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Scanning electron microscopic investigation on the scales of grass carp, *Ctenopharyngodon idellus* (Cuvier and Valenciennes) exposed to lead nitrate

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Abstract
The aquatic ecosystem is the most affected ecosystem due to contamination of heavy metals. The present investigation was undertaken to assess the toxicity of lead nitrate on the scales of *Ctenopharyngodon idellus*. Acute toxicity test was conducted to evaluate 96 h LC_{50} of lead nitrate to *C. idellus* and was found to be 33.27 mg/L. For Chronic bioassay studies, two sublethal concentrations (i.e. 6.65 mg/L and 11.09 mg/L) of 96 h LC_{50} of lead nitrate were selected for 45 days. Fish exposed to sublethal concentrations of the toxicant showed hyper excitability, erratic swimming, jerky movements and tendency to escape from aquaria, increase in the rate of air gulps, fin and opercular movements. Loosening of scales, excess secretion of mucus and depigmentation were the morphological alterations. The ultrastructural changes on the scales of *C. idellus* were observed using scanning electron microscope (SEM). The scales showed various degrees of deformity to the circuli and tubercles on exposure to lead nitrate.

Keywords: *Ctenopharyngodon idellus*, lead nitrate, scanning electron microscope, scales, grass carp, heavy metals

1. Introduction

Heavy metals are naturally occurring metallic element that exhibited relatively high specific gravity then water and is extremely toxic or poisonous at low concentrations [1, 2, 3]. Some of these are essential metals, such as iron, copper, zinc and manganese, which have significant biological roles as cofactors for various enzymes and proteins while as others have no biological role but are poisonous, e.g., mercury, lead and cadmium [4]. The amount of heavy metals in industrial leachates is of great concern as these chemicals are present in large quantities and are continuously discharged into aquatic ecosystem [5, 6]. Due to their toxicity, persistence and bioaccumulation potentials, heavy metals are considered as one of the most significant aquatic pollutants [7].

Among these heavy metals, lead is a non-essential metal that occurs naturally in nature. On the other hand, anthropogenic activities also increased its concentration in the natural environment [6, 8]. Lead is a persistent metal [9], has slow elimination rate therefore, accumulated in the fish body [10, 11], via respiration, adsorption and oral ingestion through the food chain [12, 13, 14].

The main objective of the environmental toxicologist is to assess the risk resulting from the presence of such substances. Fish have been used for many years to determine the pollution status of water and are thus considered as excellent biological indicator in aquatic ecosystems [15, 16]. As the exotic fishes are considered to be very hardy, most adaptable and have high reproductive potential, hence these are immune to such toxic substances present in the water. Therefore, in the present study, Ctenopharyngodon idellus (grass carp) has been selected as the test animal. They are ideally suitable for laboratory conditions for longer periods.

Scanning electron microscope is a valuable tool for evaluating the toxic effects of environmental pollution on fish structures that are vital for their fitness or survival [17]. Limited studies on surface morphology have been reported on the scales of fish treated with heavy metal using scanning electron microscope (SEM). Hence, in the present study, it was decided to determine the ultrastructural deformities induced on the scales of C. idellus treated with sublethal concentrations of lead nitrate by SEM.

2. Materials and Methods

2.1 Procurement of fish

The fish was procured from Nanoke Fish Seed Farm, District Patiala. The average length and weight of the fish was 8±2 cm and 10±2 gm respectively. Fish were acclimatized to laboratory conditions in glass aquarium for 15 days provided with aerators and filters. The fish were fed by artificial feed, manufactured by Tairopet Product Pvt. Ltd. Chennai, India, contains 32% crude protein, 4% crude fat, 6% fiber, 10% crude ash and 10% moisture. Before the start of experiment, the fish were starved for 24 hours. Lead nitrate manufactured by Qualikems Fine Chemicals Pvt. Ltd. New Delhi, India was used as a toxicant for the present investigations.

2.2 Acute toxicity test

Acute toxicity tests were performed according to the standard methods [18]. 96 h LC_{50} was calculated by Probit analysis [19]. The 96 h LC_{50} value of lead nitrate for grass carp was found to be 33.27 mg/L.

2.3 Chronic bioassay study

Chronic bioassay experiments were performed in a syntax plastic tank aquarium of 25 L capacity with 20 L of dechlorinated water in triplicates. Ten fish were exposed to two sub lethal concentrations of 1/3rd (6.65 mg/L) and 1/5th (11.09 mg/L) of 96 h LC_{50} of lead nitrate for 45 days. Along with it, a control was also maintained. The toxicant medium was changed every day so as to maintain the desired level of the toxicant concentration. Fish were sacrificed on 45th day of the experiment. Throughout the experiment, fish were regularly examined for morphological and behavioral alterations.

2.3.1 Scanning electron microscopic studies

The scales of control and treated fish were removed from the left side of the body beneath the dorsal fin from second or third row. They were washed thoroughly in tap water and cleaned gently by rubbing them between tips of fingers. Scales were subjected to sonication in double distilled water twice for 10 minutes, then dried on Whatman Filter Paper. Cleaned and dried scales were mounted on aluminium stubs with help of double adhesive tape and coated with thin layer of gold palladium alloy of thickness 100Å. The scales were then viewed under vacuum in JSM JEOL 6100 Scanning Electron Microscope.

2.4 Semiquantitative scoring

The ultrastructural changes in the scales were analysed in the randomly selected three fish from each group per replicate. Three scales were studied from each fish. The following parameters were analysed: reabsorbed focus, damaged circuli, damaged tubercles and damaged intercircular space. The mean occurrence of each morphological changes was categorized as none (−), mild (+, <25% of sections), moderate (++, 25–50% of sections) and severe (+++, >50% of sections) [20].

2.5 Ethical clearance

The experiments were performed according to the guidelines of Institutional Animal Ethics Committee, Panjab University, Chandigarh (PU/IAEC/S/14/159).

3. Results and Discussion

3.1 Acute toxicity test

Acute toxicity test, for determination of 96 h LC_{50} of lead nitrate to *C. idellus* were carried out according to the Standard Methods [18]. After performing exploratory experiment, five concentrations (31, 32, 33, 34 and 35 mg/L) of lead nitrate were selected for the experiment. After 96 h exposure to the toxicant observations were made on the percentage mortality in each concentration. 96 h LC_{50} of lead nitrate for *Ctenopharyngodon idellus* were calculated by Probit analysis [18] and the data has been presented in the table 1.

After 96 hrs exposure to lead nitrate the percent mortality was calculated and the values were transformed into probit scale. LC_{50} Graph was plotted by taking log concentration of the toxicant on X- axis and probit kill on Y-axis (Figure 1). The correlation coefficient 'r' was 0.98 and was significant ($p<0.05$). Regression line was drawn (Figure 2). Thus, the LC_{50} of lead nitrate to *Ctenopharyngodon idellus* was found to be 33.27 mg/L.

Table 1: LC_{50} of lead nitrate for *Ctenopharyngodon idellus.*

Group	Conc. (mg/L)	Log conc.	Mortality	% mortality	Probit kill
1	31	1.49	0/10	0	3.4
2	32	1.50	1/10	10	3.72
3	33	1.51	4/10	40	4.75
4	34	1.53	7/10	70	5.52
5	35	1.54	9/10	90	6.28

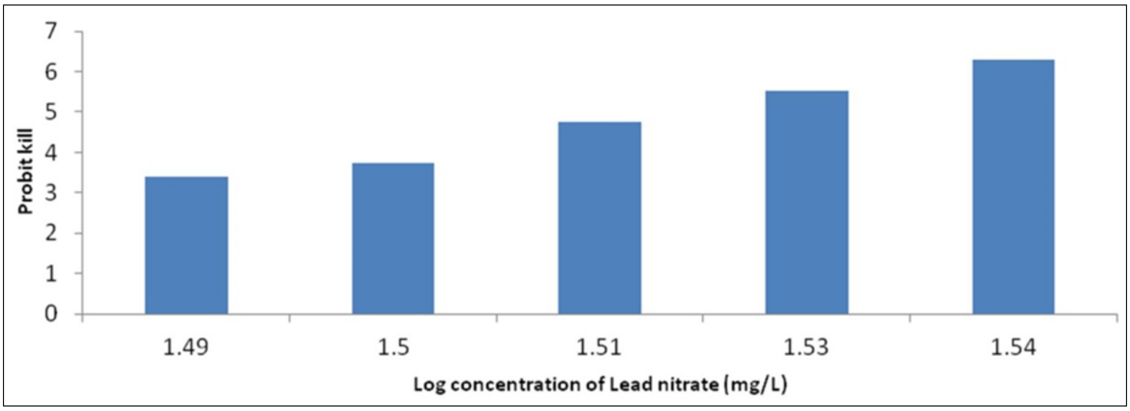


Fig 1: Relationship between conc. of lead nitrate and probit kill of *Ctenopharyngodon idellus* for 96 hrs.

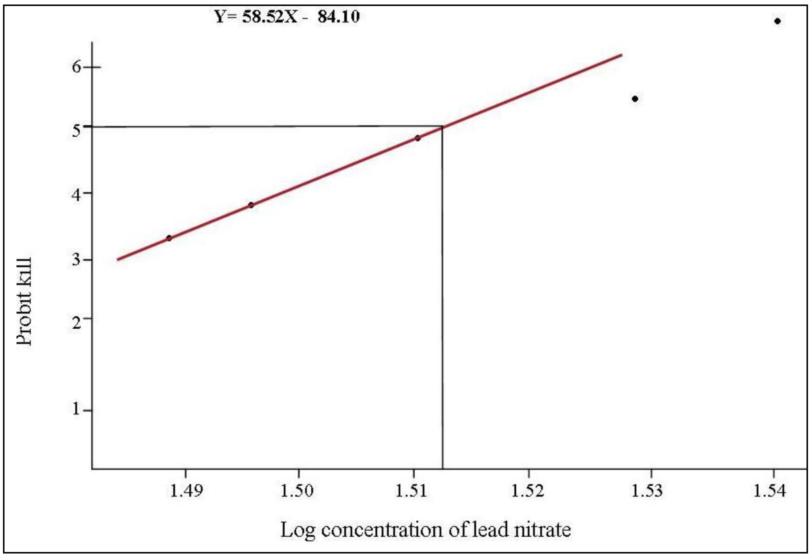


Fig 2: Regression line between the probit kill of *Ctenopharyngodon idellus* and Log concentration of lead nitrate.

3.2 Chronic toxicity test

During the experiment, various physiochemical characteristics of water was analysed and were temperature 24±2 ^{0} C, pH 7.2±0.2, dissolved oxygen 8-10 mg/ml and hardness 175±5 mg/ml (Table 2).

Table 2: Physicochemical characteristics of test water

Parameters	Experimental tank
Temperature	24±2 ^{0} C
pH	7.2±0.2
Dissolved oxygen (mg/ml)	8-10
Hardness (mg/ml)	175±5

3.2.1 Morphological studies

During present investigation, morphological changes were observed in fishes exposed to the toxicant comparison with that of control condition. There was a significant change i.e. loss of pigmentation, excessive mucous secretion all over the body, increase in locomotive activity, hyperactivity and pigmentation was recorded in the cephalic region of the fish.

3.2.2 Behavioral studies

The opercular, fin and air gulps movements of fish were observed on exposure to sublethal concentrations of the toxicant (6.654 mg/L and 11.09 mg/L) for 15, 30 and 45 days exposure. Toxicant exposed fish resulted in increased opercular movements whereas decreased fin and air gulps movements when compared to control (Figure 3, 4, 5).

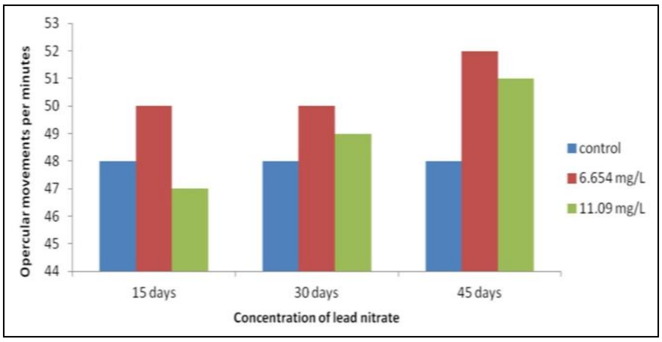


Fig 3: Variation in opercular movements per minute of *C. idellus* on exposure to lead nitrate

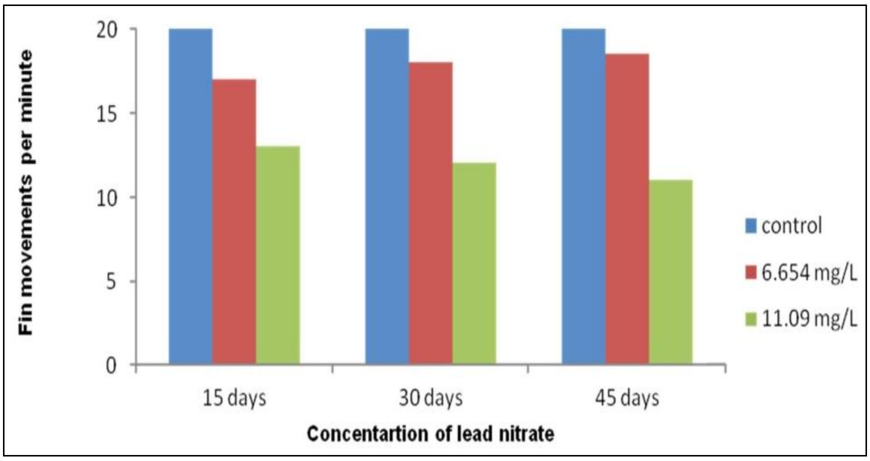


Fig 4: Variation in fin movements per minute of *C. idellus* on exposure to lead nitrate

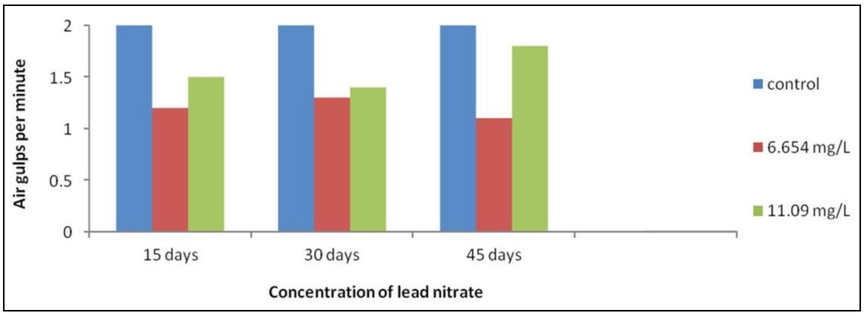


Fig 5: Variation in air gulps movements per minute of *C. idellus* on exposure to lead nitrate

In the present study, increased opercular movements were observed in toxicant treated fish. Such increased in opercular movement at high doses was also reported in *Cyprinus carpio communis* on exposure to organophosphates [21] and dimethoate [22]. This increased opercular movement might be an adaptive reaction of the fish for proper aeration of the gills. Rapid opercular movements, leaping out of water of the fish and thick mucus covering over the body surface, leading to coughing in common carp have been reported on exposure to industrial effluents [23, 24, 25]. From the present observations, it is evident that the fin movements showed a decrease in the treated fishes. The decrease in fin movements which results in lowering of the swimming has also been reported in *Jenynsia multidentata* exposed to endosulphan, *Gambusia affinis* exposed to mercury and *Gambusia affinis* exposed to monocrotophos [26, 27, 28]. On addition of the toxicant to the experimental tank, the fish tried to come up on the surface to engulf air bubbles to utilize oxygen from the thin layer of the water that is supersaturated with dissolved atmospheric oxygen. This increased surfacing and air gulping under the stress created by the toxicants has also been reported in *Cyprinus carpio* [29], *Rasbora daniconius* [23] and *Anabas testudineas* [30].

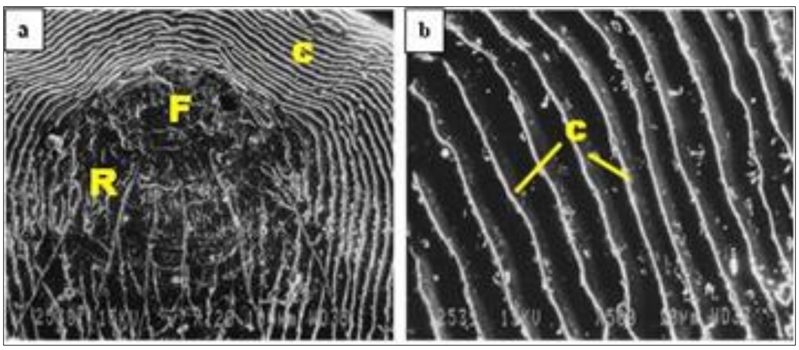
3.2.3 Scanning electron microscopy of scales

The scales of control and treated groups of fish were observed for any structural anomalies. It was observed that the scale of control group has well marked circuli, focus, radii and tubercles. The circuli were intact with no damage (Figure 6 a, b). The circuli seem to be irregular and breaks were observed in the circuli after 45 days treatment of lead nitrate (Figure 6 d, f). Some parts of focus were reabsorbed with evidently more damage which means more number of breaks and irregularities (Figure 6 c, e). The magnified view of scale showed the presence of tubercles on the circuli of anterior region with absence of lepidonts. The tubercles are small triangular projections which meant for attachment of scale to the fish body. In control scale, the tubercles were having serially arranged curved pattern and no damage have been observed in circuli (Figure 7 a, b). However the effect of heavy metal was well marked in 45 days treated scales which showed damaged circuli and tubercles. The damage was observed to be more in scales treated with higher concentration (11.09 mg/L) of the toxicant than in the lower concentration (6.654 mg/L) (Figure 7 c, d, e, f). On exposure to toxicant concentration the tubercles and circuli lost their shape and damage was evident (Figure 7 c, d, e, f). Semiquantitative evaluations of alterations on the scales of *C. idellus* exposed to sublethal concentrations of lead nitrate have been represented in Table 3. Scales have been successfully employed as pollution indicator [31]. In the present investigation, lead nitrate treated fish showed various degrees of morphological changes in scales. Similar ultrastructural changes in the fish scales was observed on exposure to fly ash which contains arsenic, cadmium, zinc, chromium, cobalt, lead and mercury [32]. Numerous workers have reported the damage to the lepidonts upon exposure to toxicants [29, 33, 34].

Table 3: Semi quantitative evaluation of Scanning electron microscopic observations of scale of grass carp from control and sublethal concentrations of lead nitrate

Concentration of toxicant (mg/L)	Reabsorbed focus	Damaged circuli	Damaged tubercules	Damaged intercircular space
6.654	+	++	++	+
11.09	+++	+++	+++	++
control	-	-	-	-

none; +, mild; ++, moderate; +++, severe.



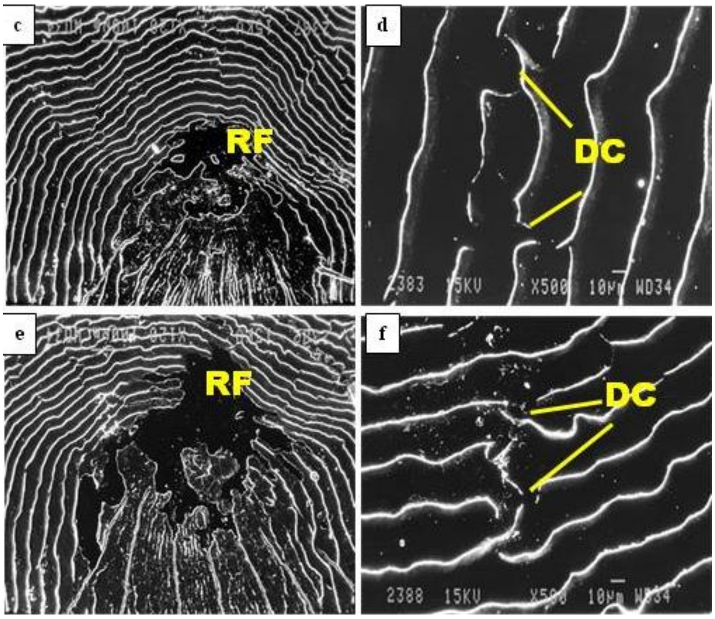


Fig 6 (a-f): Scanning electron micrographs of scales of *Ctenopharyngodon idellus (*Cuvier and Valenciennes) showing the normal and lead treated fish. a and b, Control; c and d, treated (1/5th dose i.e. 6.6mg/L); e and f treated (1/3rd i.e. dose 11mg/L) for 45 days. Abbreviations: C-Circuli; F- Focus; DC-Damaged circuli; R-Radii; RF- Reabsorbed focus

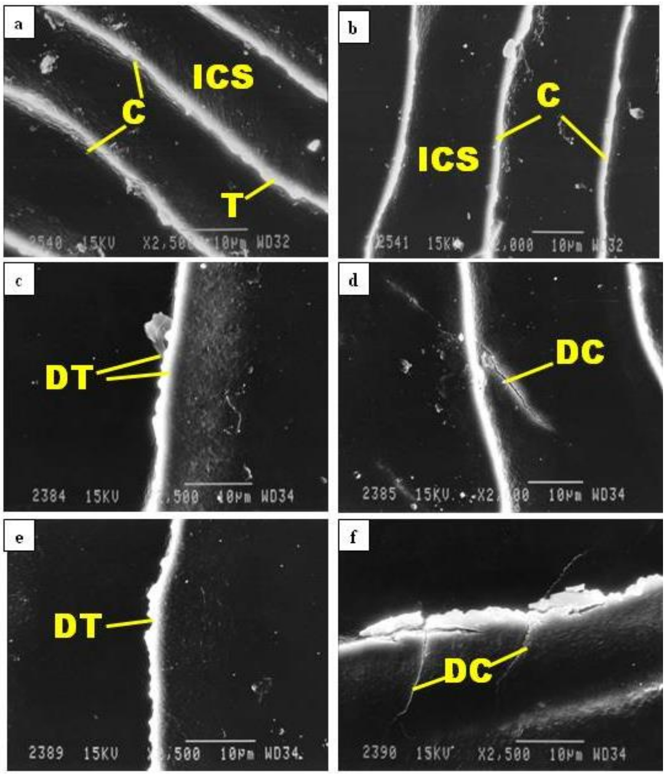


Fig 7 (a-f): Scanning electron micrographs of scales of *Ctenopharyngodon idellus* (Cuvier and Valenciennes) showing the normal and lead treated fish. a and b, Control; c and d, treated (1/5th dose i.e. 6.6 mg/L); e and f treated (1/3rd dose 11mg/L) for 45 days.

Abbreviations: C- Circuli; ICS- Intercirculi space; T-Tubercles; DC-Damaged circuli; DT- Damaged tubercles

4. Conclusion

Scanning electron microscopic observations indicated that exposure to sub-lethal concentrations of lead nitrate results in deleterious effect on the scales of *C. idellus*. The present findings reveal a direct correlation between heavy metal exposure and microstructural abnormalities in the scales. Also, suggesting the use of alterations in scale morphology as reliable biological markers for heavy metal pollution with additional advantage that these can be used without sacrificing the animal.

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