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Evaluation of Acute Toxicity and Histopathological Impact of Diclofenac Exposure in Freshwater Fish *Channa punctatus*

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Abstract

Pharmaceuticals have emerged as priority pollutants of the environment all over the world in recent times. Diclofenac is a non-steroidal anti-inflammatory drug that has been usually detected in surface waters in the range of ng/l to µg/l. There is much experimental evidence of its toxicity in the aquatic flora and fauna. The objective of the present study is to investigate the acute toxic effect of the drug, Diclofenac in the freshwater fish, Channa punctatus. The fish were exposed to ten different concentrations of Diclofenac for 96 hours. The median lethal concentration value was evaluated by Probit analysis. The histological effect of Diclofenac was observed in the gill tissue on exposure to sub-lethal and median-lethal concentrations of diclofenac for a period of 96 hours. The fish exposed to sublethal concentrations of Diclofenac have shown marked pathological changes in the gills. Several cuts were also observed in secondary gill lamellae. There was a tendency for lamellar fusion and disorganised secondary gill filaments. The lamellae have turned into club shape which indicates the progressive degeneration of cells in the gill. The pathological changes observed in the fish exposed to lethal concentration are lamellar fusion, disruption of the epithelial layer, hyperplasia and cellular necrosis. There was bulging in the tip of primary gill filaments, distortion of the shape of secondary filaments, necrosis in pillar cell nucleus and vacuolization in the secondary gill epithelium. The findings of the study reveal that the drug Diclofenac has toxic potential and can alter the structural integrity of the tissues in the fish. Diclofenac in aquatic environments would cause negative impact on non-target organisms like fish.

Keywords: *Diclofenac, acute toxicity, enzymes, Channa punctatus*

1. Introduction

Pharmaceuticals are biologically active compounds that are designed to exert specific action on the target molecules of human and veterinary animals. The erroneous use of pharmaceuticals has resulted in the continuous discharge of pharmaceuticals and their metabolites into the environment. Pharmaceuticals enter the aquatic environment through different pathways and cause untoward effects on biota. Human excretion is the primary source of pharmaceuticals in the environment (Williams, 2005). The pharmaceuticals administered are incompletely absorbed and most of the parent drug or its metabolites are excreted through urine and feces. These chemicals enter the wastewater treatment plants and subsequently into surface waters because the waste treatment plants do not completely remove these chemicals (Zhang *et al*., 2008).

The improper disposal of unused medication also contributes to aquatic pollution. The unused drugs are directly disposed of into the domestic sewage system (Bound and Voulvoulis, 2005). The use of sewage sludge

on land causes pharmaceuticals to leach into surface and ground waters. The veterinary drugs also enter into the water through animal excretions. The antibiotics and hormones used in aquaculture practices also cause pharmaceutical pollution. The hospital wastes and effluents from manufacturing industries are released into the waters and contaminate the waters (Larsson *et al*., 2007).

Most pharmaceuticals are designed to target specific metabolic pathways in humans and animals. As most of the drug targets are evolutionarily conserved across different phyla, they may show an impact on non-target organisms like invertebrates and lower vertebrates. Pharmaceutical residues are highly potent and as they are continuously released, even low-level exposure may lead to chronic effects on a diverse range of organisms. The adverse effects caused by pharmaceutical compounds include acute toxicity, behavioural abnormalities, physiological changes, development of resistance in pathogenic bacteria, genotoxicity and endocrine disruption.

Pharmaceuticals comprise a wide spectrum of therapeutic classes which are used for the treatment of various disorders in humans. Diclofenac IUPAC name is 2(2,6- dichloroaniline) phenylacetic acid. Its generic name is Diclofenac Sodium, Diclofenac Potassium and Diclofenac Epolamine. Diclofenac is the widely prescribed non-steroidal anti-inflammatory drug for treating both acute and chronic pain in various disorders like rheumatoid arthritis, osteoarthritis, spondylitis, ocular inflammation, gout and dysmenorrhea (Skoutakis *et al*., 1988). It is available in the form of tablets, capsules, suppositories, intravenous solutions and injections. It is usually supplied in the form of either sodium or potassium salt.

The non-steroidal anti-inflammatory drug, Diclofenac was found to induce many potential toxic effects in aquatic flora and fauna. Diclofenac was found to inhibit the growth of bacteria, fungi and algae (Paje *et al*., 2002) and reduce the bioluminescence in bacteria (Farre *et al*., 2001). Carlsson *et al*., (2006) have compiled the acute toxic effects of pharmaceuticals including diclofenac and concluded that diclofenac is potentially dangerous to the environment.

Fish are sensitive to many toxicants and are the best bioindicators for assessing the environmental risk caused due to their pollution (Chovanec, 2003). *Channa punctatus*, an omnivorous freshwater fish, has been selected as the animal model for the toxicological evaluation of Diclofenac. Several characteristics of *Channa punctatus* such as its wide distribution in the freshwater environment, availability throughout the year, easy acclimatization to the laboratory conditions and commercial importance make this species an excellent test animal for evaluation of toxicity.

The available literature has revealed that diclofenac could cause potential adverse effects on non-target organisms and bioaccumulate in fish. The subchronic and chronic studies have reported the toxicity of diclofenac in aquatic flora and fauna. However, the acute toxicity data in fish is very scarce. There are no reports on acute exposure of diclofenac on biochemical, molecular and histopathological parameters in fish. Therefore, histopathological parameters have been taken up to find out the acute toxicity of diclofenac. The present study may contribute to the formulation of policy-making in reducing pharmaceutical contaminants in the aquatic environment.

2. Materials and Method

The freshwater fish, *Channa punctatus*, were collected from the waters of Hasanparthy village of Warangal district, Telangana, India. Analytical grade of Diclofenac sodium (2- [(2-6 Dichlorophenyl) amino] benzene acetic acid sodium salt, 99% pure (CAS 15307- 86-5) was purchased from Sara Exports, Ghaziabad, Uttar Pradesh, India. Diclofenac stock solution was prepared with acetone and ten different concentrations 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 30 ppm, 35 ppm, 40 ppm, 45 ppm and 50 ppm were prepared from the stock solution.

2.1 Experimental Procedures for Acclimatization and Test

The experiments were performed according to the standard methods to determine the LC50 of *Channa punctatus*. The healthy fish weighing about 100-110g and 20±1.21cm in length were transported to the laboratory in large plastic tanks and filled with water. The fish were washed in 1% potassium permanganate to free them from microbial infections. The fish were acclimatized in 50-litre capacity plastic tubs filled with dechlorinated water before experimentation. The fish were fed ad libitum with commercial feed rice bran and oil cake twice a day. Proper aeration was provided with the help of aerators. The fish were maintained in tanks under a 12:12 hour light: dark period. The dead fish were removed immediately to keep the water afresh. During the acclimatization and test period, water was renewed every 12 hours followed by the addition of the desired concentration of the test compound. The fish were starved one day before experimentation.

2.2 Evaluation of Median Lethal Concentration

The concentration of the toxicant at which 50 per cent of the test animals die during a specific period of time is referred to as Median lethal concentration (LC50) or Lethal concentration. A group of 10 healthy fishes were exposed to 10 different concentrations of the drug Diclofenac to calculate the LC50 value. One set of fish was maintained as control and was kept in tap water. The level of dissolved oxygen, pH, alkalinity, hardness and other parameters were monitored and maintained constantly. The mortality of fish was recorded every 24 hours during the exposure period in control and ten different concentrations of Diclofenac. The whole experiment was carried out six times with each concentration and control. The median lethal concentration (LC50) value was calculated after 96 hours using the probit analysis method (Finney, 1971).

The calculation was done by probit analysis using Microsoft Excel Windows 10. The per cent mortality, their logarithm values and probit values were incorporated into Excel sheets. Regression analysis was done with the help of Windows 10.

Acute toxicity is usually studied by exposing fish to a chemical for 96 hours as it is the standard duration. The literature also defines the acute toxicity of diclofenac at 96 hours of exposure. Therefore, the fish were exposed for 96 hours to determine the acute toxicity of diclofenac.

2.3 Histopathological examination

The histopathological studies were performed using the standard methods. (Humason, 1972). The freshwater fish, *Channa punctatus,* were exposed for 96 hours to sublethal and lethal concentrations of Diclofenac. At the end of the exposure period, fish were randomly selected for histopathological examination. The live fish was sacrificed and the tissues from the brain were collected. The tissues were stored in Bouin's fixative medium for 24 hours to immobilize the structure of the cell while maintaining morphological identity. After fixation, the tissues were washed under tap water to remove traces of picric acid as it hinders the staining processes. Later the tissues were dehydrated to remove water from the tissues. The gradual removal of water from the tissues is done by using increasing concentrations of alcohol overnight gradually. They were dehydrated with 30%, 50%, 70%, 90% and 100% absolute alcohol for one hour in each concentration as it prevents the putrefaction of tissues. Later the tissues were impregnated with paraffin wax to make it firm for section cutting. Before this clearing of the tissues is done by clearing agent xylene. Later the tissues were immersed in xylene for ten minutes. Two changes of xylene were given at a ten-minute time interval. Later, the tissues were kept for cold infiltration in a mixture of xylene and wax for two hours. Xylene brings about the infiltration of paraffin into the tissues and makes the tissue transparent. For embedding, the tissues were soaked

in paraffin wax melted at 57°C. All the tissues were given three to four changes for better impregnation. After the tissues were embedded with wax they were cast into blocks of paraffin. The tissues were fixed as blocks and were prepared, trimmed and kept overnight. The block was coated with lubricating gel to prevent the wax block from sticking. The blocks were left overnight in cold water to ensure that the wax had completely solidified. The wax block should be trimmed with a razor blade. The sides were cut to leave about 2-3 mm of wax around the tissue. The tissue sectioning was done with a rotary microtome, and serial sections were cut with an average of 7µ of thickness. The sections were deparaffinized in xylene and brought down to the water via alcohol grades. The staining was done with Hematoxylin Eosin. They were washed under running tap water for fifteen minutes. They were dehydrated via graded alcohol to 90%. They were counterstained in 0.49% eosin in 90% alcohol. The slides were rinsed in two changes of absolute alcohol. They were cleared in two changes of xylene of half an hour each and were mounted in DPX.

3. Results and Discussion

3.1 Acute Toxicity:

The results of the mortality of *Channa punctatus* on exposure to ten different concentrations of Diclofenac are presented in Table 1 and graphically represented in Fig 1.

Table: 1 Mortality of *Channa punctatus* on exposure to different concentrations of Diclofenac for 96 hours

S.No.	Concentration of Diclofenac	Log Concentration	No. of fishes exposed	No. of fishes died at 96 hr	Probit Kill	Percent Kill
1	5 ppm	0.698	10	0	0	0
2	10 ppm	1	10	1	3.72	10
3	15 ppm	1.176	10	1	3.72	10
4	20ppm	1.301	10	2	4.16	20
5	25ppm	1.397	10	4	4.75	40

6	30ppm	1.477	10	5	5	50
7	35ppm	1.544	10	6	5.25	60
8	40ppm	1.602	10	8	5.84	80
9	45ppm	1.653	10	9	6.28	90
10	50ppm	1.698	10	10	7.33	100

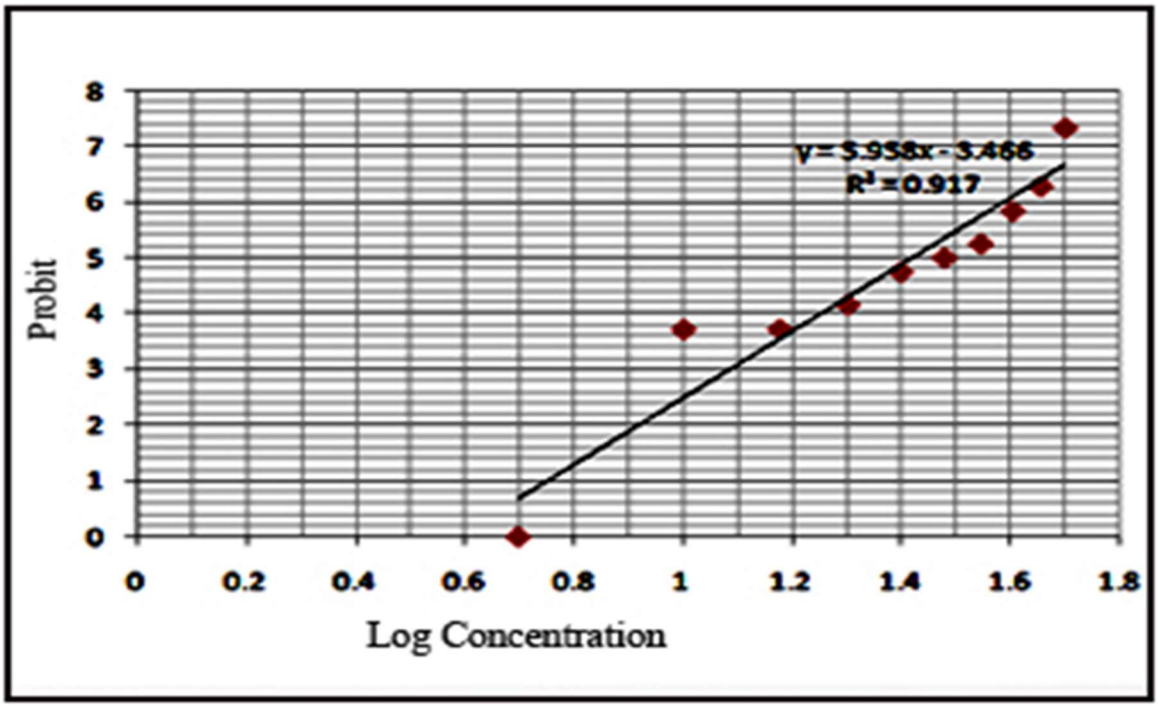


Fig:1 Mortality of *Channa punctatus* on exposure to different concentrations of Diclofenac expressed through Probit kill and log concentration

There was no mortality in a 5ppm concentration of Diclofenac. There was 10 % mortality in both 10ppm and 15ppm concentrations of diclofenac. 20% mortality was observed in 20 ppm and 40% mortality in 25 ppm concentration. Fifty per cent mortality was noticed in 30 ppm and 60% mortality in 35 ppm. There was 80% mortality in 40ppm, 90% mortality in 45ppm and 100 % in 50ppm of Diclofenac. The 96 hour LC_{50} value of diclofenac in *Channa punctatus* was found to be 25.28mg/L or 25.28ppm. One-third of the median lethal concentration, 8.42 mg/L or 8.42ppm was taken as sublethal concentration for further evaluation.

The present study has shown a positive relationship between mortality and level of concentration, as the concentration has increased, the rate of mortality also has increased. The same has been evidenced in various studies.

The acute toxicity values of diclofenac for different fishes were reported by earlier workers. Ajima *et al*., (2015) have studied the acute toxicity of Diclofenac in *Clarius gariepinus* and 96 hrs. LC_{50} value was found to be 25.12 mg/L. Praskova *et al*., (2011) have studied acute toxicity in both juvenile and embryonic stages of *Danio rerio* and the LC_{50} mean values of diclofenac were found to be 166.6 ± 9.8 mg/L and 6.11 ± 2.48 mg/L respectively.

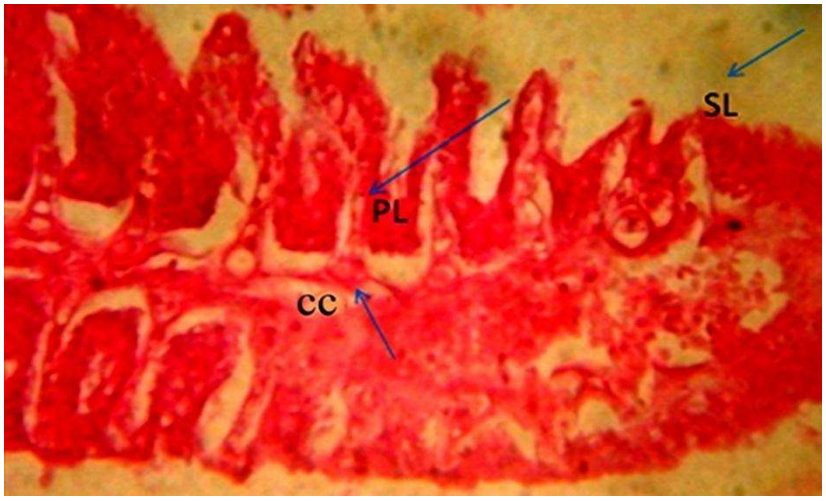
The acute toxicity study has indicated that the rate of mortality for the fixed time increases with an increase in concentration and for a particular concentration with an increase in exposure time (Nilkhant and Sawant, 1993). The death of fish in higher concentrations of diclofenac may be due to hypoxemia or due to impaired oxygen uptake by the gills. While in lower concentrations, the slow intrusion of the drug might have primarily induced alterations in physiology and ultimately led to death. Das and Sahu (2005) have reported that the major cause of mortality might be due to the damage of the respiratory epithelium by oxygen consumption during the formation of a mucus covering over the gills of fish.

3.2 Histological changes in gill:

Gill is an important organ of fish as it performs multiple functions. It participates in respiration, osmoregulation, excretion and homeostasis. Gills are the most sensitive organs to environmental contamination due to various reasons. The fish's gill is separated from the external environment by only a few microns of delicate gill epithelium. Gill has a large surface which facilitates greater absorption of the toxicant. The detoxification system of the gill is also less efficient than that of the liver (Mallatt, 1985). Moreover, absorption of toxic chemicals through gills is rapid and toxic response occurs instantly through gills. Hence, gill is employed as a biomarker in most toxicological studies.

Channa punctatus has four pairs of gills situated in the gill chambers. They are covered externally by the operculum and accessory respiratory organs. The wall of the pharynx is perforated by five-gill slits on each side and separated by four-gill arches. The primary gill lamellae are flat leaf-like structures with a central rod-like supporting axis. A row of secondary gill lamellae is situated laterally on either side of the interbranchial septum. The primary gill lamellae have blood vessels on either side of the supporting axis. The surface of secondary gill lamellae is covered with simple squamous epithelial cells and mucous cells. Numerous blood vessels are extended into each of the secondary gill filaments. There is an interlamellar region between the two adjacent secondary gill lamellae.

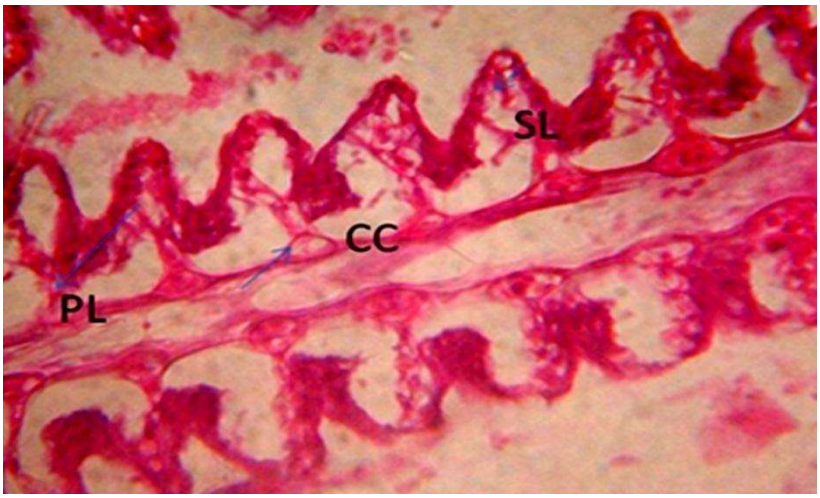
The gills of control fish have shown a typical structural organisation of the gill. The primary gill lamellae and secondary gill lamellae were intact. The blood vessels and epithelial cells were normal in control fish (Fig:2).



PL- Primary lamellae, SL- Secondary lamellae, CC- Chloride cell,

Fig: 2. Photomicrograph of gill tissue of *Channa punctatus* in control

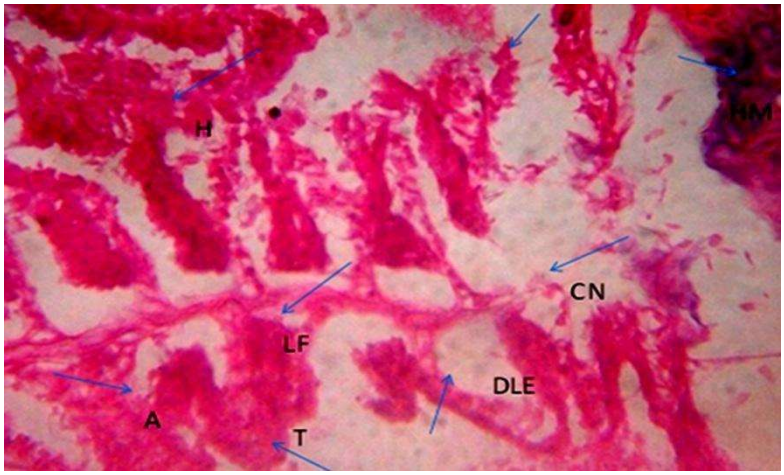
The fish exposed to sublethal concentrations of Diclofenac has shown marked pathological changes in the gills (Fig. 3). Several cuts were also observed in secondary gill lamellae. There was a tendency for lamellar fusion and disorganised secondary gill filaments. The lamellae have turned into a club shape which indicates the progressive degeneration of cells in the gill.



SL- Secondary lamellae, CC-Chloride cell, PL- Primary lamellae,

Fig: 3. Photomicrograph of gill tissue after exposure to sublethal concentration of Diclofenac.

The pathological changes observed in the fish exposed to lethal concentration are lamellar fusion, disruption of the epithelial layer, hyperplasia and cellular necrosis. There was bulging in the tip of primary gill filaments, distortion of the shape of secondary filaments, necrosis in the pillar cell nucleus, aneurysm and vacuolization in the secondary gill epithelium (Fig:4).



H- Hyperplasia, LF- Lamellar fusion, DLE- Dissociation of lamellar epithelium, A- Aneurysm, H - Haemorrhage, CN- Cellular necrosis

Fig: 4 Photomicrograph of gill tissue of *Channa punctatus* after exposure to a lethal concentration of Diclofenac.

The results of the present study are similar to some of the earlier investigations done on fish with diclofenac. Schwaiger *et al*., (2004) have reported necrosis of pillar cells leading to damage of the capillary wall within the secondary lamellae on exposure to diclofenac in rainbow trout. Triebskorn *et al*., (2004) have noticed pillar cell necrosis, hypertrophy of chloride cells and lifting of epithelium in the secondary lamellae of rainbow trout (*Oncorhynchus mykiss*) on exposure to 1µg/L, 5µg/L, 20µg/L, 100µg/L and 500µg/L of diclofenac. Hoeger *et al*., (2005) have observed telangiectasis in the gills of Brown trout (*Salmo trutta* f. fario), after exposure to 0.5µg/L, 5µg/L and 50µg/L of diclofenac for 21 days, while long term exposure for 10, 20 and 30 days has shown congestion with infiltration by the chronic inflammatory cellular exudates and collapsed secondary lamellae. There were no histopathological alterations in the gill of Zebrafish *(Danio rerio*) after subchronic exposure to sublethal doses of diclofenac (Praskova *et al.,* (2014).

Vijaya Lakshmi and Tilak (1996) have reported epithelial proliferation, congestion of blood vessels and hyperplasia on exposure to organophosphate pesticide monocrotophos. The results are in parallel with the works of Kapila and Ragothaman (1999) who have reported that *Boleophthalmus dumieric* exposed to sublethal concentrations of cadmium. Jiraungkoorskul *et al*., (2002) have noticed filament cell proliferation, lamellar cell hyperplasia, lamellar fusion, epithelial lifting and aneurysm in the Nile tilapia, *Oreochromis niloticus* after exposure to glyphosate for 96 hours. Tilak *et al*., (2005a) have reported dropsy, vascular degeneration, cloudy swelling and necrosis in epithelial and pillar cells of the gills upon chlorpyrifos intoxication in *Catla catla*.

Butchiram *et al.*, (2009) have found histopathological changes in the gill-like necrosis, vacuolar degeneration, fusion and atrophy of primary and secondary gill lamellae in *Channa punctatus* after exposure to sublethal concentration of a chloroacetanilide herbicide alachlor technical grade and lasso 50% EC for 10 days. Patnaik *et al*., (2011) have reported shrinkage and fusion at the lamella suggesting that lead intake mostly occurs via the gills, while cadmium-treated gill has shown marked hyperplasia of the branchial arch, pilaster cell vacuolization and congestion of blood vessels. Siva Kumar *et al*., (2015) have reported disruption of the epithelial layer, disintegration and fusion of primary lamellae, marked hyperplasia of the branchial arch and vacuolization in the gill on exposure to tannery effluent.

It is evident from earlier studies and the present study that Diclofenac induces pathological changes in the gill of exposed fish. The alterations like hyperplasia, hypertrophy, epithelial lifting and fusion of the adjacent secondary lamellae in the diclofenac-exposed fish may be defence mechanisms of the gills which serve as a barrier to the entrance of toxicants. These histopathological changes may lead to a great disturbance of gas exchange and ion regulation. The toxicant exposure causes a reduction in the supply of oxygen-enriched water to lamellar tissues which ultimately leads to curtailment in the performance capacity of fish (Caldwell, 1997). The lamellar epithelial lining reacts with toxicants creating tissue osmoregulatory imbalance. Bradbury and Coats (1989) have reported that histological damage to gill surfaces may be attributed to high accumulation of pesticides in gills, irritation due to elevated mucus secretion, increased ventilation volume and decreased gill oxygen uptake efficiency.

The rupture of pillar cells and capillaries due to pollutants may lead to an accumulation of erythrocytes in the distant portion of the secondary lamellae resulting in lamellar telangiectasis. The collapse of the pillar cell system and breakdown of the vascular integrity cause a change in the appearance of the secondary lamellae and release large quantities of blood that push the lamellar epithelium outward. Epithelial edema increases the distance between the contaminant and the bloodstream while secondary lamellar fusion reduces the gill surface thereby decreasing the contact between the pollutant and gill epithelium.

The pathological changes observed may be a response of the gills to toxicant intake or an adaptive response to pollutants and also may be due to increased capillary permeability (Olurin *et al*., 2006). The alterations in the gill like lifting of epithelium, hyperplasia hypertrophy of the epithelial cells and fusion of some secondary lamellae are the defence mechanisms to combat toxic stress.

Edema along with the lifting of lamellar epithelium serves as a mechanism of defense. The separation of the epithelial surface of lamellae increases the distance across which waterborne pollutants diffuse to reach the bloodstream (Arellano *et al*., 1999). Lamellar fusion and clubbing of lamellae could diminish the vulnerable surface area of the gill (Ortiz *et al*., 2003). The fusion of secondary lamellae leads to a great disturbance of gas exchange and ionic regulation (Eckert *et al*., 1990). It also reduces the total available respiratory surface area of the gill resulting in decreased oxygen uptake (Adhikari *et al*., 1998). The dilation of lamellar blood vessels and the presence of edematous fluid in the secondary lamellae may be due to increased permeability induced by prolonged exposure to the metals (Balah *et al*., 1993).

Hyperplasia would decrease the surface area of the gill available for oxygen consumption and also would increase the distance between water and blood. Hyperplasia hinders the respiratory, excretory and secretory function of the gills (Kumaraguru *et al.,* 1982). Miron *et al*., (2008) have suggested that the lesions oedema and fusion of the secondary lamellae in gills may reduce gill functional surface for gaseous exchange and impair respiratory function.

4. Conclusion

The results of the present study have depicted that the drug Diclofenac alters the histological integrity of the gill tissue. The drug is potentially harmful to non-target organisms like fish. This results in the deterioration of the health status of fish and may lead to an imbalance in the ecosystem. Measures have to be taken to reduce pharmaceutical residues at different stages like manufacturing, consumption and waste management.

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