Effects of waterborne Diclofenac on guppy (*Poecilia reticulata*): behavioural, histopathological, developmental and haematological approach under chronic laboratory exposure

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Abstract *- Environmental presence of diclofenac, an anti-inflammatory drug, may pose threats to non-target organisms. Present study investigated the effects of chronic exposure of waterborne diclofenac on laboratory reared guppy (Poecilia reticulata) at two life history stages; juveniles (experiment I) and fries (experiment II). Both experiments consisted of one control group and three diclofenac exposed groups with concentrations of 1 µg/L, 10 µg/L, and 100 µg/L. Experiment I and II were conducted for 28 and 14 days respectively. Data were collected in the areas of behavioural endpoints (food detection time, physical avoidance response and ventilation rate), erythrocyte nuclear abnormalities (ENA) and gill histology in the experiment I, while it was done on predator avoidance response, osteology, and RNA/DNA ratio in the experiment II. For the behavioural endpoints there was no significant difference among groups (p>0.05) in juveniles. Diclofenac caused significant induction of ENA types except bi-nucleated cells and micronuclei compared to control group. Also, diclofenac exposed juveniles developed histopathological lesions in gills as inter-lamellar hyperplasia and dilated secondary lamellae. Fries exposed to diclofenac showed significantly lower (p<0.05) predator avoidance response than control group. Also, diclofenac caused alteration of the skeletal structure of guppy fries. There was no significant effect on the RNA/DNA ratio of fries. The present study revealed that 28-day exposure to waterborne diclofenac caused alterations in erythrocyte nuclear morphology and gill histology of juvenile guppy. The behavior was unaffected. Further, 14-day exposure of guppy fries to diclofenac caused alterations in predator avoidance behavior and osteology, but no significant effect on RNA/DNA ratio. Results collectively show that diclofenac in water is detrimental to developing guppy fries.*

Keywords - guppy fry, behavioural changes, ENA, pharmaceuticals

I. INTRODUCTION

Pharmaceuticals are considered as emerging contaminants in the environment and they are comprehensively and exceedingly being used in medical treatments of human. Also pharmaceuticals are used in veterinary medicine (Fent *et al*., 2006). Environmental presence of pollutants like pharmaceuticals poses serious threats to non-target organisms including fish (Kermiche *et al*., 2016). Pharmaceuticals are biologically active substances and affect control mechanisms in living organisms such as by regulating metabolism (Patel and Sen, 2013), influencing hormonal balance (Kidd *et al*., 2007) or alleviating signal transmission between cells (Aggarwal *et al*., 2007). But when these compounds are released into the environment, the biological activity of them causes adverse effects on non-target organisms (Weber *et al*., 2014). Diclofenac is a widely used non-steroidal anti-inflammatory drug (NSAID). It was introduced in 1973 (Altman *et al*., 2015). This mainly acts as inhibitor of the cyclooxygenase (COX) responsible for the synthesis of prostaglandins (De Felice *et al*., 2012). People use diclofenac to relieve pain and inflammation caused by diseases like arthritis, gout, dysmenstruation and menorrhagia (Chan *et al*., 2001). Diclofenac, is a known environmental pharmaceutical released by people (Tixier *et al*., 2003). Diclofenac which present in aquatic ecosystem have direct negative impact on organisms (Ewellina, 2014). Among them fish take important place. As an aquatic organism they are in direct contact with waterborne diclofenac. Therefore they are directly

impacted by these pollutants (Roberts, 1989). Present study investigated the effects of chronic exposure of waterborne diclofenac on laboratory reared guppy fish (*Poecilia reticulata*) in relation to behaviour, development and RNA:DNA ratio of guppy fries, and behavior, erythrocyte nuclear abnormalities and gill histopathology of guppy juveniles. No previous research using controlled laboratory experiments of diclofenac exposure on guppy are found, and therefore, the present study is designed to assess the effects under several related endpoints using guppy as the test organism.

II. METHODOLOGY AND EXPERIMENTAL DESIGN

The study comprised of two experiments as experiment I and experiment II. Experiment I was done with guppy juveniles as the test organism investigating behavioural endpoints, erythrocyte nuclear abnormalities, and gill histopathology upon waterborne diclofenac exposure. Similarly, against the same toxicant, the experiment II involved guppy fries (24-36 hours post hatch) and aimed at following behavioural endpoints and developmental markers (osteology and RNA:DNA ratio). Both experiments consisted of one control group (C) and three groups with dissolved diclofenac as 1 µg/L (T_1), 10 µg/L (T_2) and 100 µg/L (T_3) in three replicates (Plate 1 and Plate 2). Experiment I (n= 10 juveniles per tank) was conducted for 28 days and experiment II (n= 7 fries 24-36 hours post hatch) was conducted for 14 days with static renewal every fifth day. Feeding regime and aeration were similarly maintained in all groups, with regular monitoring of water quality. Data were obtained under three behavioural endpoints (food detection time, physical avoidance response and ventilation rate), erythrocyte nuclear abnormalities (ENA), and gill histology in the experiment I, while it was done on predator avoidance response, osteology, and RNA:DNA ratio as developmental markers in the experiment II.

Plate 01: Experimental setup with guppy juveniles

Plate 02: Experimental setup with guppy fries

In the statistical analysis, all quantitative data were analyzed by using the software STATISTICA, Version 10 (StatSoft, USA). Initially data were tested for normality. When the data were normally distributed further analysis were done by using Parametric ANOVA test. When the data were not normally distributed further analysis were done by using Non-parametric Kruskal-Wallis test. For the group comparisons, simple pairwise comparison and multiple comparison mean ranks tests were used. For the all statistical analysis significance levels were set to $p<0.05$.

III. RESULTS

In experiment I, The data obtained from behavioural tests and erythrocyte nuclear abnormalities of guppy juveniles were statistically analysed to find out whether there are significant effects caused by the long term exposure (28 days) to waterborne diclofenac compared to the control groups. However, the gill histology results were shown only descriptively. For the behavioural endpoints there was no significant difference among groups for any parameters ($p>0.05$) in juveniles.

Chronic exposure of waterborne diclofenac caused significant induction of all ENA types (notched nuclei, lobed nuclei and nuclear buds) except bi-nucleated cells and micronuclei compared to control group (Plate 3).

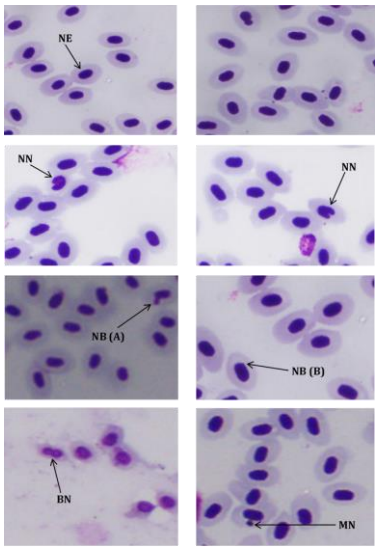

Plate 03: Images showing different types of erythrocyte nuclear abnormalities found in fish under diclofenac exposure. (NE: normal erythrocytes, LN: lobed nuclei, NN: notched nuclei, NB (A): cell with nuclear bud (Type A - well developed nuclear bud), NB (B): cell with nuclear bud (Type B – undeveloped nuclear bud), BN: bi-nucleated cell and MN: micronuclei)

For the ENA test, nine fish juveniles were selected from each group (n=9). Then the frequencies of each type of erythrocyte nuclear abnormalities were calculated (per 1000 RBC's). Descriptive statistics of the data on erythrocyte nuclear abnormalities of the guppy fish are shown in the Table 1 with their median and range within parentheses.

Table 01: Summary of descriptive statistics of the frequencies of erythrocyte nuclear abnormalities of guppy juveniles (*Poecilia reticulata*) upon 28-day exposure to diclofenac in water (n=3 fish per group in three replicates).

Group[a]	N	BN	NN	LN	MN	NB (A)	NB (B)
C	9	0 (0 - 0.2)	0.40 (0.2 - 1.2)	0.60 (0.2 – 0.6)	0 (0 – 0.2)	0.60 (0.2 – 0.8)	0.60 (0 – 1.2)
T_1	9	0 (0)	0.80 (0.2 – 2.2)	1.40 (0.2 – 2.4)	0 (0 – 0.2)	1.00 (0.2 – 1.8)	0.60 (0 – 1.2)
T_2	9	0 (0)	0.60 (0.4 – 1.2)	1.20 (0.8 – 2.0)	0 (0 – 0.2)	0.80 (0.4 – 1.0)	1.00 (0.4 – 1.6)
T_3	9	0 (0-0.2)	1.20 (0.8 – 1.6)	1.40 (1.0 – 2.0)	0.20 (0 – 0.6)	1.20 (0.8 – 1.60)	1.00 (0.4 – 1.4)

[a] C = Control, T_1, T_2, and T_3= 1, 10 and 100 µg/L - diclofenac respectively
N = Number of fish per group
BN, NN, LN, MN, NB (A) and NB (B) = bi-nucleated cells, notched nuclei, lobed nuclei, micronuclei, nuclear buds (type A) and nuclear buds (type B) respectively

According to the results, fish in control group had relatively low frequency of nuclear abnormality types such as, notched nuclei, lobed nuclei, nuclear buds (Type A) and nuclear buds (Type B) than diclofenac exposed groups. The median values of frequencies of bi-nucleated cells were 0 in all four groups. And also among all erythrocyte nuclear abnormality types, lobed nuclei had highest frequency than other types (per 1000 RBC's).

In gill histopathology, gills which have normal architecture consist with good integrity of the central axis, well organized secondary lamellae and evident interlamellar space. The gill tissues obtained from fish in control group showed normal gill architecture (Plate 04). But gill sections obtained from diclofenac sodium exposed groups (T_1, T_2 and T_3) showed several alterations in gill structure than normal structure. They showed moderate to intense histopathological alterations in gill structure. Among these alterations, inter-lamellar hyperplasia, epithelial lifting and dilated secondary lamellae were prominent (Plate 05). All these types of pathological alterations were presented in all three treatment groups and they do not showed dose dependent pattern. That means they do not showed regular increment of alterations according to the increasing of drug concentrations.

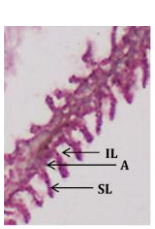

Plate 04: Image showing gill filaments of fish in control group. (SL: secondary lamellae, A: area without hyperplasia and IL: evident interlamellar space)

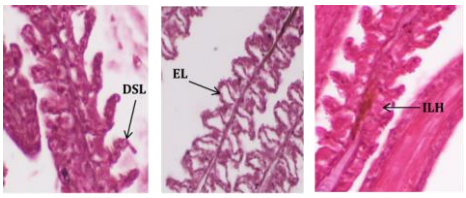

Plate 05: Images showing different types of pathological alterations in gill filaments found in fish under diclofenac exposure. (DSL: dilate secondary lamellae, EL: epithelial lifting and ILH: interlamellar hyperplasia)

In the experiment II, the data obtained from behavioural test and RNA:DNA of guppy fries were statistically analyzed to find out whether there are significant effects caused by the long term exposure (14 days) to waterborne diclofenac compared to the control group. However, the osteology results were shown only descriptively. Fries exposed to diclofenac showed significantly lower ($p<0.05$) predator avoidance response than control group (Table 2).

Table 02: Comparison of the predator avoidance response of guppy fries (*Poecilia reticulata*) upon 14-day exposure to diclofenac in water.

Group[a]	T_1	T_2	T_3
C	0.036*	<0.0001*	0.004*
T_1	-	0.020*	0.014*
T_2	-	-	0.431

[a] C= Control, T_1, T_2, and T_3= 1, 10, and 100 µg/L - diclofenac respectively
*p<0.05= significant difference

Accordingly, there is a significant difference between control and T_1 and control and T_2 and control and T_3, T_1 and T_2, T_1 and T_3 group for predator avoidance response ($p<0.05$). There is no significant difference between, T_2 and T_3 group for predator avoidance response ($p>0.05$).

The fries' specimens of the control group (n=9) showed typical skeletal structure with well-arranged vertebral column, caudal fin rays and the other skeletal structures (Plate 6). But, fries in the diclofenac exposed groups (T_1, T_2 and T_3) showed several altered skeletal structures compared with control group. Among them presence of curved vertebral column and malformed tails were prominent (Plate 7). However, both these alterations found in all three diclofenac exposed groups. That means they do not showed regular increment of alterations according to the increasing of concentrations of drug (Table 3).

Table 03: Summary of osteology of guppy fries (*Poecilia reticulata*) upon 14-day exposure to diclofenac in water.

Group[a]	N	Number of fries that have altered skeletal structures	Percentage (%)
C	9	0	0
T_1	9	2	22.22
T_2	9	4	44.44
T_3	9	3	33.33

[a] C= Control, T_1, T_2, and T_3= 1, 10, and 100 µg/L - diclofenac respectively
N= Number of fish per group

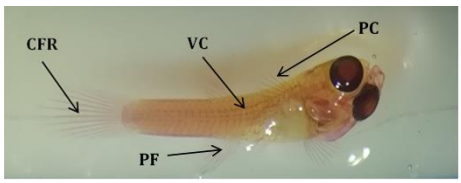

Plate 6: Image showing typical skeletal structure of fish in control group. (VC: vertebral column, CFR: caudal fin rays, PC: pectoral fin rays and PF: pelvic fin rays)

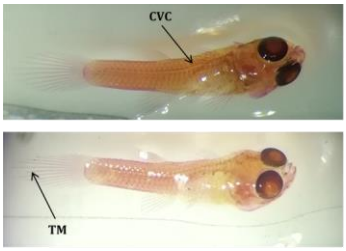

Plate 7: Images showing different types of alterations in skeletal structure found in fish under diclofenac exposure. (CVC: curved vertebral column and TM: malformed caudal fin rays)

However, There was no significant effect on the RNA:DNA ratio of guppy fries as a result of exposure to diclofenac.

IV. DISCUSSION

Pharmaceuticals are designed to target specific metabolic and molecular pathways in human and other organisms. But these pharmaceuticals also have important side effects. When the pharmaceuticals enter to the environment they may affect the same pathways in animals having identical or similar target organs, tissues, cells or biomolecules (Fent *et al*., 2006). Diclofenac is one of the analgesic drugs which belong to non-steroidal anti-inflammatory drug group. Here, within the human body, when it became over dose, it caused to severe side effects such as, an increased chance of serious cardiovascular thrombotic events, myocardial infraction, stomach ulceration, stroke and renal failure (Chan *et al*., 2002). Extensive presence of diclofenac in the aquatic environment causes toxicity on aquatic organism including fish (Acuna *et al*., 2015). The present study mainly designed to investigate the effect of waterborne diclofenac on guppy fish (*Poecilia reticulata*). Diclofenac was selected as an experimental drug because of its wide and frequent usage, and reported environmental occurrence especially in the aquatic environment (Chan *et al*., 2002; Acuna *et al*., 2015). For this study, guppy fish was selected as a test organism. The main reasons for this selection are; they are one of the most famous tropical ornamental fish in the world, have short life cycle, easy to maintain and handle in laboratory as well as their responsiveness to the environmental toxicants (Nakajima and Taniguchi, 2001; Hawkins *et al*., 2003).

Researchers found that waterborne diclofenac caused genotoxic effects on fish. These genotoxic effects can be studied and evaluated by different kinds of techniques such as by analysis of nuclear alterations in peripheral erythrocytes and cytogenetic analysis (Summak *et al*., 2010; Barbosa *et al*., 2010). There is a main mechanism of action of diclofenac to the induction of genotoxicity. It is mainly due to an increase of oxidative stress (Gomez – Lechon *et al*., 2003). Erythrocyte nuclear abnormalities are considered as excellent markers of genetic instability, because of their advantages like simplicity, reliability and sensitivity (Al-Sabti and Metcalfe, 1995). According to Yadhav and Trivedi (2006) erythrocyte nuclear abnormalities mainly occur due to genotoxic and cytotoxic damages such as chromatid breaks, chromatid deletions, fragments, acentric fragments, chromosome breaks, dicentric chromosome along with chromatid and chromosome gaps. There were several studies done for evaluate the effect of waterborne diclofenac on erythrocyte nuclear morphology of fish. Vasanthi *et al*. (2016) reported that short term and long term exposure of freshwater fish, *Cirrhinus mrigala* on drug diclofenac caused several erythrocyte nuclear abnormalities including micronuclei and notched nuclei.

Fish gills are the most important organ which is help to respiration of the fish. As aquatic organisms fish gills are always in direct contact with aquatic environment and they are very sensitive to changes in water quality (Lujic *et al*., 2013). Fish gills have a very thin epithelium. The total area of this epithelium is larger than total are of the skin epithelium (Roberts, 1989). Because of the delicate structure, different kinds of important functions and

direct contact with water, fish gills are the first organ which is affected by waterborne pollutants (Bernet *et al*., 1999; van der Oost *et al*., 2003). According to study of Bernet *et al*. (1999) they classified the histological alterations of gill tissue under three groups as progressive changes, regressive changes and circulatory disturbances. Under progressive changes there are several alterations such as, epithelial hypertrophy, epithelial hyperplasia, mucous cell hyperplasia, chloride cell hyperplasia, chloride cell hypertrophy and fusion of secondary lamellae. Under regressive changes there are several alterations such as, atrophy, epithelial lifting, necrosis and other structural alterations include clubbing of distal parts of lamellae, curving and branching of lamellae and rupture of epithelium. As well as hyperaemia, haemorrhage and telangiectasia belongs to circulatory disturbances category. In the present study diclofenac exposed groups showed moderate to intense histopathological alterations when compared to the normal gill structure. Among them inter-lamellar hyperplasia, epithelial lifting and dilate secondary lamellae were prominent. The inter-lamellar hyperplasia belongs to progressive changes category and sometimes which may be lead to complete fusion of lamellae. Epithelial lifting and dilate secondary lamellae are the kinds of regressive alterations. These alterations may lead to respiratory impairments. And also degenerative changes of gill histology may be the direct effect of accumulation of diclofenac on gills of guppy juveniles (*Poecilia reticulata*). The occurrence of inter-lamellar hyperplasia caused reduction in the respiratory surface of fish and it act as barrier for the water flow in the lamellae. The occurrence of epithelial lifting caused increase in the water-blood barrier present for defensive mechanisms of gills when exposed to pollutants. As well as dilate secondary lamellae caused accumulation of high amount of blood within it (Lujic *et al*., 2013). In mammals prostaglandins are the major regulators of blood circulation and ion concentration of kidney. In the mammals' body diclofenac is act as inhibitor of cyclooxygenase which is responsible for the synthesis of prostaglandins. Therefore, it can be assumed that, in fish prostaglandin may also play same mechanism in gills because, the blood circulation and ion regulation are essential biological mechanisms of fish body. Therefore chronic exposure of low levels of diclofenac caused adverse effects associated with the inhibition of cyclooxygenase and prostaglandins (Hoeger *et al*., 2005).

Predator avoidance response behavior of fish is most important for protection from predators, foraging, mating and territorial defense (Brown *et al*., 2009). This predator avoidance response behavior is clearly ecologically relevant behavior because; it is directly related to the growth and survival of the fish (Weis *et al*., 2001). This behavior of fish is mainly dependent on individual personality (Brown *et al*., 2014). Also, this behavior is directly correlated with swimming ability of the fish. But, waterborne contaminants affect the swimming performance and ability to escape from predators (Weis *et al*., 2001). They also reported that the waterborne pollutants alter the predator avoidance behavior of fish and they make changes in predator-prey interaction. This can be result in population changes of the predator species, prey species or both. The guppy fish exhibit strong behavioral response to both aquatic and potential aerial predators (Templeton and Shriner, 2004). The primary behaviors used by guppies to avoid aquatic predators are schooling, inspecting, hiding and jumping from the surface (Magurran and Seghers, 1994; Seghers, 1974; Seghers, 1970). The altered predator avoidance behavior occurred as a result of toxicant damage to the nervous system. Here, these toxicants can alter the synthesis and release of neurotransmitters, which may associated with behavioral changes of the fish (Weis *et al*., 2001). In fish dopamine, acetylcholine, serotonin and GABA like neurotransmitters are involved in predator avoidance behavior (Smith, 1984). Some fish also use olfaction to avoid predators (Tierney *et al*., 2010). Several studies showed that the different types of pollutants affect the predator avoidance response behavior of fish. Barry (2013) reported that the exposure of Arabian killifish (*Aphanius dispar*) on fluoxetine which is an antidepressant, alter the swimming ability of fish and it affect the predator avoidance response. Bromazepam, which is a psychiatric drug, alter the activity of zebrafish (*Denio rerio*) (Gebauer *et al*., 2011). However, the present study clearly reveals that there is an impact of chronic exposure of waterborne diclofenac on the predator avoidance response of guppy fries (*Poecilia reticulata*).

Fish early life stage test is one of the accredited methods for evaluating the acute and chronic toxic effects of aquatic pollutants on fish. This method provides information about several developmental parameters such as the endpoints of toxicants effects. This includes developmental delay, development of somites, eyes and deformations of the skeletal structure etc. (Fraysse *et al*., 2006; Luckenbach *et al*., 2001). The skeletogenesis of fish is starts in their embryonic development. Here, first calcified structures are normally associated to feeding and movement of them. The bony skeletal structures form by both endochondral and intramembranous ossification. Formation of the axial skeleton begins later in larval life and parallels the formation of unpaired fins. However,

waterborne pollutants can alter the gene expressions of fish in their development. Therefore, abnormal gene expression and protein accumulation may cause malformations of skeletal structure. There are several kinds of skeletal malformations can be seen in fish such as, lordosis and kifosis in vertebral column, vertebral fusion and vertebral malformation in vertebrae, supra/subnumerary rays and fused rays/ pterigophores in fins, mandibulal malformations and operculum malformations (Gavaia and Cancela, 2006). There are several studies showed that the effect of waterborne pollutants on osteogenesis of fish. Agathon *et al.* (2003) reported that the diclofenac affect the expression of Wnt8a gene of zebrafish (*Danio rerio*) and it prevents tail development of fish. Exposure of zebrafish on dexamethasone and hydrocortisone caused alteration in craniofacial development and altered somitogenesis (Hillegass *et al*., 2007; Hillegass *et al*., 2008). Gorman *et al*. (2007) reported that, the mutant curve back of guppy fish exhibit a skeletal dysplasia and it is resemble to idiopathic scoliosis (spinal deformity) of human. In this mutation, affected gene of fish is not determined. In the present study, the results clearly reveal that, there is an effect of waterborne diclofenac on osteology of guppy fries.

V. CONCLUSION

In conclusion, the present study reveals that, chronic exposure (28 days) of waterborne diclofenac at 1 µg/L, 10 µg/L and 100 µg/L concentrations caused significant effects on erythrocyte nuclear morphology and gill histology of guppy juveniles (*Poecilia reticulata*). Chronic exposure of waterborne diclofenac did not cause significant effects on behavior of guppy juveniles (*Poecilia reticulata*) at the above concentrations. Diclofenac exposed guppy juveniles showed higher frequencies of erythrocyte nuclear abnormalities in notched nuclei, lobed nuclei and cells with nuclear buds (Type A and Type B) than the control group, and had histological alterations in gills. Among them inter-lamellar hyperplasia, epithelial lifting and dilate secondary lamellae were prominent. The chronic exposure (14 days) of waterborne diclofenac at 1 µg/L, 10 µg/L and 100 µg/L concentrations caused significant effects on behaviour and osteology of guppy fries (*Poecilia reticulata*), while there was no significant effect on RNA:DNA ratio. Diclofenac exposed fries showed less number of positive responses to the predator than fries in control group. Also they had significant alterations in skeletal structure. Among them curved vertebral column and malformed caudal fin rays were prominent. Therefore, it can be concluded that chronic exposure of diclofenac in water causes changes in erythrocyte nuclear morphology and gill histology but it does not cause alterations in behaviour of guppy juveniles. Also, chronic exposure of diclofenac in water causes behavioural alteration and changes in osteology and it does not cause significant effect on RNA:DNA ratio of guppy fries.

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