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Diclofenac Induced Genotoxicity in the Gill Tissue of *Channa Punctatus*

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ABSTRACT

Pharmaceuticals have emerged as priority pollutants in recent times. The enormous usage of these drugs has led to pollution of the aquatic systems all over the globe. Diclofenac is the most widely prescribed non-steroidal anti inflammatory drug. The drug Diclofenac has been detected in surface waters in the magnitude of ng/L to µg/L. There are many investigations on the toxicity of the drug Diclofenac in aquatic flora and fauna. This study aims to examine diclofenac induced genotoxicity in the gill tissue of *Channa punctatus*. The acute toxicity test was conducted by exposing the fish to ten different concentrations of diclofenac for 96 hours. The median lethal concentration was found to be 25.28 ppm. Genotoxicity was evaluated by exposing the fish to median lethal concentration (25.28 ppm) and sub lethal concentration (8.42 ppm) of Diclofenac. DNA damage was estimated by Comet assay (Singh *et al*., 1988). There was an increase in the percentage of tail DNA, tail length and tail moment in the gill cells of the exposed fish in both the set concentrations of Diclofenac. The present study clearly indicates that the drug Diclofenac damages DNA of the aquatic organisms like fish. This study also signifies that genotoxic studies are effective biomarkers in the assessment of pollution.

Key words: Diclofenac, genotoxicity, *Channa punctatus*, DNA, Gill

INTRODUCTION

Pharmaceutical residues have become prominent emerging pollutants in recent times. The increased consumption of these drugs has led to their discharge into the environment. A large number of pharmaceuticals both prescription and over the counter drugs have been detected in influents and effluents of wastewater, surface water, ground water and even in drinking water. They enter into water bodies through many routes and the foremost being excretion from treated patients either in the form of parent compound or its metabolites, direct release from manufacturing units, hospitals, disposal of unused drugs and leaching from terrestrial deposits.

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Though pharmaceuticals have been detected in traces from ng/L to µg/L they show adverse effects on aquatic life. There are several experimental evidences on the toxicity of different classes of pharmaceuticals. They are known to cause behavioral alterations, changes in biochemical constituents, genotoxicity, endocrine disruption in non-target organisms like fish and selection of antibiotic resistance in pathogenic microorganisms.

Diclofenac is the widely prescribed 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. It is sold with the common brand names as Voltaren, Pennsaid, Arthrotec, Flector, Solaraze etc,. Diclofenac has been detected in surface waters of rivers, lakes, seas, influents and effluents of wastewater treatment plants, groundwater, drinking water, soil and sediment worldwide (Nikolaou *et. al.,* 2007). It was detected in the magnitude of high ng/L to µg/L in many countries in the world including India.

Fish is the best bioindicator for assessing environmental risk caused due to various pollutants (Chavonec, 2003). *Channa punctatus* was selected as the test animal due to its wide distribution, availability throughout the year and easy maintenance in the laboratory.

Diclofenac was found to induce many potential toxic effects in aquatic flora and fauna. The subchronic and chronic studies have reported the toxicity of diclofenac in the aquatic organisms. However, the acute toxicity data in fish is very scarce. There are little evidences of genotoxicity in fish. Therefore, DNA damage has been taken as parameter to find out acute toxicity of diclofenac. Several investigations have shown that gill was the suitable tissue for conducting genotoxicological studies in fish. (Masud *et al*., 2003). Hence gill tissue has been taken up for study of DNA damage.

Materials and Methods

Warangal district, Telangana, India. 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 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 litres capacity plastic tubs filled with dechlorinated water prior to 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 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.

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 stock solution. The fish were exposed for 96 hours to ten different concentrations and median lethal concentration was analysed. The experiment was carried out for six times.

The molecular abnormalities were evaluated by exposing the healthy fish to sub lethal (8.42ppm) and median lethal concentrations (25.28ppm) of Diclofenac for 96 hours along with a control. The live fish were sacrificed after 96 hours and the tissues were isolated from gill to examine DNA damage.

The alkaline Single Cell Gel Electrophoresis or comet assay was performed by Singh *et al*. (1988) with minor modifications. Microscopic slides were precoated with 1% NMP agarose on pre cleaned and methanol treated dry slides. The 30 µL of cell suspension was gently mixed with 70 µL of 0.1% Low Melting Point Agarose (LMPA) and was layered on a frosted slide which was precoated with a layer of 1% 200 µL normal agarose. Then again, it was layered with LMPA (100 µL) and covered with a coverslip and kept for 5 minutes at 4°C. Then coverslip was removed and slides were kept submerged in freshly prepared, prechilled lysis buffer overnight at 4°C. The slides were positioned side by side in horizontal unit of gel electrophoresis, dipped in fresh cold alkaline electrophoresis buffer and left for 20 min. at 4°C in the same solution in order to unwind DNA and convert alkali-labile sites to single strand breaks. The same alkaline buffer was used for carrying out alkaline electrophoresis using 300 mA, 15 V (0.8 v/cm) for 20 min. at 4°C. Then the slides were gently neutralized using 0.4 M tris buffer (pH 7.5). The slides were stained with ethidium bromide and were inspected under an epifluorescent microscope. The microscopic images of comets were scored using Comet IV computer software. Fifty nuclei were analysed per slide.

The data obtained from the experiment was expressed as Mean±SE. The data was analysed by using one way analysis of variance (ANOVA) followed by Tukey pair wise multiple comparison test. The value of $p<0.05$ was considered statistically significant.

Results and Discussion

There was significant DNA damage in the gill tissue of fish exposed to both 8.42ppm and 25.28ppm concentrations of Diclofenac on comparison to control. The comet images of gill from control group have shown circular DNA without any tail formation. (Fig:1a). The results of DNA damage are expressed as percentage of DNA damage, tail length and olive tail moment. The results are shown in the Tables 1, 2 and 3 respectively.

Gill cells of fish exposed to 8.42ppm and 25.28ppm concentrations of diclofenac have shown significant increase in percentage of tail DNA, tail length and olive tail moment on comparison to control. Nuclear DNA with tail formation was observed in gill cells of the exposed fish. The percentage of DNA damage, tail length, and olive tail moment are given in the tables below and graphically represented.

Table: 1 Percentage of DNA damage in gill cells of *Channa punctatus* on exposure to Diclofenac

Diclofenac exposure	% of DNA Damage
Control	4.21 ± 2.4
8.42ppm	14.22 ± 0.8
25.28ppm	48.64 ± 1.6

DNA damage is the most serious consequence of aquatic pollution. DNA damage is primarily a kind of chemical damage which involves changes in molecular structure of DNA. It may be breakage in single strand or double strand of DNA, alkali-labile sites, single base alterations or two base alterations, intra strand cross linkages or inter strand cross linkages. DNA damage during replication process will lead to base mismatch or absence of base and some other related damages.

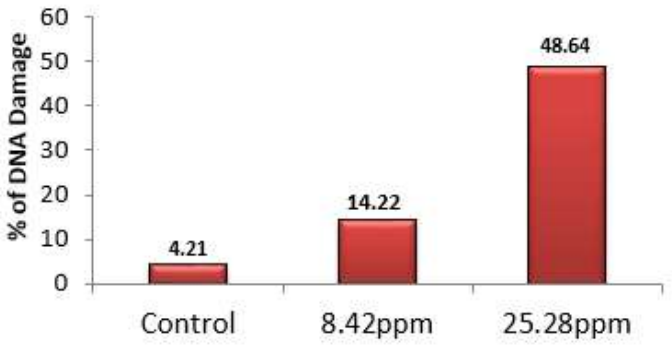

Figure-1. DNA damage in gill cells of *Channa punctatus* on exposure to 8.42 ppm and 25.28 ppm concentrations of Diclofenac against Control

Single cell gel electrophoresis or Comet assay is a versatile, sensitive and cost effective technique used to measure DNA damage and repair in individual cells (Nandakumar *et al*., 2011). The comet assay helps to measure single or double strand DNA breaks, alkali labile sites (apurinic/ apyrimidinic sites), DNA cross-links, base or base-pair damages and apoptotic nuclei in the cells.

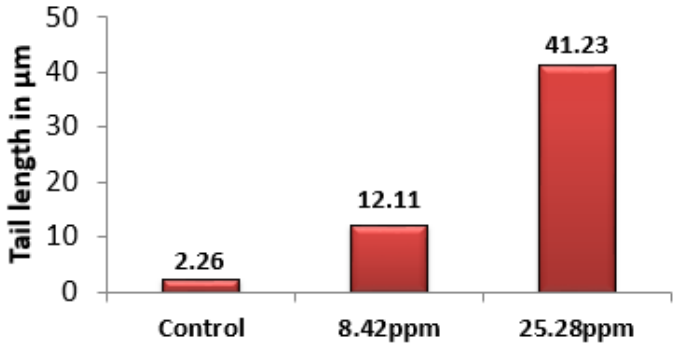

Figure-2. Tail length observed in µm in gill cells of Channa punctatus on exposure to Diclofenac

The three parameters of genotoxicity usually used to evaluate DNA are tail moment, tail length, and tail intensity. The shape, size and amount of DNA in the comet are crucial in the determination of the level of damage of DNA. The extent of DNA migration positively corresponds with the DNA damage present in the cells. It is quantified in terms of an increased amount of determined fluorescence in the tail region, as well as by tail length. The resulting comet like structure is quantified by measuring the length of the tail and or tail moment (the intensity of the migrated DNA multiplied by the respective tail length with respect to DNA (Akpoilih, 2012).

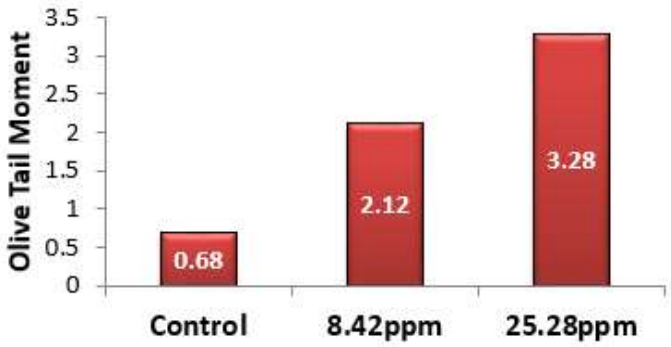

Figure-3. Olive Tail Moment observed in gill cells of *Channa punctatus* on exposure to Diclofenac

A few studies have reported DNA damage in different fish on exposure to Diclofenac. Pandey *et al*., (2017) have reported DNA damage in the liver of *Oreochromis niloticus* after 30 days of exposure to sub lethal doses of diclofenac. Ghelfi *et al*., (2016) have reported that there was no DNA damage in the liver, kidney and blood of *Rhamdia quelen* on exposure to 0.2 µg/L, 2 µg/L, and 20 µg/L concentrations of diclofenac for 96 hours.

Bolognesi *et al*., (1999) have reported a statistical increase of DNA damage in mussels on exposure to Cadmium and also an increase of DNA single strand breaks and micronuclei frequency on exposure to Copper and Mercury. Abbas and Ali (2007) have revealed that there was concentration dependent increase in percentage of DNA damage in liver and kidney cells on exposure to sub lethal concentrations of Cr (VI) in *Oreochromis species*. Sharma *et al*., (2007) have noticed DNA damage in gill, kidney, and erythrocytes of *Mystus vittatus* on exposure to sublethal and nonlethal concentrations of Endosulfan.

Goodale *et al*., (2008) have noticed DNA double strand breaks in medaka fin cell lines exposed to Cr (VI) and chromosome damage in a concentration dependent manner. Ali and Kumar (2008) have reported significant DNA damage in gill followed by kidney and lymphocyte cells in *Channa punctatus* on exposure to different sublethal and nonlethal concentrations of monocrotophos. Ali *et al*., (2009) have reported DNA damage in lymphocyte and gill cells of *Channa punctatus* exposed to chloropyrifos.

Jin *et al*., (2011) have studied the effects of cypermethrin exposure on the induction of hepatic oxidative stress and DNA damage in adult zebra fish and found that even low concentration of pesticide can cause heavy DNA damage and defects in gene expression. Pandey *et al*., (2011) have reported DNA damage in gill cells of *Channa punctatus* on exposure to profenofos. There was an increase in DNA damage in gill and liver on

exposure to Roundup, a glyphosate based herbicide in *Anguilla anguilla* (Guilherme *et al*., 2012).

Kumar *et al*., (2013) have reported the genotoxic potential of Arsenic at different exposure concentrations in *Channa punctatus* and *Carassius auratus*. Ismail *et al*., (2014) have noticed more DNA damage in gill than blood in *Labeo rohita* on exposure to chlorpyriphos. Kousar and Javed (2014) have reported higher percentage of DNA damage in *Cirrhinus mrigala* on exposure to Arsenic, Copper, Zinc and their mixtures in peripheral blood erythrocytes. Arsenic has induced DNA damage in terms of percentage of damaged cells, genetic damage index and cumulative tail length of comets in peripheral blood erythrocytes of *Labeo rohita, Cirrhinus mrigala, Catla catla and Ctenopharyngodon idella* (Kousar and Javed, 2015).

Ullah *et al*., (2016) have noticed DNA damage in the gill tissue of *Labeo rohita* on exposure to Malathion and revealed that there was a linear relation between exposure and DNA damage. Cypermethrin exposure has induced DNA damage in erythrocytes of *Labeo rohita* (Gadhia *et al*., 2016). Sharma and Chada (2017) have reported DNA damage in *Channa punctatus* after subchronic exposure to 4- Nonylphenol. Vieira *et al*., (2018) and Ashwini Ravichandra Jatap (2013) have reported DNA damage in liver, kidney and gills of *Prochilodus lineatus* exposed to imidacloprid.

The decrease of DNA contents in kidney may be due to reduction or absence of the essential factors controlling DNA synthesis which are the substrates (4-Deoxyribonucleoside triphosphates), enzymes (polymerase), template activity of deoxyribonucleic-protein and activators like Mg^{2+} and other divalent ions (Jaya and Shettu, 2015). Velma and Tchounwou (2010) have opined that DNA damage at higher test concentrations in the liver and kidney could be due to elevated levels of hydroperoxides compared to control. Induction of ROS under metallic stress could attack DNA and damage its integrity. The oxidative damage due to reactive oxygen species causes DNA strand breakage.

The higher DNA damage in gills might be due to the fact that the gills are constantly, directly and continuously exposed to the toxicant (Pandey *et al*., 2006). There are several reports on various species of fish which reported higher sensitivity of gills to DNA damage than other cells including lymphocytes, erythrocytes, liver and kidney (Ali *et al*., 2009). The toxicants cause DNA damage in cells through different mode of action such as cellular transformation, gene amplification, breaking DNA protein crosslinks and rupturing of DNA strand (Ullah *et al*., 2016a).

The biotransformation of xenobiotic leads to an increase in the production of Reactive Oxygen Species (ROS) which is highly toxic to fish. ROS can directly break DNA through OH and H_2O_2 resulting in oxidized bases of DNA (Akcha *et al*., 2004). Fish have an antioxidant defence system against ROS but when higher production of ROS surpasses the defence systems of fish, cellular lesions and DNA damage occur (Jha, 2008).

Oxidative DNA damage due to production of ROS is attributing for variable and higher DNA damage in the cells of the gills (Pavlica *et al*., 2001). Sureda *et al*., (2006) have established that there is a close correlation between oxidative stress and DNA damage. Pollution induced ROS can provoke oxidative damage of DNA including strand breaks and base and nucleotide modifications, particularly in sequences with high guanosine content (Viarengo *et al*.,1990).

Conclusion

The present study has clearly indicated that the drug diclofenac would cause DNA damage in the aquatic organisms like fish. The pharmaceuticals should be rationally used and properly disposed before releasing into the aquatic environment at different stages like manufacturing, consumption and waste management.

Conflicts of Interest

Authors declare that there is no conflict of interests regarding the publication of this paper.

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