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SEM STUDY OF ULTRASTRUCTURAL CHANGES IN BRANCHIAL ARCHITECTURE OF *CTENOPHARYNGODON IDELLA* (CUVIER & VALENCIENNES) EXPOSED TO CHLORPYRIFOS

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Abstract: We evaluated structural modifications in the branchial architecture of grass carp, *Ctenopharyngodon idella*, chronically exposed to chlorpyrifos (an organophosphate) using scanning electron microscopy (SEM). Static renewal tests were conducted for 96 h to determine the LC_{50} of chlorpyrifos to the fish. Physicochemical analysis of water was done using standard methods. To assess the effect of chronic toxicity, fish were exposed to two sublethal concentrations (1.44 μg/L and 2.41 μg/L) of chlorpyrifos for 15, 30 and 60 days, after which gills were examined by SEM, which revealed changes in gill ultrastructure. Branchial alterations included distorted secondary lamellae in the form of curling and shortening, erosion in a few primary filaments and a wrinkled and denuded epithelial surface. Excessive mucosal openings (mucoid hyperplasia) on the surface were observed in the gills of fish exposed to both concentrations of chlorpyrifos. Alteration in the microridge pattern of pavement cells and cracks on the gill rakers were also observed, and the intensity of the damage was found to be directly related to the toxicant concentration and exposure period. The present study revealed that the assessment of surface morphology can serve as a novel bioindicator of pollution, disease and toxicity.

Key words: Chlorpyrifos; *Ctenopharyngodon idella*; gills; toxicity; scanning electron microscopy

INTRODUCTION

Pesticides are considered a core group of aquatic pollutants due to their toxicity and accumulation in the ecosystem. Pesticides exhibit toxicity through the formation of coordination complexes and metabolites in the animal cells. A low concentration of pesticide may induce chronic stress, which may not kill the fish but affects its size and body weight adversely, thus reducing the potential to compete for food and habitat. Fish also have a tendency to bioaccumulate pesticides, and humans can therefore be at great risk through contamination of the food chain. In general, pesticides are non-biodegradable and bioaccumulate in aquatic organisms. Pesticide uptake by aquatic organisms is a two-phased process, involving rapid adsorption or surface binding, followed by slower transport into the cell interior and metabolite transport into the intracellular space, which may be aided by either diffusion across the cell membrane or active transport by a carrier protein. Chlorpyrifos (CPF) enters the aquatic environment through surface run-off, rain and leaching from soil as a result of its indiscriminate use as an agrochemical. In natural waters, the chlorpyrifos concentration generally ranges between 0.05 and 10.00 mg/L [1]. CPF is known to accumulate in fish tissues [2].

Gills are target organs for toxicants because of their large surface area, direct contact with the external environment and thin membranes, which separate the internal medium from the external medium, thus causing morphological and functional disturbances. Changes in gill structure affect the normal functioning of vital physiological processes, such as osmoregulation, gaseous exchange, ionic balance, excretion of nitrogenous wastes and acid-base equilibrium [3,4]. Gills represent an appropriate model

for studying the effects of environmental stressors on fish [5]. In the present study we evaluate and describe ultrastructural alterations in the branchial architecture of *Ctenopharyngodon idella* induced by sublethal concentrations of chlorpyrifos.

MATERIALS AND METHODS

Experimental organisms

Ctenopharyngodon idella (weight: 8.2±2 g, length: 9.8±0.5 cm) collected from Nanoke Fish Seed Farm, Patiala district (Punjab, India), were brought to the laboratory in well-packed polythene bags containing aerated water. Care was taken to minimize physical stress during transportation. Fish were acclimatized to laboratory conditions for 15 days in a glass aquarium (temperature 25±2 °C, pH 7.2±0.1, dissolved oxygen 8.0±0.3 mg/L, total alkalinity 110±8 mg/L and total hardness 122±5 mg/L). During the stocking period, the fish were fed with palletized supplementary feed once a day at the rate of 2% of the body weight. Feeding was given at least 1 h prior to replacement of water; 3/4 of the water was renewed daily, and the left feed and fecal material was removed. Chlorpyrifos (20% EC), commercial grade purchased from Shivalik Insecticide Pvt. Ltd., India, was used as the toxicant.

Acute and chronic toxicity testing

To test acute toxicity, range-finding tests were conducted by exposing the fish to various concentrations of CPF. Static-renewal, acute toxicity bioassays were conducted by exposing the fish to 6, 7, 8 and 9 µg/L (w/v) concentrations of CPF for 96 h in 50-L colorless plastic tanks. Physiochemical analysis of water was done following standard methods [6]. Ten fish were exposed to each concentration and each experiment was conducted in duplicate. Fish mortality was noted at 24, 48, 72 and 96 h for each pesticide concentration. On the basis of fish mortality, the 96-h LC_{50} of CPF to *C. idella*, determined by Probit analysis [7], was found to be 7.24 µg/L.

For chronic toxicity tests, three groups (I – control; II – lower sublethal concentration (1.44 µg/L); III – higher sublethal concentration of the toxicant (2.41 µg/L)) were set up, and to each group, 10 healthy fish were introduced to 50-L tanks and maintained for 15, 30 and 60 days. Fish from control and toxicant-treated tanks were collected randomly and killed at the end of each exposure period for ultrastructural analysis according to the guidelines of the Institutional Animal Ethics Committee (Panjab University, Chandigarh).

Ultrastructural studies

Ctenopharyngodon idella have four pairs of gills located on the lateral sides of the head and covered by operculum. The operculum was removed carefully and the gills were dissected. For the present investigation, the 2nd or 3rd gill arch was preferred for study as they have large gill filaments, providing a larger surface area. The 4th pair of gill arches is reduced in size and is modified. The 3rd pair of gill arches was excised and immediately immersed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1% M phosphate buffer for 10-12 h (pH 7.2-7.4, 4°C) for primary fixation, then post-fixed with 1% OsO_4 in 0.2 M phosphate buffer for 1 h at 4 °C and dehydrated in solutions of increasing acetone concentration. The dehydrated gill tissues were cleared in amyl acetate and dried in a CPD unit with liquid carbon dioxide. The dried tissues were sputter-coated with gold coating in an ion sputter unit (JEOL JFC-1110) and examined under a scanning electron microscope (JEOL-JSM 6100) at C.I.L.

RESULTS

Control group

Each gill is composed of equally spaced secondary lamellae, intact cellular layers and no sign of fusion between neighboring lamellae (Fig. 1a). The external boundaries of both primary and secondary lamellar epithelia were lined by simple squamous pavement cells, which were pentagonal or hexagonal in shape. Small microridges of these cells were observed to run

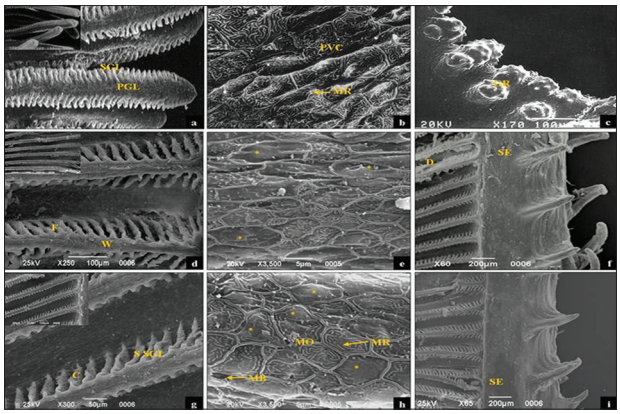

Fig. 1. Scanning electron micrographs of gills of *Ctenopharyngodon idella* of control group (a-c) showing equally spaced primary filament, secondary lamellae (a) pentagonal pavement cells with clearly demarcated boundary and concentric microridges interspersed by mucous cell openings (b), and gill arch with gill rakers (c); on exposure of the fish to chlorpyrifos for 15 days at 1.44 µg/L CPF (d-f) and 2.41 µg/L CPF (g-i) showing fusion of lamella (d) with their curling and shortening (g) and wrinkled filamentous surface, (*) degeneration and loss of protruded microridges with their distorted pattern (e, h), denudation of surface of gill arch with sloughing off of epithelium (f, i). C – curling, D – degeneration, F – fusion, GR – gill raker, MB – marginal breakage, MO – mucosal opening, MR – microridges, PGL – primary gill lamellae, PVC – pavement cells, SE – sloughed-off epithelium, SGL – secondary gill lamellae, SSGL – shortening of SGL, W – wrinkling.

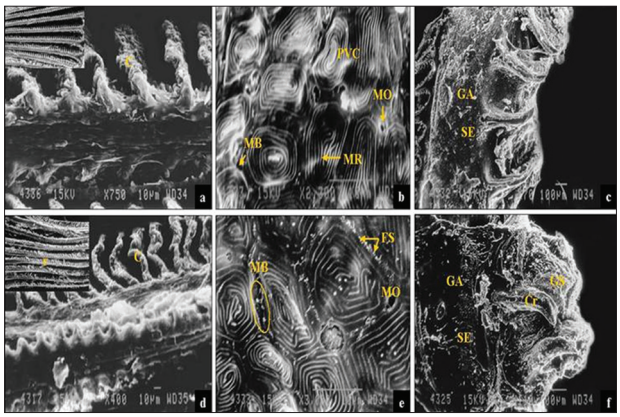

Fig. 2. Scanning electron micrographs of gills of *Ctenopharyngodon idella* on exposure to chlorpyrifos for 30 days at 1.44 µg/L CPF (a-c) and 2.41 µg/L CPF (d-f) showing marked damage of lamellar structure in form of fusion, curling and necrosis (a, d), marginal breakage (MB) and swollen microridges (MR) of pavement cells (b), severe marginal breakage and presence of filamentous strands on the border of PVC (e), presence of globular structures and cracks near gill raker (f). C – curling, Cr – cracks, D – degeneration, F – fusion, FS – filamentous strands, GA – gill arch, GR – gill raker, GS – globular structure, MB – marginal breakage, MO – mucosal opening, MR – microridges, PVC – pavement cells, SE – sloughed-off epithelium.

parallel to the cell limits and were clearly demarcated. A double-ridged or single-ridged border between pavement cells was also observed (Fig 1b). Microridges were organized into whorls over the pavement cells of the gill arch.

On exposure of the fish to chlorpyrifos for 15 days

After a 15-day exposure of the fish to 1.44 µg/L chlorpyrifos, some of the microridges were disorganized and degenerated. A wrinkled epithelial surface of the secondary gill lamellae was seen along with broken primary gill lamellae. Cracks on the gill rakers were also found. Fusion of a double-ridged border among the microridges of pavements cells was a common occurrence (Fig. 1d-f). At 2.41 µg/L CPF concentration, there was gradual necrosis of the lamellar surface. A pronounced wrinkled surface of the secondary gill lamellae was observed and due to wrinkling the lamellae were shortened. Sloughing-off of the epithelium was commonly found with broken lamellae. Microvilli were found to be degenerated and these were more pronounced at the higher concentration of the toxicant (Fig. 1g-i). Chlorpyrifos toxicity also altered the microridge pattern of pavement cells of the primary gill filament and arch. In addition, the whorls on the pavement cells of the gill arch were found to be prominently laden with mucous, which were less at low-concentration than at exposure to high-concentration of the toxicant (Fig. 1h).

On exposure of the fish to chlorpyrifos for 30 days

On the 30th day of exposure of the fish to the toxicant, excessive degeneration has been seen on the epithelial surface of secondary lamellae along with their curling and shortening. Gill rakers of the gill showed marked lesions on their surface (Fig. 2c). After exposure to the higher concentration, some of the pavement cells also showed necrosis, the margins of cells broken and separated. Filamentous strands appearing from the

lateral sides of the microridges of pavement cells occupying the portion between the walls of microridges were also observed (Fig. 2e). The borders of some pavement cells were found to be broken at the higher concentration of CPF.

On exposure of the fish to chlorpyrifos for 60 days

On the 60th day of exposure of the fish, the direct effect of the toxicant was seen on the epithelial layer covering the gill arch, filament and lamellae. A raised epithelial lining was observed on the primary filament and gill arch. This could be attributed to degeneration and wrinkling of the gill epithelium. The number of mucous cell openings increased, being more on the gill arch, followed by the primary filament. These effects had a direct proportionality to the increase in CPF concentration. There was also a loss of uniformity of the secondary lamellae, characterized by their fusion and formation of epithelial swellings and ridges, which resulted in the primary gill filament breaking from the gill arch and pronounced shortening of secondary lamellae at the high concentration (Fig. 3c). Furthermore, the gill arch exhibited a necrotic appearance (Fig. 3f). Also, at the high concentration, the pavement cells shrank and were found to be a little raised (Fig. 3e). In the fish exposed to the 2.41-µg/L concentration of CPF for 60 days, cracks were seen on the pavement cells, on the surface of the primary gill filament. Rakers on the gill showed cracks in the basal region. An abundance of mucous cells and their secretary activity were noticed in our study. Exposure of the fish to increasing concentrations of CPF resulted in an increase in mucous-cell density. A copious amount of mucous was released, which enveloped almost all of the respiratory surface, being more on the lamellae and microridges of the gill rakers.

DISCUSSION

Gills serve as sensitive indicators of the toxic effects of pesticide because of their direct contact with ambient conditions, large respiratory surface area, high permeability and characteristic responses of lamellar epithelial cells to toxicants. During the present study, it was found that there was loss of uniformity of lamellae, characterized by fusion and swellings [8], which might be due to a loss in structural integrity of the epithelial cells, causing the breakage of primary gill filament from gill arch and pronounced shortening of the secondary lamellae [9-11]. The degeneration of epithelial lining resulted in the direct contact of the toxicant with blood vessels, and was responsible for the entry of the toxicant into the blood stream. Detachment of the lamellar epithelium resulted in increased diffusion distance, leading to impaired gaseous exchange [12].

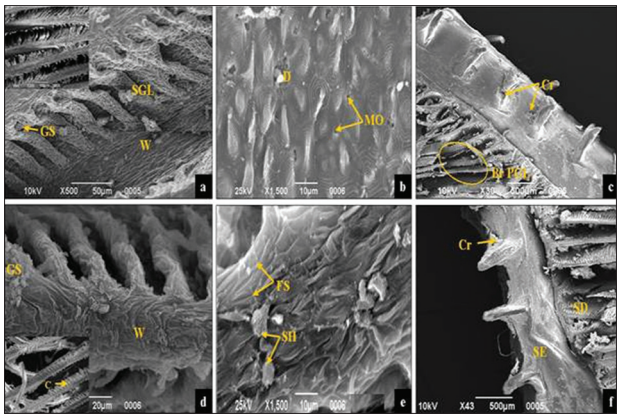

Fig. 3. Scanning electron micrographs of gills of *Ctenopharyngodon idella* on exposure to chlorpyrifos for 60 days at 1.44 µg/LCPF (a-c) and 2.41 µg/L CPF (d-f) showing severely curled lamella & wrinkled filamentous surface with presence of globular structure (a, d), highly shrunk pavement cells (b), severe marginal breakage and presence of filamentous strands on the border of PVC (e), presence of globular structures and cracks near gill raker (e) breakage of filaments from gill arch (encircled) (c) and severely damaged filaments, denudation of gill arch surface and uprooting of gill raker (f). C – curling, Cr – cracks, D – degeneration, FS – filamentous strands, GR – gill raker, GS – globular structure, MO – mucosal opening, Br PGL – broken primary gill lamellae, SE – sloughed-off epithelium, SGL – secondary gill lamellae, SH – shrinkage, SD – severe damage, W – wrinkling.

Pavement cells were found to be shrunken and slightly raised at the higher pesticide concentration. Pavement cells provide structural integrity to the epithelial surface, increase the respiratory lamellar surface and provide an anchoring mechanism to mucous [13]. In the present study, a ridged border between pavement cells was observed. Such microridge borders have also been reported in trout gill epithelia [14].

A progressive reduction in microridge length and complexity in epithelia located over the arch and primary and secondary lamellae was observed and these observations are in conformity with those of the earlier research [15,16].

The interposition of the mucous coat between the environment and epithelium is protective and insulative in nature. The barrier function of mucous may be to prevent mechanical abrasion or decrease the coefficient of drag for water flow across the gill [17,18]. This would minimize the tendency for disruptive turbulence and reduce the resistance of the gill sieve [19]. Our study revealed that mucous-cell openings were scattered over the epithelia of arch, filament and lamellae. The present findings are in agreement with those of Prasad [20]. Similar structures have been labeled as mucous-cells opening by Olson [19]. The filamentous strands present on the lateral sides of the microridges of pavement cells were also observed and their formation could probably be due to glycocalyx or mucous condensation [21].

Epithelial lifting and lamellar fusion have been reported earlier as a protective measure in different species of fishes (*Salmo trutta* [22]; *Boleophthalmus dussumieri,* [23]. According to these studies, these effects decreased the vulnerable surface areas of the gills to the toxicant, so as to maintain osmoregulation. Increased detachment and wrinkling of the epithelium was also observed. The present study is in conformity with the findings of Machado and Fanta [9] and Akaishi et al. [24].

It could be assumed that CPF provided a tactile stimulus due to its abrasive action on the gills, thereby stimulating the mucous-cell population on the lamellae. Release of a profuse amount of mucous has also been reported in other fishes (exposure of *Carassius auratus* to carbaryl, [25]; *Anabas testudineus* to cypermethrin, [11]; *Anabas testudineus* to deltamethrin and permethrin, [26]. Epithelial detachment was followed by hyperplasia characterized by cellular proliferation in the interlamellar region, which has been observed as the most deleterious effect of the toxicant. Also there was considerable loss of microridges on pavement cells in the gill filament [27-30].

From the present findings, it could be inferred that even a sublethal concentration of CPF has deleterious effects on fish gills. The result of such exposure leads to impaired cellular function, which can be a cause of sudden disease or death in fish. CPF was found to be highly toxic to *Ctenopharyngodon idella*, even at a very low concentration. Its administration severely damaged the lamellar epithelium and pavement cells. Scanning electron microscopy provides useful information for evaluating the pathological and toxicological effects of chlorpyrifos in the fish.

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