight were collected locally at Chidambaram, Tamilnadu, India and were acclimated to the laboratory condition for 30 days (d). Water was renewed after every 24 hours (h) with routine cleaning of the aquaria. Fish were fed liberally with minced goat liver everyday. For the analysis of sublethal toxicity, 7 groups of 10 fish each were exposed separately to 100 l of 0.3 ppm (96 h LC_{50} = 68 ppm) cadmium chloride (Glaxo India Ltd.) solution prepared in well water having dissolved oxygen 5.5 ppm, pH 7.2, water hardness 54 mg/L and water temperature 26 ± 1 °C. Parallel control groups were also kept without the addition of cadmium chloride. Media were renewed after every 24 h. Feeding was allowed in the experimental and control groups everyday for a period of 3 h, before the renewal of the medium.

Histopathology and morphometry

After the expiry of 5, 10, 20, 30, 40, 50 and 60 days of exposure, 3 fish each from the respective experimental as well as control groups were sacrificed. The AROs were excised and fixed in 10% neutral formaldehyde and aqueous Bouin's fluid, processed and 6 µm sections were stained with Ehrlich's haematoxylin and eosin (H/E) for routine histopathological analysis. Periodic acid Schiff (PAS) method, Alcian blue pH 1.0 (AB 1.0), Alcian blue pH 2.5 (AB 2.5), AB 2.5/PAS and Bismarck brown (BB) techniques (Pearse, 1985) were used for the histochemical visualization of various glycoprotein moieties. Stained whole mount (W.M) preparations of ARO were also made. Thickness of the respiratory epithelium was measured from tissue sections using an ocular and stage micrometer. The density (number/unit area) of mucous cell(s) (MC(s)) and epithelial cell(s) (EC(s)) were calculated from W.M. preparations and tissue sections following Rajan and Banerjee (1992) using a camera lucida. Random samplings of five different sites from each of the three fish of the experimental as well as control groups of each sampling period were taken into account. One way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to determine whether the morphometric parameters differed significantly by the exposure periods. Since there was no significant variation within each parameter of the control groups of various

exposure periods, the average value of each of the parameters of the control groups was taken into account.

RESULTS

Behavioural (macroscopic) alterations

Upon transferring to the sublethal cadmium chloride solution, fish showed restlessness and respiratory distress, which was evident by the increased gulping activity and ventilatory movements of operculum. Excessive secretion of mucus into the medium was observed especially at the earlier stages of exposure. Control groups were not showing any of the behavioural changes. No death was noticed either in the experimental or in the control groups.

Microscopic alterations

Control ARO

In *H. fossilis*, the ARO (= air sac or branchial diverticulum) appeared like a pair of tubular sac like backward extensions from the supra-branchial chamber and were embedded deeply in the body myotomes one on each side of the body through which the fish respired aerially. The various morphometric measurements of the epithelium of ARO are given in Fig. 1.

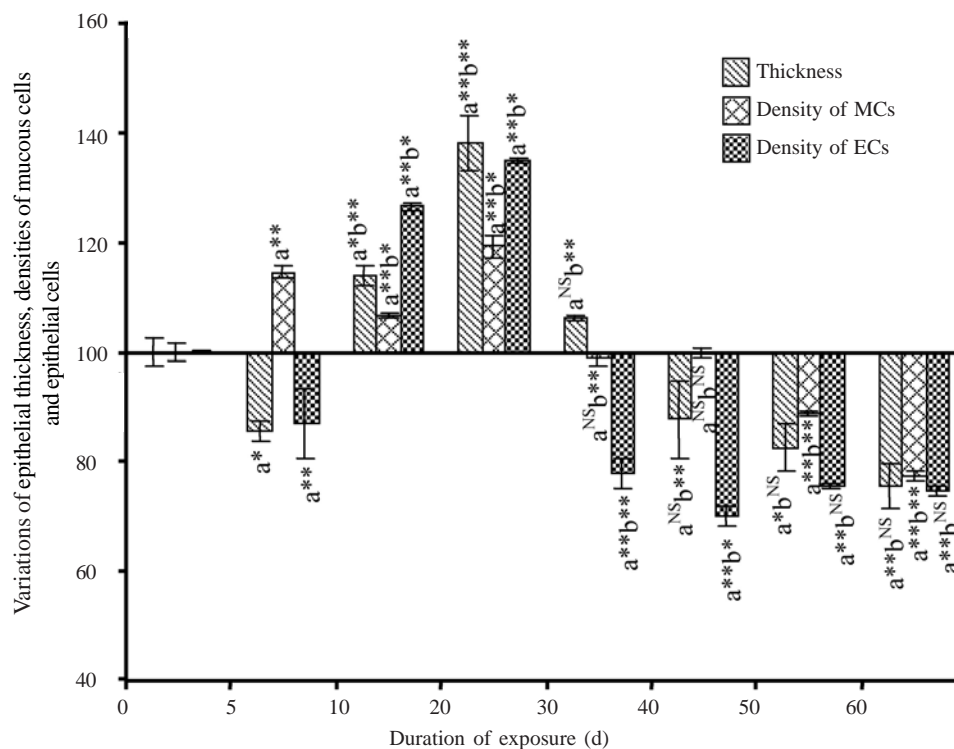
The respiratory epithelium of ARO was a typical mucous membrane and next to which serially arranged were the basement membrane, connective tissue layer, a thin membrane and the muscular coat (Figs 2 and 3). The respiratory epithelium was thrown into several ridges and grooves and consisted of vascular areas containing large and small respiratory islets (primary lamellae) and non-vascular areas with 7 to 8 layers of epithelial cells (ECs) and several mucous cells (MCs) (Fig. 4). An islet was composed of double row of modified secondary lamellae (SL) facing the lumen of ARO. Each of the SL was enclosing serially arranged pillar cells (Pilaster cells). The MCs were seen in the inter-lamellar spaces (Figs. 3 and 5) also and their histochemical staining properties are given in Table 1.

Experimental ARO

After 5 d of sublethal exposure, the tips of the SL were bulged out prominently into the lumen of the ARO (Fig. 6). At many places the inter-lamellar epithelium was not seen at the bulged ends. Large sized MCs were seen at the inter-lamellar regions

(Figs. 6 and 7) as well as in the non-vascular areas of the respiratory epithelium. The thickness of the respiratory epithelium and the density of ECs at this stage were less when compared to the control tissue (Fig. 1). However, the density of MCs was greatly

increased than that of the control epithelium and their carbohydrate moieties were more alcianophilic (Table 1) and less PAS positive. Tenth day of exposure witnessed fusion of SL (Fig. 8) and hyperplasia of ECs (Figs. 1 and 9).



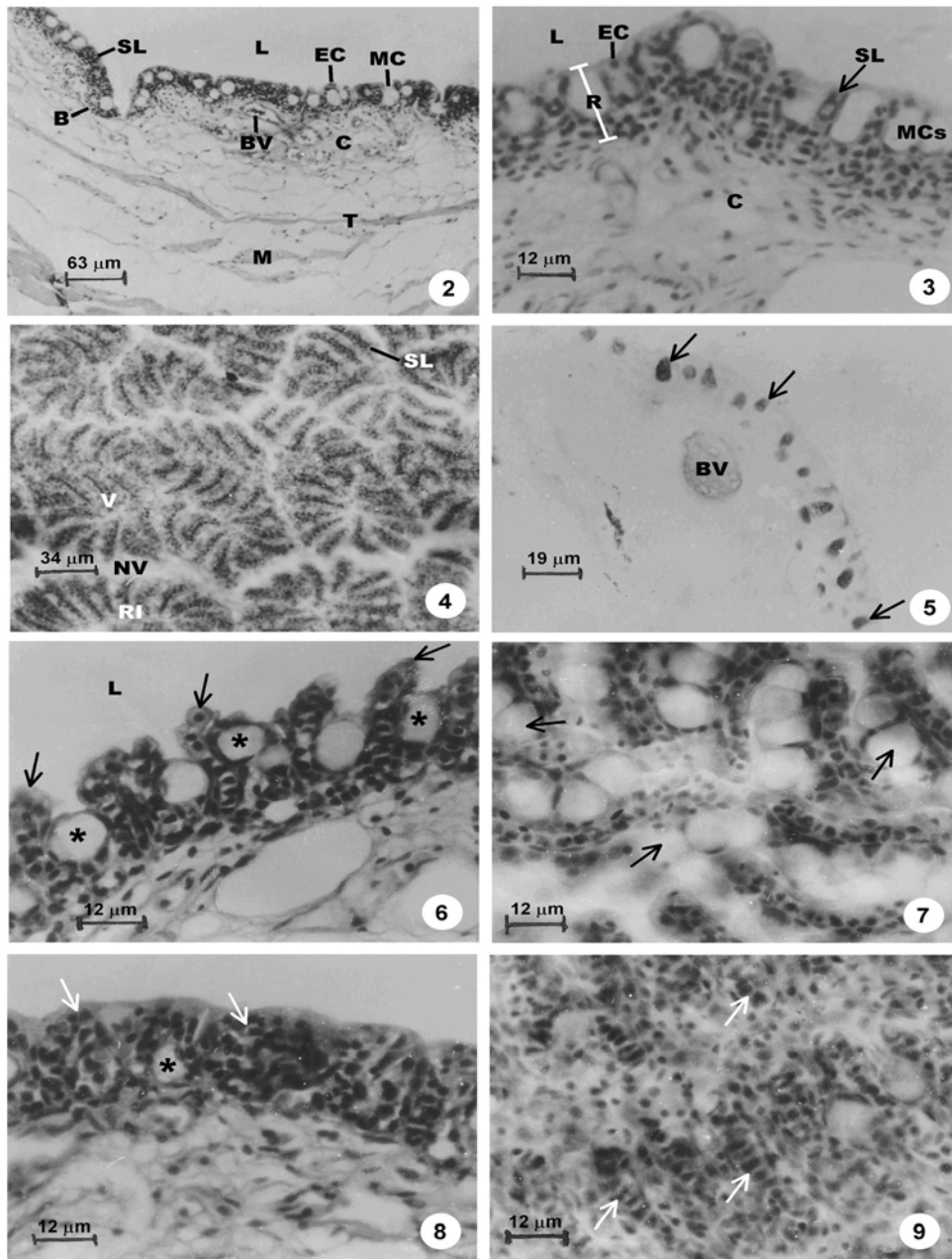
Note: $\bar{x} \pm$ SEM (based on ANOVA and Duncan's multiple range test); a = between the respective experimental group and control group; b = between the respective experimental group and preceding experimental group; d = days; NS = not significant; * = $p < 0.05$; ** = $p < 0.01$; control values taken as 100%.

Fig. 1: Variations with percentage of epithelial thickness, densities of mucous cells (MCs) and epithelial cells (ECs) of the respiratory epithelium of the accessory respiratory organ of *H. fossilis* at various stages of sublethal cadmium chloride exposure in comparison to the control level.

Table 1: Summary of the histochemical alterations in the carbohydrate moieties of the mucous cells (MCs) of the respiratory epithelium of the accessory respiratory organ of *H. fossilis* at various stages of 0.3 ppm cadmium chloride exposure

Staining technique	Control	Exposure periods						
		5 day	10 day	20 day	30 day	40 day	50 day	60 day
PAS for neutral glycoproteins (1,2 glycols)	2 ~ 4	2	1	1	1 ~ 2	3	1 ~ 2	2 ~ 3
AB 1.0 for sulphated glycoproteins (glycosaminoglycans)	1	2	2 ~ 3	3	2	\pm ~ 1	0	0
AB 2.5 for acidic glycoproteins	2	2 ~ 4	3 ~ 4	4	3	2	\pm	0
AB 2.5 /PAS for acidic and neutral glycoprotein*	2 ~ 4	2 ~ 4	2 ~ 3	4	2 ~ 3	3	1 ~ 2	2 ~ 3
BB for water stable mucoprotein	\pm	1	1 ~ 2	\pm	0	1	0	0

Note: AB 1.0 = Alcian blue pH 1.0; AB 2.5 = Alcian blue pH 2.5; BB = Bismarck brown; d = Days; PAS = Periodic acid-Schiff; ~ = to; 0 = Negative reaction; \pm = Faint reaction; 1 = Weak reaction, 2 = Moderate reaction; 3 = Strong reaction; 4 = Very strong reaction.
*Staining intensity ranges from magenta, red, violet to bluish purple due to the differential distribution of various types of carbohydrate moieties in the same or different MCs.



Figs. 2 and 3: T.S. showing the structural organization of the respiratory epithelium of control fish. H/E

Fig. 4: W. M. from control fish showing the normal distribution of RI (primary lamellae) bearing SL. H/E

Fig. 5: T. S. showing the distribution of MCs (arrows) in control fish. A.B. 2.5/PAS

Fig. 6: T. S. showing bulging of SL (arrows) with gorged pillar cell system after 5 d of exposure. Note the presence of large sized MCs (*) in between the SL. H/E

Fig. 7: W. M. showing hyperplasia of MCs (arrows) after 5 d of exposure. H/E

Fig. 8: T. S. showing fusion of SL after 10d of exposure. Note the decrease in the density of MCs (*) and increase in the density of ECs (arrows). H/E

Fig. 9: W. M. showing hyperplasia of ECs after 10d of exposure. Note the less visible SL (arrows). H/E

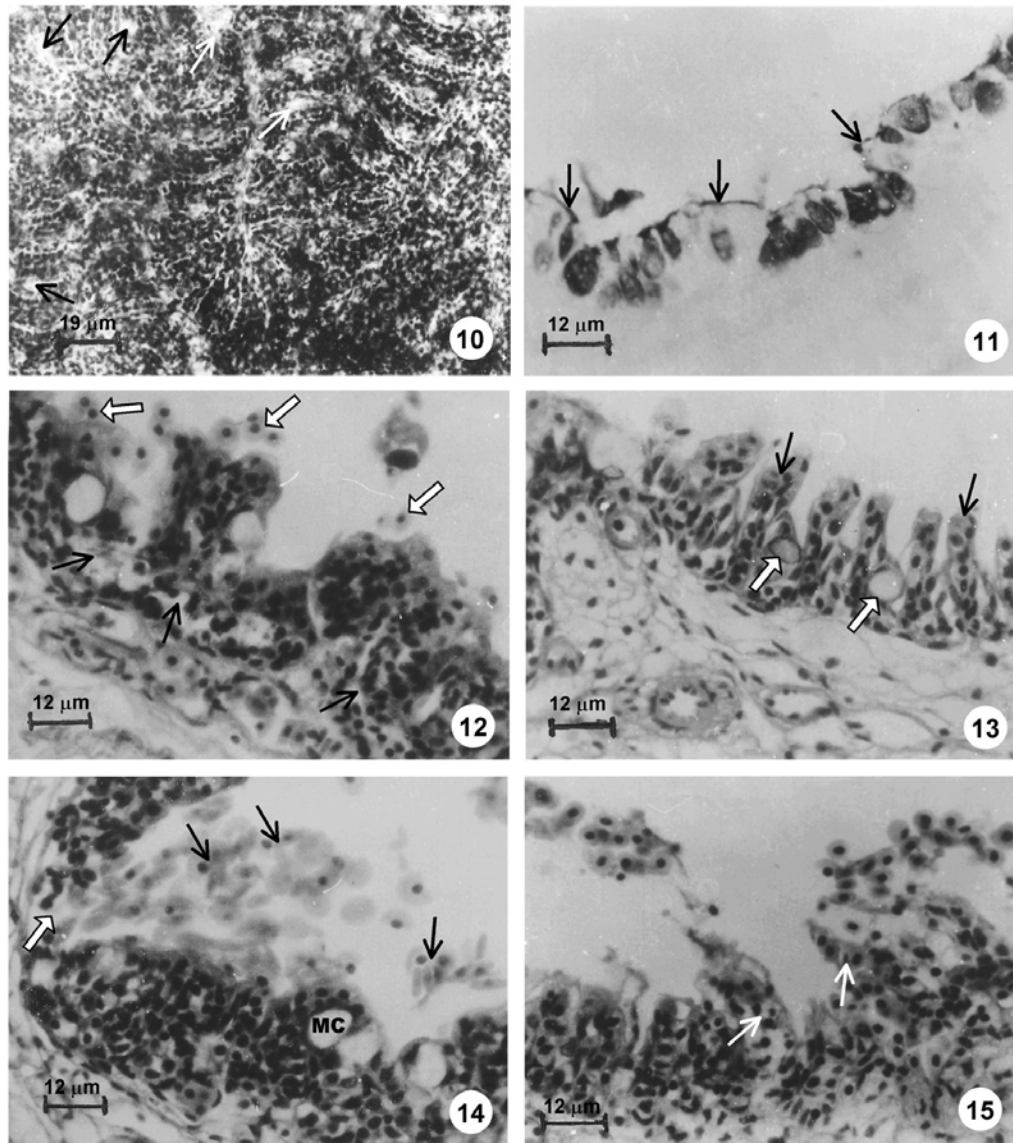


Fig. 10: W.M. showing extensive fusion of SL due to hyperplasia of ECs after 20 d of exposure. Note the increase in the number of MCs (arrows). H/E

Fig. 11: T.S. showing increased density of AB 2.5/PAS positive MCs after 20 d of exposure. Note the mucous coating (arrows) on the surface of the epithelium due to emptying of slime by MCs. AB 2.5/PAS

Fig. 12: T.S. showing necrosis after 30 d of exposure. Note the development of non-tissue spaces (arrows) and sloughing of ECs (open arrows). H/E

Fig. 13: T.S. showing fully bulged out SL after 40 d of exposure. Note the decreased density of MCs (open arrows) and gorged SL (arrows). H/E

Fig. 14: T.S. showing severe haemorrhagic lesions (open arrow) and seepage of blood into the lumen (arrows) after 50 d of exposure. H/E

Fig. 15: T.S. showing massive sloughing of the respiratory epithelium including SL (arrows) after 60 d of exposure. H/E

Even though the thickness of respiratory epithelium was significantly more than that of 5 d (Fig. 1; $p < 0.01$), the density of MCs was less than that of 5 d ($p < 0.01$). The alcianophilia of the MCs along with the BB reaction increased further after 10 d of exposure and most of them were AB 1.0 positive also (Table 1). After 20 d of exposure, fusion of the SL became more prevalent (Fig. 10). The epithelium became thicker than the previous stages due to the hyperplasia of MCs and ECs (Figs. 1 and 11; $p < 0.01$) along with the fusion of the SL. While the intensities of AB 1.0 and AB 2.5 reactions increased still further, the intensities of PAS and BB reactions decreased after 20 d (Table 1). Many of the MCs actively emptied their contents to the surface to form an AB/PAS positive mucous coating (Fig. 11). After 30 d of exposure, necrotic damages became more prominent and ECs were exfoliated singly or in groups (Fig. 12). Prominent non-tissue spaces were also seen along the epithelium. The epithelial thickness along with the densities of MCs and intact ECs (Fig. 1) were decreased significantly ($p < 0.01$) than the previous stage. Even though the MCs predominantly remained alcianophilic, the intensity of PAS reaction was also increasing. After 40 d of exposure, SL were more or less fully bulged out into the lumen of ARO due to sloughing of ECs in the inter-lamellar regions (Fig. 13). The bulged out lamellae were gorged with blood cells. The mean thickness of the epithelium and the density of ECs were greatly decreased when compared to that of 30 d (Fig. 1; $p < 0.01$). However the density of MCs remained more or less same as that of 30 d. They showed weak BB and increased PAS reactions (Table 1). After 50 d, severe necrotic lesions resulted in haemorrhage (Fig. 14) into the lumen of ARO. While the mean thickness and density of ECs remained more or less same as that of 40 d (Fig. 1), the density of MCs was decreased drastically from that of 40 d ($p < 0.01$). The MCs were BB negative and PAS positive. They were either negatively or feebly stained with AB methods (Table 1). Sixty days of continued sublethal cadmium chloride exposure resulted in massive sloughing of ECs. Even the entire SL at many places were sloughed (Fig. 15) leading to severe haemorrhage and thereby affecting the gross morphology of many respiratory islets. While the mean width of the respiratory

epithelium and density of the MCs decreased drastically (Fig. 1; $p < 0.01$) in comparison to the control, density of ECs remained at the level of the previous stage of exposure but less than the control level. The sparsely seen MCs were only PAS positive (Table 1).

DISCUSSION

H. fossilis is an active air breather and according to Munshi (1962) it meets around 40% of its oxygen demand by aerial respiration through ARO, indicating the vital role played by this organ for the sustenance of the species. In the present study, the increased gulping activity and opercular movement by the exposed fish may be the reflection of an attempt by the fish to extract more oxygen to meet the increased energy demand to withstand the cadmium toxicity. On the other hand, it may also be correlated to the formation of a hypoxic condition due to the interference in gaseous exchange caused by the accumulation of mucus on the gill epithelium. In order to overcome this, the fish might also be actively resorting to the aerial mode of respiration through increased gulping activity. The prominent bulging of SL into the lumen of the ARO, at various stages of the present study could also be correlated to the attempt on the part of the organism to facilitate maximum oxygen absorption to withstand the cadmium stress. As a result, the pillar cells systems of the SL become hyperemic and it causes stretching and dilation of the blood channels. The extreme load on these thin walled tubular structures along with the membrane damaging necrotic effect of cadmium (Versteeg and Giesy, 1985) might be responsible for the rupture of the lamellar system leading to haemorrhage into the lumen of ARO in the exposed fish. Seepage of blood materials into the lumen could not only choke it but may also result in anaemia leading to the reduced oxygen carrying capacity of the blood. Thophon *et al.*, (2003) have also noticed aneurism with rupture of the respiratory epithelium of the SL and breakdown of pillar cell system in the gills of cadmium exposed *Lates calcarifer*. The absence of such histopathological alterations in the control ARO confirms the deleterious effect of cadmium exposure. Even though the ARO never comes in direct contact with the toxicant medium as in the

case of gills (Munshi, 1962), the hyperplasia of ECs and MCs at various stages of the present study are some what similar to the alterations reported by Thophon *et al.*, (2003) in the gills of cadmium exposed *L. calcarifer*. This may be due to their common embryological origin. The primary consequence of the EC and MC hyperplasia is the increased thickness of the respiratory epithelium. More or less same effect is also produced by the epithelial lifting, development of non-tissue spaces or intercellular vacuolization. All these pathomorphological changes result in the increased barrier distance between the blood in the pillar cell system and the air in the lumen of ARO leading to impaired respiration.

The fusion/clubbing of SL of the ARO in the present study might be the result of the combined effect of hyperplasia of ECs and MCs, necrotic lesions and the compositional changes in the mucus because according to Daoust *et al.*, (1984), the lamellar adhesion in gills might be result of contact stress, which causes erosion of mucous coating and epithelial lining leading to alterations in the chemical composition and thickness of the mucous layer due to interaction with xenobiotics. Erosion of the epithelial lining and alteration in the chemical composition of mucus (Table 1) has been observed in the present study also. These events could disturb the normal ability of the cells to recognize different cell types resulting in the fusion of the SL. The immediate physiological consequence of the lamellar fusion is the reduction in the surface area available for gaseous exchange, which could adversely affect respiratory physiology of the fish. A wide range of functions has been attributed to fish mucus including protection against environmental contaminants and UV radiation (McKim and Lien, 2001; Häkkinen *et al.*, 2003). Many workers are of the opinion that some components of the fish mucus, probably the acidic and/or sulphated glycoprotein moieties have a metal binding (Pärt and Lock, 1983) and ameliorative effect against ambient toxicants (Arillo and Melodia, 1990). The present study also shows the predominance of acidic/sulphate mucoproteins in the mucus, especially in the earlier stages of cadmium exposure. The presence of these glycoproteins in the mucus is indicative of its metal binding capacity as cadmium specially binds -SH

groups (Moore and Ramamoorthy, 1984). In this context, the qualitative and quantitative alterations observed in the mucogenic activity at various stages of the present study (Fig. 1; Table 1) assume great significance because the cadmium ions may also be eliminated along with the mucous discharge. The decreased density (Fig. 1) of MCs at various stages of exposure may be due to their exhaustion and degeneration. To compensate this new MCs are formed at the lower layers of the epithelium and are gradually migrated to the superficial layers. But the acute demand for the slime does not spare these developing MCs also. These newly formed MCs in the deeper layers of the epithelium are more PAS positive and less alcianophilic. However, on reaching the surface layers, they turnout to be more alcianophilic indicating a progressive incorporation of acidic/sulphated glycoprotein moieties. The differential staining pattern of MCs at various stages of exposure may be attributed to the presence of a mixture of neutral, acidic and/or sulphated glycoproteins in them. The protective role of mucus is also evident from the fact that there occurred more intensive histopathological damages to the ARO especially at the fag end of the experiment, which witnessed a drastic reduction in the density of MCs (Fig. 1).

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