Microscopic Studies on Erythrocytes of *Channa punctata* Exposed to Commercial Grade Lindane

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ABSTRACT

Light and scanning electron microscopic studies of erythrocytes of Channa punctata exposed to two sublethal doses of lindane over a period of 21 days revealed myriads of anomalies. High frequency of micronucleus appeared progressively in the erythrocyte, ranging from 3.8-7.5%, when monitored weekly for 21 d at 0.25 g/L and 1.0-3.0% at 0.025 g/L lindane. Prominent structural anomalies of erythrocytes included abnormal shapes, vacuolation, membrane invagination and disintegration. At higher dose and at later duration, a unique tendency of abnormal cells to stick together was observed. It could be concluded that commercial formulation of lindane could induce genotoxicity and structural anomalies in the erythrocytes of fish.

Key words: RBC, SEM, light microscopy, micronucleus

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INTRODUCTION

Lindane (ɤ-hexachlorocyclohexane) is a broad spectrum, environmentally persistent organochlorine pesticide that has been banned or restricted for use in several countries, including India [1,2] . However, lindane and its residues have been regularly detected in drinking water, industrial effluent and sewage, predominantly due to its continued usage via domestic stockpile or due to its tendency to bioaccumulate in natural waters [3,4]. Often such residues in water effects non-target organisms such as fish, consequently effecting human health. Fish can take up lindane or its residue from water and sediments and bioaccumulation in their fatty tissues [5], which could be associated to its hydrophobicity and persistency in the environment [6]. Blood is an excellent indicator of lindane stress in fish [7] and the use of erythrocyte alterations in fish are considered a useful tool to evaluate pathological processes resulting from exposure to environmental pollutant [8]. Both light (LM) and scanning electron microscopic (SEM) studies have been used to detect cellular alteration in erythrocytes in response to aquatic pollution [9]. LM aids in the identification of micronuclei (MN), nuclear abnormalities, and histopathological alterations, while, SEM is used for the assessment of alterations in cells at surface microstructural and internal fine structural level. Generally, MN is formed by the condensation of whole or fragmented chromosomes that are not incorporated into the main nucleus during mitosis, due to aneugenic or clastogenic effects [10]. MN test has been found to be a sensitive assay to evaluate the effects of genotoxic compounds in fish under controlled conditions [11]. Thus, the present study aims to analyse the temporal dynamics of erythrocyte morphology in a freshwater teleost, *Channa punctata* exposed to sublethal commercial formulations of linadane (effective concentration of 6.5%) using both light and scanning electron microscope.

MATERIAL AND METHODS

EXPERIMENTAL SET-UP

Healthy *Channa punctata* (length, 16.58 ± 0.3cm, and weight, 25.7 ± 0.5 g) were procured from a local fishery with no reported pollution load. They were acclimatized for 3 weeks in 1000 L cement tank, under laboratory conditions with tap water free from chlorine, with the physicochemical characteristics such as temperature, pH, dissolved oxygen, and hardness (as $CaCO_3$) to be 29.2 ± 0.13ºC, 6.8 ± 0.3, 5.5 ± 0.24 mg L-1 and 30.5 ± 0.5 mg L-1, respectively and fed with minced goat liver at the rate of 3% body weight. Stock solution of commercially available lindane was prepared from Gamma BHC 6.5% (trade name -Kunahex) procured from Kundu Agro Chemicals Ltd., Kolkata, India and purchased from a local shop (Tuhina Seeds).
In an initial study, 96 h LC50 was found to be 2.55 g L-1. Stock solutions of the test substance were prepared by dissolving the insecticide in the tap water. These solutions were further diluted to obtain the experimental concentrations in aquariums. Adult fish were exposed to two different sub-lethal concentrations of lindane (0.025 g L-1 and 0.25 g L-1) for 7, 14 and 21 days after acclimatization period. The concentration of lindane in the water samples was determined thrice during the course of 21 days of experimentation by gas liquid chromatography (Nucon 5765) equipped with 63Ni electron capture detector (ECD) as per Singh and Singh[4].
The mean concentration was always within 5% of the intended concentration. A total of 45 fishes, divided into three groups (one control and two experimental groups),

were kept in three separate containers, each of 200 L capacity. Lindane free tap water served as control media. Control and experimental fish were fed daily
as described earlier. No mortality was observed during the experiments. Experimental and control water was refreshed every day to minimize loss of pesticide concentration. From treatment and control tanks, 5 fish were sacrificed every 7, 14 and 21 days.

LIGHT MICROSCOPY

Blood was drawn with a heparinized syringe from the caudal peduncle. The smears on grease free slides were fixed in absolute methanol for 10 min after drying at room temperature. Slides were stained with haematoxylin and eosin, followed by dehydration in ascending grades of alcohol [12]. Several slides were selected on the basis of staining quality, then coded, randomized and scored blindly. In each group 10,000 cells (a minimum of 2000 per slide, n=5) were examined [10] at 40× objective and 10× eyepiece for morphologically altered erythrocytes and micronucleus. Semi quantitative scoring of morphological anomalies were performed and the mean prevalence of each parameters was categorized as none (-), mild (+), moderate (++) or severe (+++) as per Velmurugan et al. [13]. The established criteria for identifying micronucleus [14] were strictly followed to ensure authentic scoring.

SCANNING ELECTRON MICROSCOPY

Two to three drops of blood were put inside a vial containing 2.5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer. The sample was centrifuged at 1,500 rpm for 5 min, washed, and re-suspended in distilled water, and the process was repeated two to three times. A thin film was decanted and applied to a cover slip after resuspension in distilled water [15]. The samples were then air dried and coated with gold in a JFC-1100 (JEOL Ltd., Tokyo, Japan) ion sputterer. Observations were made on a JSM-6360 (JEOL) SEM at an accelerating voltage of 15–20 kV, using the secondary electron emission mode. Blood smear samples from 5 control and 5 lindane-exposed fish were studied, and the average percentage of abnormal cells were calculated.

ETHICAL CLEARANCE

Ethical guidelines from the Canadian Council on Animal Care [16] were followed that include the following: a minimum number of fish were used for experimentation, fish were maintained in properly aerated aquaria in a quiet and well-ventilated room, crowding was avoided; an adequate amount of nutritious food was provided; fish were handled gently and only when necessary; aquaria were covered with nets as *C. punctata* has the habit of jumping out and suffer mortality. The above mentioned process has the necessary recommendations of Assam University Institutional Ethical Committee.

STATISTICAL ANALYSIS

Basic statistics such as mean and standard error were computed. One-way analysis of variance (ANOVA) was performed to determine whether treatments were significantly different from the control group ($P<0.05$).
The Tukey test was considered for multiple comparisons. In all the cases, SPSS 19 statistical software for windows was used.

QC/QA

Certified reference standard from Sigma (USA) was used for the quantification of lindane. The target analytes were identified in the sample extract by comparing the

retention time from the standard mixture and quantified using the response factors from five level calibration curves of the standard. Procedural blanks (analyte concentrations were less than method detection limit), random duplicate samples (Standard deviation <5), and matrix spike recovery of 100±20% was practiced.

RESULTS AND DISCUSSION

Erythrocytes in *C. punctata* are nucleated, biconvex and are the dominant cell type in the blood. Table 1 summarized the semi quantitative analysis of erythrocyte deformities from lindane treated *C. punctata* at various exposure durations and concentrations. The common deformities were various abnormal shapes of
erythrocytes such as elongated with membrane invagination, which showed variable degree of abnormalities.
At 0.25 g/L, at the end of 21 days of exposure, the abnormal cells showed a tendency towards sticking to each other. Appearance of MN was another prominent feature observed in the cells during later exposure durations. Some of the cells showed vacuolation.

Table 1 Semi quantitative analysis of erythrocyte abnormalities from lindane treated *C. punctata* at various exposure
durations and concentrations

Lesion	Control	7-d	14-d	21-d		7-d	14-d	21-d
		0.255 g/L				0.025 g/L		
Abnormal shapes	-	+	++	+++		+	++	++
Membrane invagination	-	++	+++	+++		+	++	+++
Micronucleus	-	+	++	+++		-	+	++
Vacuolation	-	-	+	+		-	-	-
Cytoplasmic blebs	-	-	+	+		-	-	-
Lobopodium	-	-	-	++		-	-	-
Acanthocyte	-	-	-	+		-	-	-
Membrane disintegration	-	-	+	+++		-	-	-
Sticky cells	-	-	-	++		-	-	-

- none, + mild, ++ moderate,   +++ severe occurence

The scoring of MN was performed and percent values were shown in Table 2. High statistical significance (p<0.01) were observed between MN frequencies of control and lindane treated groups. Besides, statistical significance (p<0.05) were observed between exposure durations at a particular dose, indicating progressive appearance of MN in erythrocytes as the duration of exposure increased. The highest MN frequency was observed after 21 days of exposure to 0.255 g/L lindane, which accounted for around 7.5%. Similarly, for this dose, there was around 4.5% and 3.8% MN frequencies at 14 and 7 days of exposure, respectively. At 0.025g/L, there were appearance of around 1.0%, 2.3% and 3.2% MN after 7, 14 and 21 days of exposure.

Table 2 Micronucleus frequency (mean $\pm$ SD) % in erythrocyte of *C. punctata* at various exposure durations and concentrations of lindane

Test	7-d	14-d	21-d
Control	0.15±0.23	0.1±0.008	0.12±0.03
0.255 g/L	3.8±0.12*	4.5±0.11*	7.5±0.3*
0.025 g/L	1.0±0.05*	2.3±0.21*	3.2±0.03*

*indicates significance at $p<0.05$

Figures 1A and 3A indicated the light and electron micrograph of the blood of fish under controlled condition, respectively. Under, LM, prominent changes observed at 0.25 g/L lindane included abnormal shapes (hooked and irregular membrane), vacuolation and appearance of MN after 7d. At later exposure durations, some blebs in the cytoplasmic, severe membrane invagination and slight lobopodial projections appeared in the erythrocytes. Some of the abnormal cells developed a tendency towards sticking to each other followed by membrane disintegration (Figs. 1B-D). At 0.025 g/L lindane exposure (Figs. 2A-C), similar abnormalities were observed although, with lesser severity. Besides, sticky cells were not found at this dose, at any duration. Under SEM, at 0.25 g/L, erythrocytes showed various abnormal shapes, membrane invagination, echinocytes, lobopodial projections and disintegrated membranes (Figs. 3 B-F). At 0.025 g/L lindane, some typical abnormalities were the appearance of echinocytes and severe membrane invaginations in erythrocyte membranes (Figs. 4 A-D).
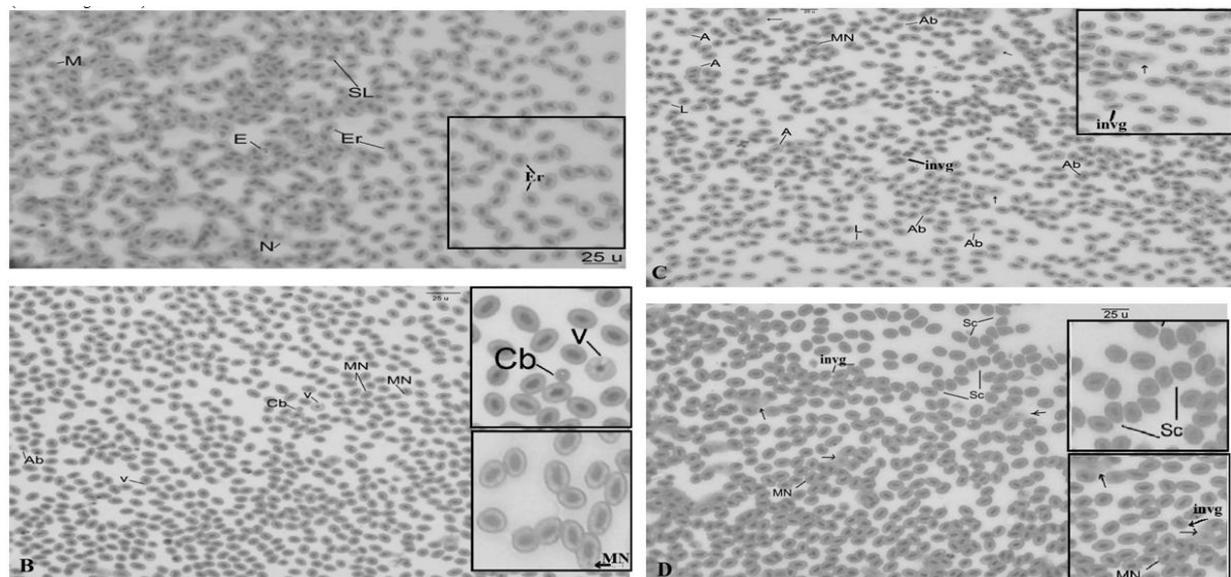
Fig. 1 Light micrograph of blood of *C. punctata* (H & E, × 400): (A) control- nucleated erythrocytes (Er), eosinophil (E), neutrophil (N), small lymphocyte (SL) and monocyte (M); (B) abnormal shapes (Ab), vacuolation (v), micronucleus (MN) and cytoplasmic bleb (Cb) after 7 d; (C) abnormal shapes (Ab), erythrocyte membrane invagination (invg), acanthocytes (A), lobopodium (L), micronucleus (MN) and membrane disintegration (arrow) after 14 d; (D) sticking (Sc), abnormal shapes (Ab), membrane invagination (invg), micronucleus (MN) and disintegrated membrane (arrow) after 21 d of exposure to 0.25 g/L lindane (inset-enlarged view).
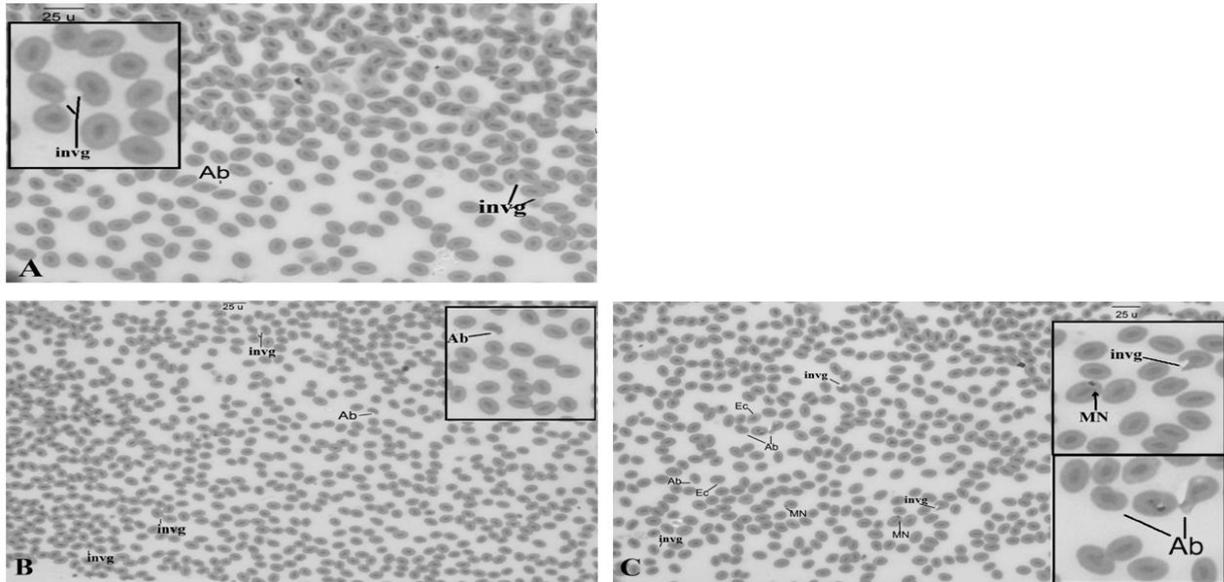
Fig. 2 Light micrograph of blood of *C. punctata* (H & E, × 400): showing (A) abnormal shapes (Ab) and erythrocyte membrane invagination (invg) after 7 d; (B) after 14 d; (C) abnormal shapes (Ab), membrane invagination (invg), echinocytes (Ec) and micronucleus (MN) after 21 days of exposure to 0.025 g/L lindane (inset-enlarged view).
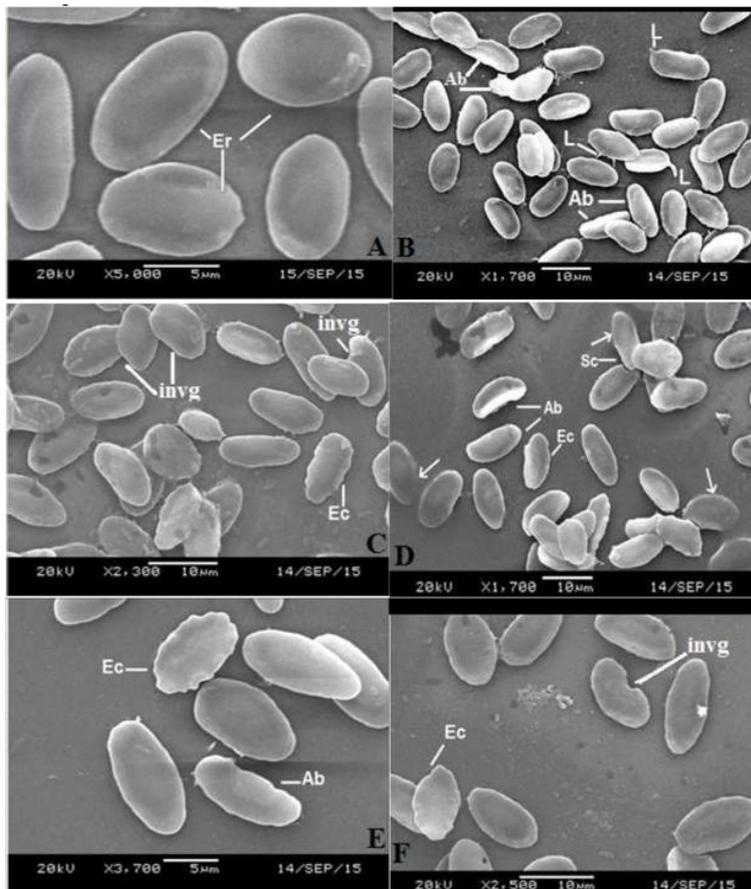
Fig. 3 SEM of blood of *C. punctata* (A) under control condition showing oval erythrocytes (× 5000); (B) abnormal shapes (Ab) and lobopodial projections after 7 days (×1,700); (C) membrane invagination (invg) after 14 d (× 2,300); (D) stickiness (Sc), abnormal shapes (Ab), membrane invagination (invg), and disintegrated membrane (arrow) (× 1, 700) after 21 d; (E) Echinocytes (Ec) and slender erythrocyte (Ab) (× 3,700) after 21 days; (F) membrane invagination (invg) and echinocytes (×2, 500) after 21 days of exposure to 0.25 g/L lindane.
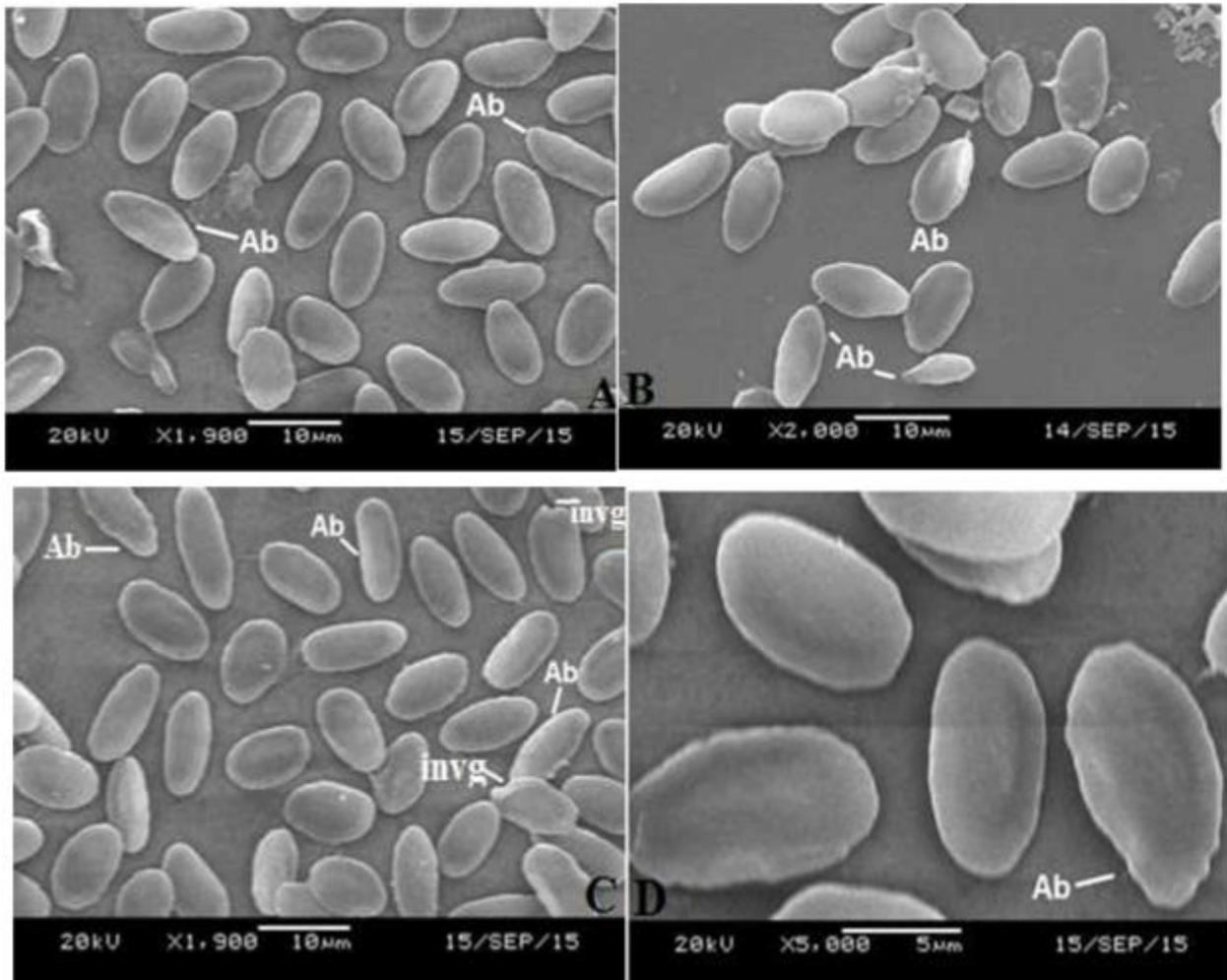
Fig. 4 SEM of erythrocytes of *C. punctata* showing (A) abnormal shapes (Ab) after 7 days (×1, 900); (B) abnormal shapes (Ab), and clumps after 14 days (× 2, 000); (C) abnormal shapes (Ab) and membrane invagination (invg) (×1,900) after 21d; (D) enlarged abnormal shape (×5,000) after 21 days of exposure to 0.025 g/L lindane.

Erythrocytes are chiefly responsible for gaseous exchange and excretion of nitrogenous wastes. Hence, any morphological alterations are bound to impair the vital functions, thereby resulting in hypoxaemia, hypercapnia and blood acidosis [13]. It is predicted that abnormal nuclear morphology is an indicator of genotoxic damage [11], while abnormal erythrocyte morphology is an indicator of cytotoxicity [17].

Appearance of micronucleus in cell is an index of accumulated genetic damage and is one of the most suitable techniques to identify impacts of pesticide in fish [11]. However, the MN frequency is dependent upon the metabolic capacity, DNA repair efficiency and defense mechanisms of fish [18]. In general, fish are relatively sensitive to changes in their surrounding environment. Hence estimation of MN frequency in terms of fish health may thus reflect, and be a good indication of the health status of a specific aquatic ecosystem.

In erythrocytes of *Tilapia rendalli* exposed to pesticide, deltamethrin, similar increase in MN frequency was observed [19]. Similarly, Ansari et al. [20] also found that deltamethrin induced MN and nuclear abnormalities in erythrocytes of *Channa punctata.*

Light microscopic (LM) studies provide valuable tool for identification of pesticide induced morphological alterations in fish [21]. Svobodova et al. [22] observed elongated oval-shaped erythrocytes with the peripheral nucleus in carp exposed to nitrite. Most xenobiotics have the potential to interact with erythrocyte membrane, as seen in nonylphenol -exposed fish, consequently leading to membrane deformities [23]. In another similar study with 4-nonylphenol on adult catfish *C. gariepinus,* altered erythrocytes and micronucleus were detected with the aid of LM [21].

Previous SEM study on erythrocytes following cypermethrin exposure *Anabus testudineus* revealed abnormal erythrocytes, oozed out cytoplasmic content and lobopodial projections [13], similar to this study. Such increased membrane abnormalities may be attributed to lindane induced oxidative stress [5], which targets the cellular membrane and alter its permeability and selectivity, making the nucleus more susceptible to the formation of nuclear abnormalities and micronuclei, respectively. The vacuoles, observed in erythrocytes at higher lindane dose, could be due to unequal distribution of hemoglobin [17]. Lindane might disrupt the hemopoietin process [7] and accelerated disintegration of erythrocyte membrane may also lead to a reduction in hemoglobin and hematocrit values in the fish [24]. Oozed out cytoplasmic content and lobopodial projections were observed also in the erythrocytes after exposure to chlorpyrifos in *Anabas testudineus* [25].

In the present study, increase in altered cell frequencies was found to be the function of dose and duration of lindane exposure, as the increased concentrations or exposure duration registered a quantitative increase in erythrocyte aberrations. The blood cells of the fishes exposed to lindane consistently showed loss of the normal shapes and became more irregular. Cell membranes of fish are often subjected to peroxidative damage due to pesticide exposure [26] that might attribute towards the observed structural alterations. In another study on rat erythrocytes with SEM, fenvalerate exposure revealed various degrees of distortion in shape and ruptured membranes and echinocyte formation [27].

CONCLUSION

We conclude that morphological alterations in erythrocytes may result in severe physiological complications, ultimately leading to the death of fish. The present microscopic investigations de monstrated a direct correlation between pesticide exposure and erythrocyte morphological alterations and provided with useful and sensitive tool in the investigation of sublethal effects induced by environmental contaminants.

ACKNOWLEDGMENT

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