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EFFECT OF DICLOFENAC SODIUM ON GLYCOGEN CONTENT OF ZEBRA FISH *DANIO RERIO*

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ABSTRACT

Numbers of pharmaceuticals are commonly used now-a-days for the treatment of various ailments. Among them, Diclofenac sodium is used to relief pain and joint stiffness but the residual part of it exerts various side effects. It has been shown that the residue of diclofenac reaches in considerably high amounts in aquatic environment which causes toxicity to aquatic animals especially in fishes. Therefore, the aim of this experimental work is to observe the effect of diclofenac sodium in the glycogen content of zebra fish in three different tissues i.e. liver, gills and muscles. LC50 at 24 hours exposure of diclofenac sodium estimated by direct interpolation method was found to be 26.25mg/l. To observe the effect of diclofenac sodium on glycogen content, the fishes were divided into 4 groups. Group 1 served as untreated control. Group 2 was treated with 24hrs LC50 of diclofenac. Group 3 & 4 received 1/5th conc. of 24hrs LC50 for 7 and 15 days respectively. Fishes were sacrificed after *lethal and sub lethal* exposure. Liver, gills and muscles were separately analysed to study the glycogen content by Anthrone reagent test. It was observed that glycogen content drastically decreased within 24 hours of diclofenac treatment & then gradually increased in 7 & 15 days treatment. Thus, this study indicates that level of glycogen can greatly be altered in response to the toxic effects caused by diclofenac in zebra fish.

Keywords: Diclofenac, Zebra fish, glycogen, LC50.

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INTRODUTION

Rapid growth of human population is leading to a great exposure in all possible fields in today's world. Humans not only have discovered many new ideas about health and life but also have led to an alarming danger for both animals and the environment. Excessive chemical usage is useful on one hand and harmful on the other, while some being lethal. One such example is of Diclofenac sodium (DS) which is found in high concentrations in water bodies.

Diclofenac sodium is a Non-steroidal, anti-inflammatory drug (NSAID), which is generally used to reduce pain, stiffness and inflammation caused by rheumatoid arthritis, osteoarthritis, abdominal cramps during menstruation, etc. It is used all around the world and is available in forms of intravenous solutions, capsules, tablets, suppositories and in ointment & gel form for dermal application. The drug enters the environment when not disposed properly and poses harmful impacts to the living beings. Drastic decline in the no. of species of *Gyps* Vulture, in the last few decades is the consequence of this drug [1, 2]. Not only on land but also, through surface runoff, the residual of this drug reaches the water bodies and causes toxicity to the aquatic environment.[3-5] In long term investigations of 'Sewage and surface water samples', diclofenac was identified as one of the most active compounds present in water cycle.[6, 7] Due to its lower solubility in water, it is not readily eliminated, so fishes living in these water bodies are greatly affected as the drug gets into the aquatic food chain and affect the aquatic life via. bio-magnification. Fishes being sensitive to toxicants, respond even at low concentrations of this drug. [8]

Glucose is very important constituent as it provides energy for various metabolic processes and proper functioning of body tissues and organs. It is stored in form of glycogen in liver and muscles. In such stressful conditions of fish encountering the drug (diclofenac), liver is the main target as the drug reaches this organ along with food during digestion. Recently, diclofenac metabolites have been found in fish bile [3]. Diclofenac degenerates the cells and tissues of liver and also deplete the glycogen content as seen in rainbow trout [9].

Residuals of diclofenac have been confirmed in various vital organs of many fishes by several workers. [10, 11]

Danio rerio (zebra fish), a freshwater fish, were analysed under stress condition induced by Diclofenac sodium by many workers [12, 13]. As already stated in researches that 70% of protein coding of human genes, are related to those found in the zebra fish & also, that 84% of genes known to be associated with human diseases have a zebra fish counterpart.[14] So it is quite evident that effects of diclofenac seen in zebra fish will somewhere or the other can have similar effects on humans as well. So the present study is made to investigate the toxic effects of diclofenac sodium on zebra fish.

MATERIALS & METHODS

Collection and maintenance of fish

Young Zebra fish (*Danio rerio*) of both the sexes were brought from a local aquarium shop. To check any surface infection or any parasite, they were dipped in 0.1% conc. of $KMnO_4$ for 1 to 2 minutes. Then these fishes were acclimatized in the lab environment for about 8-10 days at room temperature in a glass aquarium in normal dechlorinated water. They were fed with commercially available fish food. Water was renewed on daily basis and cleanliness was well taken care of.

Toxicant used

The chemical (toxicant) used was Diclofenac sodium 2[(2,6-dichlorophenyl) amino] benzene acetic acid sodium salt, that is manufactured by Nitin Lifesciences Ltd 92-93, sector 3, H.S.I.D.C (Karnal, Haryana).

Experimental Design

(i) Determination of LC50

LC50 of Diclofenac sodium (DS) was determined by direct interpolation method. For this purpose, one Exploratory and one Definitive tests were conducted. In exploratory test, two groups of 5 fishes each were treated with 6.25mg/l and 75mg/l concentration of DS to estimate the supposed mortality between 0% - 100%. In the Definitive test, six groups (I, II, III, IV, V, VI) with 10 fishes each were treated with conc. (7.5, 15, 22.5, 30, 37.5, and 45) mg/l respectively of DS and mortality was recorded after 24, 48, 72and 96 hours following exposure. The toxicity after 24 hours was taken into consideration. After the definitive test, a graph was plotted between concentrations and %mortalities. LC50 after 24 hours, obtained by drawing a perpendicular from 50% (on y-axis), towards the conc. of diclofenac (on x-axis) by a vertical line to be used for glycogen estimation.

(ii) Estimation of glycogen

The fishes were divided into four groups i.e. 1,2,3,4 of 10 fishes each. Group 1 was served as untreated control. Group 2 was treated with 24hrs LC50. Group 3 & 4 received 1/5th conc. of 24hrs LC50 for 7 and 15 days respectively. After lethal and sub-lethal exposure, fishes were sacrificed, and tissues of liver, gills and muscles were extracted and separately weighed. Liver and gills (both separately) were homogenized in 1 ml and muscles in 2ml of 80% methanol. Homogenized tissues were centrifuged at 3000rpm for 10 minutes. Supernatant was removed; 1ml of 5% TCA (Trichloro Acetic acid) was added to each tissue residue. The materials (tissues) were then kept in oven for 15 minutes. They were cooled, and again centrifuged. At last, Anthrone reagent was added to each tissue supernatant as per Anthrone reagent test and optical density was measured at a wavelength of 610nm by a colorimeter. [15]

RESULTS AND DISCUSSION

The results of exploratory and definitive tests for the LC50 determination of 24hrs are summarized in table 1 and 2. During the exploratory test no mortality occurred in 6.25mg/l conc. i.e. the lower concentration while 100% mortality was seen in 75mg/l conc. of DS in 24hours, while in definitive test, a sequential increase in the percent mortality was seen with increasing DS conc.

Table 1: Exploratory test for diclofenac sodium in zebra fish

Conc. of DFC (mg/l)	No. of fish	24 hours M	%M	48 hours M	%M	72 hours M	%M	96 hours M	%M
6.25	5	0	0	_	_	_	_	_	_
75	5	5	100%	_	_	_	_	_	_

Table 2: Definitive test for diclofenac sodium in zebra fish.

Conc. of DFC (mg/l)	No. of fish	24 hours M	%M	48 hours M	%M	72 hours M	%M	96 hours M	%M
7.5	10	1	10%	1	20%	1	30%	1	40%
15	10	2	20%	1	30%	1	40%	2	60%
22.5	10	4	40%	2	60%	1	70%	1	80%
30	10	6	60%	1	70%	1	80%	1	90%
37.5	10	6	60%	2	80%	1	90%	1	100%
45	10	8	80%	1	90%	1	100%	_	_

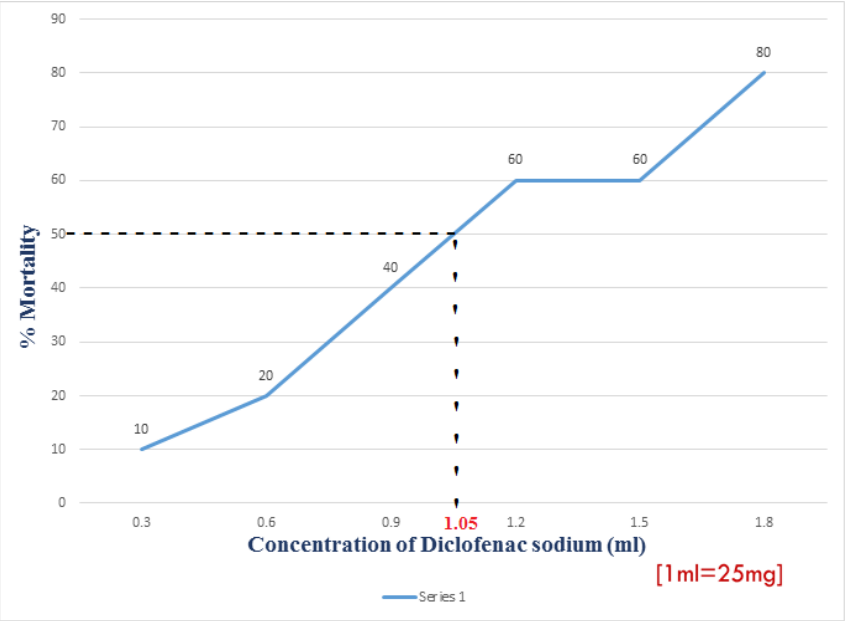

Figure 1: (LC50 determination)

Figure 1 clearly shows the LC50 of diclofenac after 24 hrs which was found to be 26.25mg/l. After 72hrs of DS exposure, the LC50 was 5.3mg/l as reported by Hallare et al, 2005. [16] Praskova et al, 2011 reported the LC50 of diclofenac to be 6.11mg/l in the embryo and 166.6mg/l in juvenile zebra fish after 96hrs and 144hrs respectively and also in 2014, they reported the LC50 of DS to be15mg/l in 96hrs. [8, 12] The LC50 of diclofenac was also reported on *Clarius garipinus* which was estimated as 25.12mg/l.[17] Our result of LC50 is in agreement with the above researchers.

The aim of this work is to check the toxic effect of diclofenac on glycogen content in three different tissues i.e. liver, muscles and gills. The results of glycogen content after diclofenac treatment for 24hrs, 7 days and 15 days in zebra fish is summarized in table 3 and figure 2. It was observed that glycogen content drastically decreased within 24 hours treatment and then gradually increased in 7 days and 15 days treatment.

Table 3: Alterations in glycogen content in tissues of zebra fishes induced by DS.

TISSUE	CONTROL (mg/gm)	24 HOURS (mg/gm)	7 DAYS (mg/gm)	15 DAYS (mg/gm)
LIVER	12.89	2.32	5.09	5.43
GILLS	8.3	1.75	8.05	8.9
MUSCLES	7.93	1.56	2.49	2.84

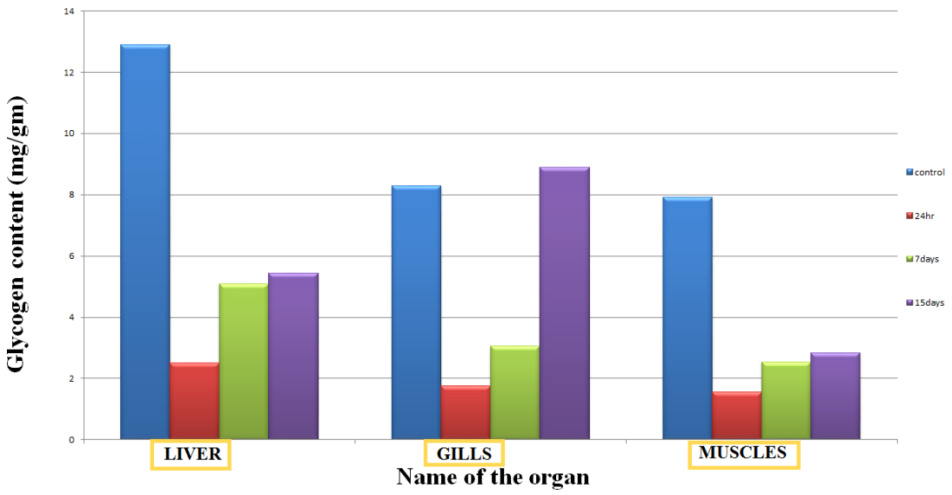

Figure 2: (Effect of diclofenac sodium on glycogen content)

Glycogen content in liver, muscles and gills suffers a great loss when exposed to diclofenac for 24 hours when compared to control but slight improvement was seen in the glycogen content of liver and muscles. Liver is the major detoxifying organ of the body and had shown a great depletion in glycogen level. The depletion in glycogen is probably due to stress induced by the diclofenac. According to Karrine [18] DS metabolites 4-OH-diclofnac and 5-OH-diclofenac can be oxidised to intermediate of a reactive quinone immine which induces stress in the tissue. So to compensate the stress induced by the diclofenac there is an elevation in glycogenolysis. The decline of glycogen in the liver may be due to the breakdown of glycogen into glucose to reduce the stress. Due to accumulation of diclofenac in the gills there may be a state of anoxia (absence of oxygen) which favour the process of glycogenolysis and decrease the content of glycogen. [19] The fishes that were exposed for 7 and 15 days show an increment in the glycogen reservoir. This shows that glycogen stores slowly replete and the rate of utilization was probably equal to the rate of supplementation. This increment may be an indication that fishes might have adapted in the toxic environment.

The transport of oxygen in the body through the gills is reduced due to the accumulation of diclofenac in gills, such that there is a shortage of oxygen in muscles also. The function of the glycogen reserve in the muscles is to provide the instant energy through glycolysis as per the need. In the present study the content of glycogen in the muscles had a serious reduction which indicates the increased glycolysis to produce glucose from glycogen due to anaerobic stress caused by toxicant. [20] During anaerobic condition in muscles there is a sudden requirement of energy which is fulfilled by the breaking of glycogen into glucose.

DS degenerates the cells and tissue of liver, muscles and gills which has been shown by many workers. [9], [21], [22] Tissue necrosis could be another reason for glycogen depletion. During stress condition there was a decrease in glycogen content which is marked by the decrease in blood glucose level for 24 hours when feeding was stopped but when the fishes were exposed for 15 days there was a slight enhancement in glucose level. [20], [23]

CONCLUSION

We can conclude that this pharmaceutical can cause toxicity in fish during lethal and sub lethal exposure. This is the preliminary work and needs further investigation taking other biochemical parameters as well.

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