



## Ultrastructural alterations in the gills of cyprinid, *Labeo rohita* (Hamilton) exposed to Lead nitrate

Manjeet Kaur<sup>1\*</sup> & Onkar Singh Brraich<sup>2</sup>

<sup>1</sup>Department of Zoology, Akal University, Talwandi Sabo, Bathinda, Punjab, India

<sup>2</sup>Department of Zoology and Environmental Sciences, Punjabi University, Patiala, Punjab, India

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Lead contaminates aquatic ecosystems and causes deleterious effects on aquatic organisms, particularly fishes, and percolates to human beings through food chain as accumulated residue. In this context, we conducted histological studies on impact of lead toxicity in Rohu, *Labeo rohita* (Hamilton), a common freshwater fish consumed by humans, exposed to sub-lethal concentrations of lead nitrate. Previously, we observed damage in the gills using light microscope, and now we studied ultrastructural alterations at cellular level in the gills using Transmission electron microscopy (TEM). The gill tissues were dissected after 15, 30, 45 and 60 days of exposure, washed, fixed in Karnovsky's fixative, and processed further for histological investigation. Normal cellular architecture was noticed in the control group while, whereas large number of alterations could be observed in different organelles in the cells of the lead nitrate treated groups. The severity of the lesions was proportional to the increased concentration of the tested pollutant in a time dependent manner.

**Keywords:** Aquatic pollution, Heavy metal toxicity, Rohu, Transmission Electron Microscopy

Water is an essential and abiotic component of nature which provides habitat a vast variety of flora and fauna. Increase in human population and subsequent industrial revolution has lead to non judicial use of natural resources and thereby polluted the environment. The domestic wastes and untreated or partially treated industrial effluents carry heavy load of pollutants like heavy metals, pesticides and many organic compounds which poses a serious threat to aquatic organisms<sup>1-5</sup>.

Some heavy metals are considered as a strong biological poisons because of their toxicity, persistence and tendency for bio-accumulation in food chains which affect fish population, reducing their

growth, reproduction and survival rate<sup>6-9</sup>. Lead is one such non-essential heavy metal which is toxic to plants, animals and human<sup>10,11</sup>. It enters the natural waters through the mining operations, smelting, coal burning, cement manufacturing, use in gasoline, batteries and paint and their waste discharge into the aquatic systems, causing mortality to aquatic animals<sup>11-13</sup>. Recently, Shalaby *et al.*<sup>11</sup> have shown the possible role of the common Water hyacinth *Eichhornia crassipes* and the macroalgae *Gelidium pectinatum* in removal of lead and cadmium from contaminated water bodies.

Fishes are one of the best biological indicators of aquatic pollution<sup>14-16</sup>. Gills are the primary target organs of the contaminants present in water as they are the first to come in contact with the environmental pollutants<sup>17,18</sup>. Though histopathological effects of lead (Pb) on gills have been studied by many workers<sup>19,20</sup>, there is a paucity of literature on ultrastructural responses which are sensitive enough to indicate the early effects of chemicals on the organism. We have earlier reported ultrastructural changes in *Labeo rohita* gills due to lead nitrate exposure as observed through scanning electron microscope (SEM)<sup>13</sup>. Here, we carried out transmission electron microscopic (TEM) study to assess the architectural changes in the cells of the gill of *L. rohita* (Hamilton) on exposure to lead nitrate. *Labeo rohita*, commonly called Rohu, is one of the low priced food fish with good market demand<sup>21</sup>. Due to its commercial importance in the area, this fish has been used in the present study to observe the effect of lead nitrate which is the common pollutant in Punjab, India. In this TEM study, we tried to compare the cellular alterations in the gill tissue of *L. rohita* with the histopathological responses observed in our earlier light microscopy study.

### Materials and Methods

#### Chemicals & Test animals

Lead nitrate (CAS NO. 10099-74-8) and concentrated nitric acid (CAS NO. 7697-37-2) were purchased from Sigma Aldrich, Mumbai, India. The test concentration of Lead nitrate was prepared just before the exposure using distilled water and was maintained as stock<sup>22</sup>. *L. rohita* weighing 10±2 g and

\*Correspondence:

E-Mail: manjeetkaur080385@gmail.com

length  $10 \pm 1$  cm were collected from a fish farm, Nanoki, District Patiala, Punjab, India. Fishes were acclimatized to the laboratory conditions for 15 days prior to experiments in well-aerated tubs. The water from the fish tanks was changed after every 24 h and the fishes were fed with pelleted feed from Toya industries twice a day<sup>19</sup>.

#### Preliminary tests & Treatments

The physicochemical features of the tap water were estimated as per the procedure given in APHA<sup>22</sup>. The average water temperature was found to be  $25 \pm 2^\circ\text{C}$ , water hardness =  $172 \pm 2$  mg/L, pH =  $7.1 \pm 0.2$  and dissolved oxygen varied from 8-10 mg/L. After attaining permission from Ethical Committee, Punjabi University, Patiala, Punjab, India, the experimentation on fish was done. The  $LC_{50}$  of 96 h lead nitrate exposure for *Labeo rohita* is reported to be 34.20 mg/L<sup>23</sup>. For experimentation, the fishes were divided into five groups, each with 10 fingerlings. One group was kept as a control and four were exposed to sublethal concentrations of lead nitrate on the basis of  $1/3^{\text{rd}}$ ,  $1/5^{\text{th}}$ ,  $1/7^{\text{th}}$  and  $1/10^{\text{th}}$  of  $LC_{50}$  value. i.e., 11.4, 6.84, 4.88 and 3.42 mg/L, respectively. All the experiments were repeated thrice.

#### Ultrastructural study

One fish from each trough was sacrificed and dissected to remove the gill tissues at the end of 15, 30, 45 and 60 days of exposure. The gill tissues were washed in saline, cut into small pieces and fixed in Karnovsky's fixative (2.5% gluteraldehyde and 2% paraformaldehyde made in 0.1M phosphate buffer) for 12 h. Then, the gill tissues were washed in 0.1M phosphate buffer and post fixed in 1% Osmium tetroxide. Further, they were dehydrated in ascending grades of acetone, infiltrated and embedded in araldite and processed for viewing under TEM (FEI Philips Morgagni, 100kV, 2,80,000X) at AIIMS, Delhi.

#### Results and Discussion

The ultrastructural study of the gills of the fish in control group showed a normal gill epithelium consisting of 4 types of cells namely pavement cells,

mucous cells, undifferentiated cells and mitochondrial rich cells or chloride cells (Fig. 1). Pavement cells were the most abundant cell type covering the major portion of the gill filament. The apical surface of the pavement cells were characterised by the presence of microvilli. These cells showed rounded nucleus, dense cytoplasmic matrix with conspicuous golgi apparatus, rough endoplasmic reticulum with spaced cisternae, smooth endoplasmic reticulum, ribosomes, vesicles and a small number of mitochondria. Chloride cells were large, round or ovoid located within the interlamellar region and on the ends of the filament. The remarkable ultrastructural features of the chloride cell were the presence of abundant mitochondria and complex tubulo-vesicular system in their cytoplasm. Mucous cells have been located on the edges of the filaments. These were large ovoid cells composed of large mucous secretory granules. The nucleus has been found to be usually flattened and cytoplasm consists of cellular machinery (endoplasmic reticulum, golgi complex, mitochondria) for producing the mucous. The pillar cell was a type of modified endothelial cell crowded with mitochondria, free ribosomes and membrane bound organelles but golgi apparatus and endoplasmic reticulum were not found common in pillar cells (Fig. 1 A-D).

The transmission electron microscopy revealed a large number of ultrastructural alterations such as loss of cytoplasm and vacuolation in the cells, deformed nucleus with rupturing of the nuclear membrane, clumping of the chromatin, swollen and ruptured mitochondria, increase in the number of lysosomes, dilated golgi bodies, dilated cytoplasm, fragmented endoplasmic reticulum, large intercellular spaces, cell debris in the cell, disorganisation of the epithelial cells in the lead nitrate treated groups of fishes. It was also found that the degree of damage in the cellular architecture of gills of *L. rohita* were dependent on concentration and duration of exposure to lead nitrate (Table 1).

On exposure to 3.42 mg/L of lead nitrate for 15 days, vacuolation in the cells, rupturing of the nuclear

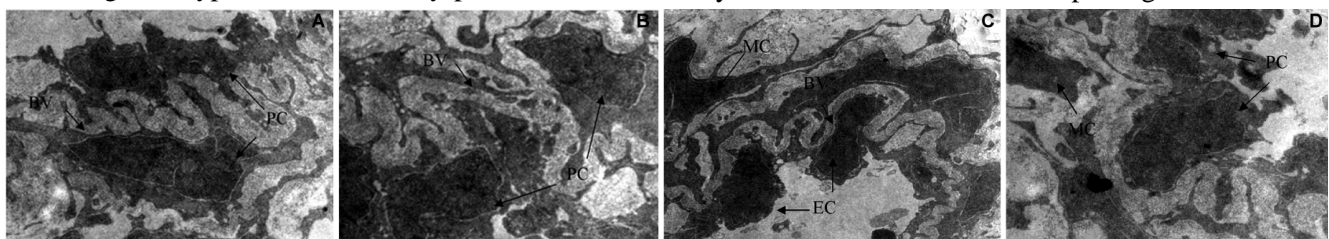


Fig. 1 — Ultrastructure of gills of fish in control group. X4000. [PC, Pillar cells; EC, Epithelial cells; MC, Mucous cells; and BV, Blood vessels]

Table 1 — The ultrastructural alterations observed in the gills of a fish, *Labeo rohita* on exposure to different doses of Lead nitrate for different number of days

No. of Days	Lead nitrate dose (mg/L)			
	3.42	4.88	6.84	11.4
15	loss of cytoplasm and vacuolation in the cells, rupturing of the nuclear membrane, disorganisation of epithelial cell	deformed nucleus, swollen mitochondria, large intercellular spaces, cell debris in the cells	increase in the number of lysosomes, dilated golgi bodies, dilated flattened sacs of golgi present	vacuolisation in the cells, dilated intercellular spaces, rupturing of the cell
30	dilated endoplasmic reticulum, dilated flattened sacs of golgi body, swollen mitochondria, increase in the number of lysosomes	deformed nucleus and nuclear membrane, swollen mitochondria with dilated structures of cristae, large intercellular spaces	dilation of cytoplasm, rupturing of the nuclear membrane, clumping of chromatin, large cell debris	dilation of cytoplasm, rupturing of the nuclear membrane, clumping of chromatin, swollen cristae of mitochondria, dilated endoplasmic reticulum
45	vacuolisation in the cytoplasm, rise of cell debris in the cell, shrinkage of the nuclear material from nuclear membrane	pillar cell necrosis, dilation of intercellular space between pillar cell and epithelial cells, cytoplasmic vacuolisation	clumping of nuclear material, turgid mitochondria, dilated tubular network of endoplasmic reticulum	loss of cytoplasm, necrosis of cellular material, deformed nucleus, with ruptured nuclear membrane
60	clumping of chromatin, swollen mitochondria, deformed nucleus, ruptured plasma membrane	degeneration of cellular material and necrosis	clumping of nuclear material, degeneration of cell, complete loss of cytoplasm, necrosis	complete loss of the organelles and cytoplasm, necrosis

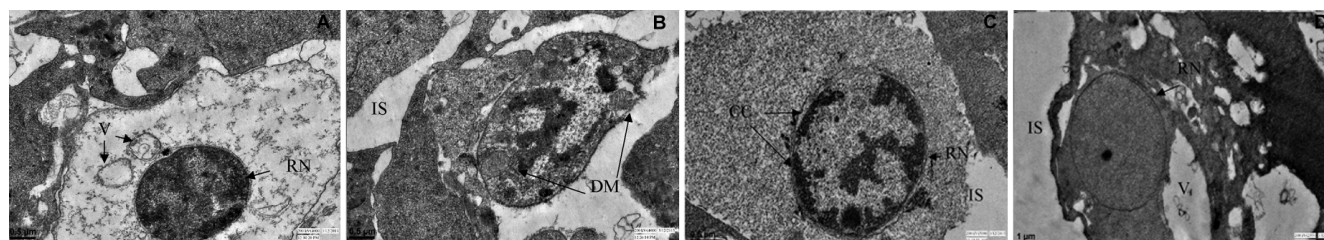


Fig. 2 — Ultrastructure of gills exposed to lead nitrate for 15 days. X5000. (A) 3.42; (B) 4.88; (C) 6.84; and (D) 11.4 mg/L lead nitrate. [V, Vacuolation; RN, Rupturing of nuclear membrane; DER, Dilated endoplasmic reticulum; DM, Dilated mitochondria; SM, Swollen mitochondria; CC, Clumping of chromatin; DC, Dilation of cilia; and IS, Intercellular spaces]

membrane and disorganisation of epithelial cell were observed (Fig. 2A). On exposure to 4.88 mg/L for same duration, the cells showed deformed nucleus, swollen mitochondria and large intercellular spaces (Fig. 2B). Exposure to higher doses 6.84 and 11.4 mg/L, increased the number of lysosomes, dilated golgi bodies, dilated flattened sacs of golgi, vacuolisation in the cells, dilated intercellular spaces and the rupturing of the cell as observed (Fig. 2 C and D). It was further observed that the lesions increased with the increase in the concentration and with the duration of exposure. Dilated endoplasmic reticulum, dilated flattened sacs of golgi body, swollen mitochondria, increase in the number of lysosomes (Fig. 3A); deformed nucleus and nuclear membrane, swollen mitochondria with dilated structures of cristae, large intercellular spaces (Fig. 3B); dilation of cytoplasm, rupturing of the nuclear membrane, clumping of chromatin (Fig. 3C) and dilation of cytoplasm, rupturing of the nuclear membrane, clumping of chromatin, swollen cristae of mitochondria, dilated

endoplasmic reticulum (Fig. 3D) were reported on exposure to different doses of lead nitrate for 30 days.

Lead nitrate exposure to 3.42 and 6.84 mg/L for 45 days resulted in vacuolisation in the cytoplasm, rise of cell debris in the cell, shrinkage of the nuclear material from nuclear membrane, pillar cell necrosis, dilation of intercellular space between pillar cell and epithelial cells, cytoplasmic vacuolisation (Fig. 4 A & B). Clumping of nuclear material, turgid mitochondria, dilated tubular network of endoplasmic reticulum, loss of cytoplasm, necrosis of cellular material, large electron dense irregular nucleus with ruptured nuclear membrane were observed on exposure to higher dosage of 4.88 and 11.4 mg/L of lead nitrate (Fig. 4 C and D). Clumping of chromatin, swollen mitochondria, large intercellular spaces, ruptured plasma membrane, irregularity in shape of nucleus with fragmented endoplasmic reticulum (Fig. 5A), degeneration of cellular material and necrosis (Fig. 5B), clumping of nuclear material, degeneration of cell, complete loss of cytoplasm, necrosis (Fig. 5C) and complete loss of

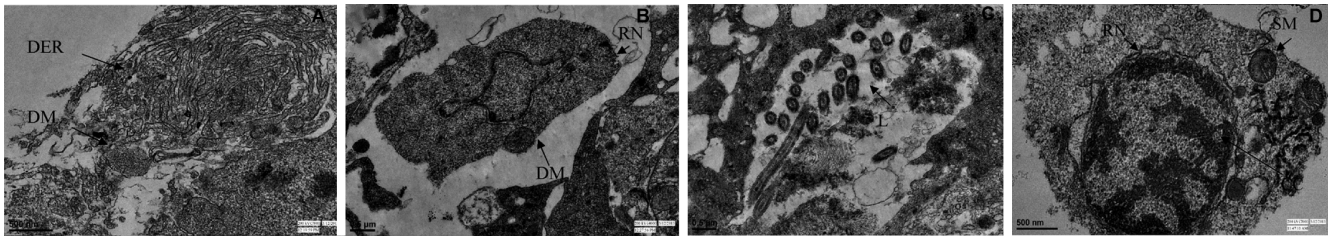


Fig. 3 — Ultrastructure of gills exposed to lead nitrate for 30 days. X5000. (A) 3.42; (B) 4.88; (C) 6.84; and (D) 11.4 mg/L lead nitrate. [V, Vacuolation; RN, Rupturing of nuclear membrane; DER, Dilated endoplasmic reticulum; DM, Dilated mitochondria; SM, Swollen mitochondria; CC, Clumping of chromatin; L, Lysosomes]

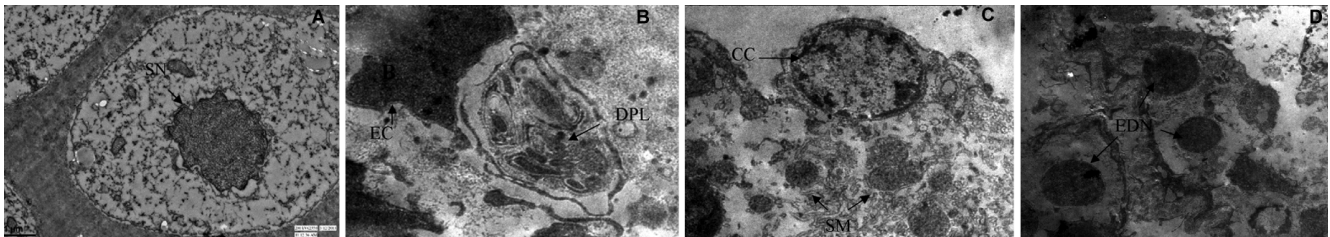


Fig. 4 — Ultrastructure of gills exposed to lead nitrate for 45 days. X5000. (A) 3.42; (B) 4.88; (C) 6.84; and (D) 11.4 mg/L lead nitrate. [RN, Rupturing of nuclear membrane; DER, Dilated endoplasmic reticulum; DM, Dilated mitochondria; SM, Swollen mitochondria; CC, Clumping of chromatin; SN, Shrunken nucleus; EDN, Electron dense nucleus; DPL, Dilated pillar cells]

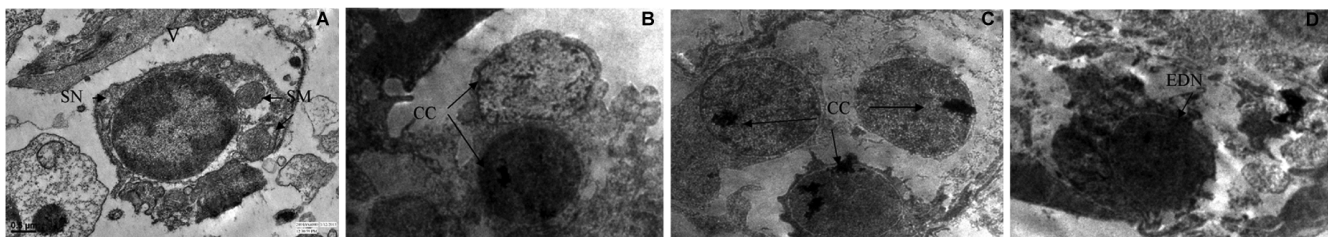


Fig. 5 — Ultrastructure of gills exposed to lead nitrate for 60 days. X5000. (A) 3.42; (B) 4.88; (C) 6.84; and (D) 11.4 mg/L lead nitrate. [RN, Rupturing of nuclear membrane; DER, Dilated endoplasmic reticulum; DM, Dilated mitochondria; SM, Swollen mitochondria; CC, Clumping of chromatin; SN, Shrunken nucleus; EDN, Electron dense nucleus; DPL, Dilated pillar cells]

the organelles and cytoplasm (Fig. 5D) were observed when exposed for 60 days.

Similar observations were reported for trout exposed to beryllium<sup>24</sup>. The dilation in the endoplasmic reticulum and cisternae of mitochondria has also been described for tropic and Nordic fish after exposure to inorganic mercury<sup>25</sup>. In the present study, the gills showed different degrees of cellular alterations depending upon the concentration and duration of exposure of lead nitrate. Similar alterations in the gills have also been reported by other workers in the fish exposed to various toxicants<sup>24,26,27</sup>.

Large number of electron dense lysosomes and electron dense endoplasmic reticulum were observed in the gill tissues of *Salmo trutta* and *Barbatula barbatula* on exposure to heavily polluted streams<sup>23</sup>. The lesions such as clumping of chromatin, swelling of nucleus and mitochondria in the cells of gills of

*Oreochromis mossambicus* on ambient exposure to sublethal concentrations of cadmium were also observed<sup>28</sup>. The present study revealed cytoplasmic disorganisation, reduced and swollen microridges on the surface of pavement cells in the gills of *L. rohita* treated with lead which was also evidently observed by other workers in different studies<sup>29,30</sup>. Pillar cell necrosis, hypertrophy of chloride cells, epithelium lifting evident on the secondary lamellae, dilated endoplasmic reticulum, swollen mitochondria, electron dense secondary lysosomes as well as cell debris appeared in the cytoplasm in the gills of *Onchorhynchus mykiss* on being exposed to different concentrations of anti-inflammatory drug diclofenac<sup>31</sup>.

Chloride cells in the lead exposed fish showed dilated vesicles and damaged mitochondria, vacuolisation and dilated vesicles. This was in agreement with the findings of earlier workers<sup>32</sup>. Chloride cells are responsible for the maintenance of the acid-base and the ionic balance. As a result of

damage to these cells, fish could die owing to metabolic acidosis or alkalosis. The chloride cells show high number of apical vesicles which may be used to excrete lead. Gill chloride cells are the most active cells of the fish gills and absorb and secrete many ions and electrolytes. Because of these functions, increase in the apical vesicles of chloride cells could be the result of increase in ion exchange activity<sup>33</sup>.

In the gills of lead nitrate exposed fish, pillar cell necrosis and epithelial lifting are cytopathological effects which affect the functionality of this organ. The formation of aneurism as a result of pillar cell necrosis has been described as a strong reaction in fish gills to organic pollutants<sup>34</sup>. As the toxicant concentration was increased, the frequency of alterations in ultrastructure was also found increased proportionally. Sahoo *et al.*<sup>35</sup> who observed similar alterations suggested that these gill alterations might interfere with normal respiratory function and might lead to an impairment of the general health conditions of the fish.

Alterations described in the gills here may not be specific to lead, but other contaminants as well, and also could be as chronic response to parasitic or bacterial infections<sup>18</sup>. However, these modifications can produce adverse effects on fish health and may increase their susceptibility to secondary infectious diseases and even death<sup>36</sup>.

## Conclusion

Our results from this study suggest that lead nitrate is severely toxic to freshwater fish, *Labeo rohita*. Exposure to different concentrations showed significant architectural changes as explained above which directly affect the metabolism of the fish and ultimately disrupt the survival of the fish in their natural environment.

## Conflict of Interest

Authors declare no competing interests.

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