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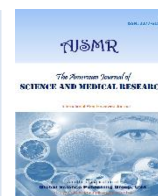

Research Article

Histological alterations in the liver tissue of *Channa punctatus* on exposure to the drug Diclofenac

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ABSTRACT

Pharmaceuticals have emerged as priority pollutants in recent times. Diclofenac is a widely prescribed non steroidal anti-inflammatory drug. It has been frequently detected in the surface waters worldwide. There are many experimental evidences on the toxicity of the drug Diclofenac in the aquatic flora and fauna. The present study aims to investigate the histological alterations caused by Diclofenac in the liver tissue of the fresh water fish, *Channa punctatus*. The acute toxicity test was conducted by exposing the fish to ten different concentrations of Diclofenac. The median lethal concentration was found to be 25.28mg/L by probit analysis. The fish were then exposed to sub-lethal (8.42mg/L) and median lethal (25.28 mg/L) concentrations of Diclofenac for a period of 96 hours. The histological alterations in liver were studied by adopting standard method. The exposed fish have shown marked pathological changes in the liver tissue when compared to control. The pathological changes noticed include degeneration of cytotoplasm in hepatocytes, severe necrosis, atrophy, vacuolization, rupture in blood vessels, disappearance of hepatocyte wall, disposition of hepatic cords, appearance of blood streaks, syncytial appearance of hepatocytes, increase of sinusoidal space and evacuation of portal systems. The findings of the study have clearly shown the toxic effect of the drug Diclofnac in the non target organisms like fish.

1. Introduction

Pharmaceuticals are the 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 continuous discharge of pharmaceuticals and their metabolites into the environment. Pharmaceuticals enter into the aquatic environment through different pathways and cause untoward effects in biota. The primary source of pharmaceuticals in the environment is human excretion (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 into the wastewater treatment plants and subsequently into surface waters because these chemicals are not completely removed by the waste treatment plants (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 application of sewage sludge to land causes pharmaceuticals to leach into surface and ground waters. The veterinary drugs also enter into 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 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, behavioral 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 is sensitive to many toxicants and is the best bioindicator 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 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 makes 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 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 Methods

The fresh water 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 LC_{50} 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 from microbial infections. The fishes were acclimatized in 50 liters capacity plastic tubs filled with dechlorinated water prior to experimentation. The fishes 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 12:12 hour light : dark period. The dead fish were removed immediately to keep the water afresh. During acclimatization and test period, water was renewed for every 12 hours followed by the addition of 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 percent of the test animals die during a specific period of time is referred to as Median lethal concentration (LC_{50}) or Lethal concentration. A group of 10 healthy fishes were exposed to ten different concentrations of the drug Diclofenac to calculate LC_{50} value. One set of fishes are maintained as control and were 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 for every 24 hrs. during 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 (LC_{50}) value was calculated after 96 hour using probit analysis method (Finney, 1971).

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

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

2.3 Histopathological examination

The histopathological studies were performed by taking 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 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 a period of 24 hours in order to immobilize the structure of the cell while maintaining morphological identity. After fixation the tissues were washed under tap water in order 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 the purpose of section cutting. Prior to 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 minutes time interval.

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

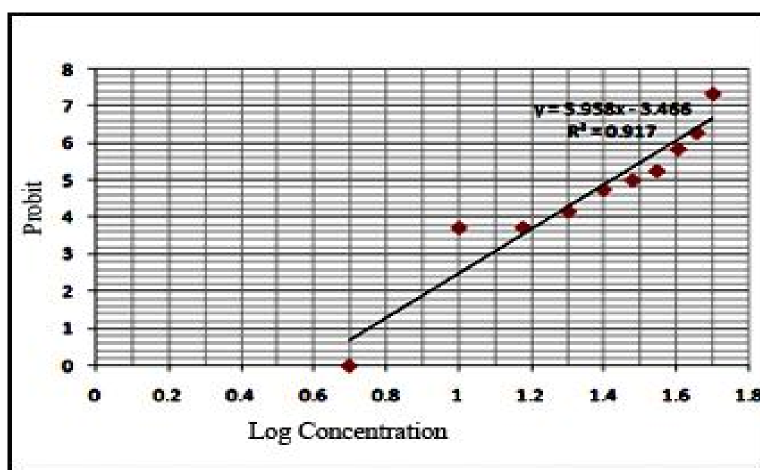

Figure-1. Mortality of *Channa punctatus* on exposure to different concentrations of Diclofenac expressed through Probit kill and Log concentration

Later, the tissues were kept for cold infiltration in a mixture of xylene and wax for two hours. Xylene brings about infiltration of paraffin into the tissues and makes the tissue transparent. For embedding, the tissues were soaked in paraffin wax melted at 57ºC. 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 wax block 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 so as 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 7µ of thickness. The sections were deparaffinized in xylene and brought down to 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 Discussions

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

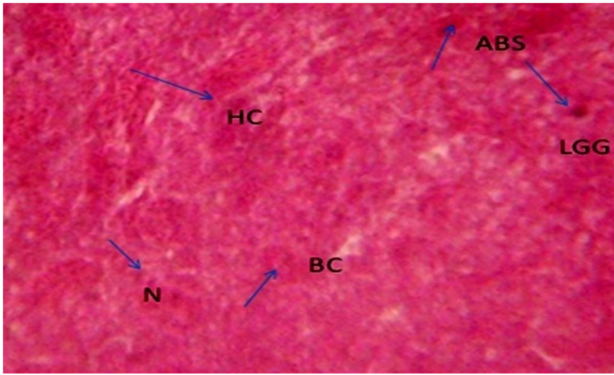

Figure-2. Photomicrograph of liver tissue of *Channa punctatus* in control group.
HC- Hepatic cell, BC – Bile canaliculi, LGG- Lipid and glycogen granules, N- Nucleus

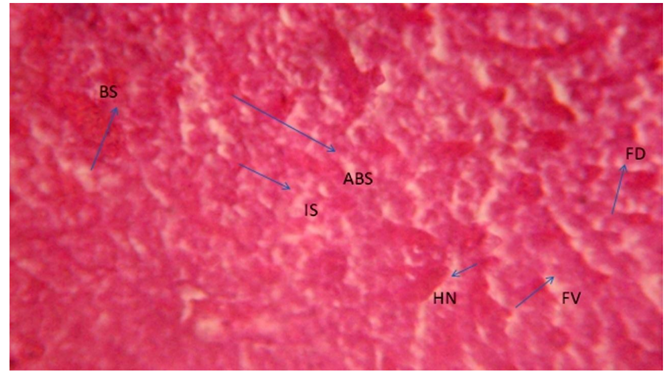

Figure-3. Photomicrograph of liver tissue of *Channa punctatus* after exposure to sublethal concentration of Diclofenac.
.BS- Blood streaks, HN- Hepatic necrosis, FV- Fatty vacuolization,
ABS- Appearance of blood streaks , IS – Irregular sinusoids

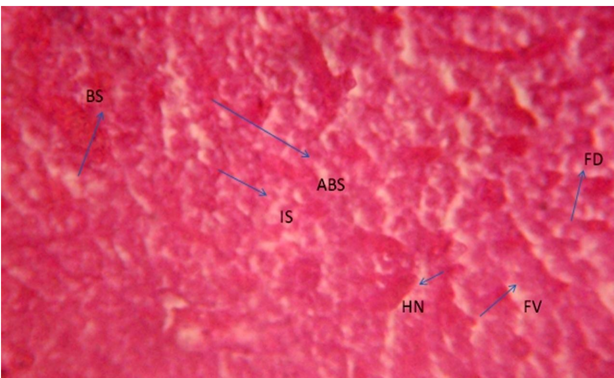

Figure-4. Photomicrograph of liver tissue after exposure to lethal concentration of Diclofenac.
FV- Formation of Vacuoles, ABS- Appearance of blood streaks among hepatocytes, FD- Degeneration of foci, HN- Hepatic Necrosis, IS- Irregular sinusoids

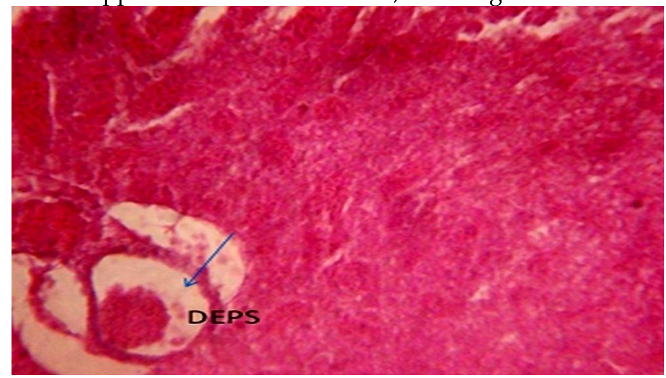

Fig: 3b. Photomicrograph of liver tissue of *Channa punctatus* after exposure to lethal concentration of Diclofenac. DEPS- Degeneration and evacuation of portal systems

There was no mortality in 5ppm concentration of Diclofenac. There was 10 % mortality in both 10ppm and 15ppm concentrations of diclofenac. 20% mortality was observed in 20ppm and 40% mortality in 25 ppm concentration. Fifty percent 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 the mortality and level of concentration, as the concentration has increased, the rate of mortality also has increased. The same has been evidenced from 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 clearly indicated that the rate of mortality for the fixed time increases with increase in concentration and for a particular concentration with 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 leading 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 consummation during the formation of a mucus covering over the gills of fish.

3.1 Histological alterations in liver

Liver is an important organ and plays a major role in the metabolism. It participates in detoxification, protein synthesis and secretion. It is easily susceptible to contamination as it can accumulate harmful components. Hence, the functional integrity of the liver can be affected by xenobiotics and is often used as an environmental biomarker in toxicity studies.

Liver is the largest gland in the body of fish. It is lined with serous membrane. The concentration of the toxicant at which 50 percent of the test animals die during a specific period of time is referred to as Median lethal concentration

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3.3 Histological changes in liver:

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Liver is the largest gland in the body of fish. It is lined with serous membrane and some connective tissue extends inward into the parenchyma. It consists of branching and anastomosing two cell thick laminae or cords of hepatocytes. Hepatic cells are polygonal with a central and distinct spherical nucleus, densely stained chromatin and a prominent nucleolus. The sinusoids are lined by distinct endothelium which are irregularly distributed between the polygonal hepatocytes. A fairly large quantities of lipid glycogen granules are present in the cytoplasm of hepatic cells of fish. Phagocytes are occasionally observed in the sinusoids. The sinusoidal lining of cells is fenestrated and overlie the Space of Disse which is the zone between sinusoid cells and hepatocytes (Fig:1).

The fish in the control group has shown typical structural organization of liver. The fish exposed to sublethal concentrations of Diclofenac has shown alterations like degeneration of cytoplasm in hepatocytes, atrophy, formation of vacuoles, rupture in blood vessels, necrosis and disappearance of hepatocyte wall and disposition of hepatic cords (Fig: 2).

The fish exposed to lethal concentration of Diclofenac has shown alteration in the structure of hepatocytes, appearance of blood streaks, degeneration of cytoplasm of hepatocytes, syncytial appearance of hepatocytes anincrease of sinusoidal space and evacuation of portal systems (Fig:3 a and Fig 3 b).

There is some literature available on diclofenac toxicity in liver of different fishes exposed for different duration. Hoeger *et al*., (2005) have observed increased monocyte infiltration in the liver of Brown trout (Salmo trutta f. fario) after exposure to 0.5 µg/L, 5 µg/L and 50 µg/L diclofenac for 21 days. Mehinto *et al*., (2010) have exposed juvenile rainbow trout to DCF (0.5 µg/L, 1 µg/L, 5 µg/L, 25 µg/L) for 21 days and has not observed any change in liver. Triebskorn *et al*., (2004) have observed collapse of the cellular compartmentation as well as the glycogen depletion of hepatocytes in the liver. Binu kumari *et al*., (2016) have reported symptoms of general necrosis, degeneration of hepatocytes, clumping of nucleus, fatty degeneration and cloudy swelling in liver after short term exposure upto 96 hours. The long term exposure for 10, 20, 30 days has shown karyolysis, rupture in blood vessels and portal triads unfiltered with chronic inflammatory cells. The degree of structural heterogeneity was enhanced with increasing concentration of the toxicant (Hawkes,1980).

The histological alterations in the liver is often associated with degenerative necrotic condition (Myers *et al.* 1987). Das and Mukherjee (2000) have reported swelling of the hepatocytes with diffuse necrosis and marked swelling of blood vessels in liver of *Labeo rohita* on exposure to hexachlorcylclohexane. Similar degenerating changes were observed in liver of both sexes of *Cyprinus carpio* after exposure to $HgCl_2$ at 0.1 ppm for 45 and 60 days (Masud *et al.,* 2001, 2003).

Degenerative and necrotic changes were also observed in liver of white bass (*Lates calcarifer)* exposed to cadmium (Thophon *et al.,* 2003). The irregular sinusoidal structures show congestion in blood vessels (Ozturk *et al*., 2005). Butchiram *et al*., (2009) have found degeneration of cytoplasm in hepatocytes, atrophy, formation of vacuoles, rupture in blood vessels and disposition of hepatic cords in *Channa punctatus* after exposure to sublethal concentration of chloroacetanilide herbicide alachlor technical grade and lasso 50% EC for a period of 10 days. Kumar and Nandan (2014) have reported degeneration of liver cells, congestion of central vein, clumped erythrocytes and haemorrhage in the exposed fishes. Udotong (2015) has noticed extensive hepatocyte necrosis with chronic inflammatory changes in the liver of fishes exposed to Copper solution, but, Lead and Iron have caused less damage in the liver of *Tilapia guineensis*.

Conflicting Interests

The authors have declared that no conflicting interests exist.

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