Blood cell enumeration assay on Channa striatus exposed to sub lethal concentration of Diazinon

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Abstract : Diazinon [O, O-diethyl-O-(2-isopropyl-6-methylpyrimidin-4-yl) phosphorothiate] is one of the most widely used pesticides in agriculture for pest control in crops and animals. The present study was conducted to determine toxicity and effects of diazinon on the haematological parameters of juvenile Channa striatus. The lethal concentration of diazinon (96 h LC50) was 24.16 mgl−1 for test fish under a semi-static system. Fish exposed to diazinon showed behavioural abnormalities such as hyperactivity, erratic swimming, convulsion, loss of balance, depigmentation and surfacing to gulp air. Fish were exposed to two sublethal concentrations of the pesticide (LC10, LC20, LC30, LC40, LC50, LC60, LC70, LC80, LC90 and LC99), which were selected on the basis of LC50. The blood samples from the challenged fishes were taken after every 20, 40 and 60 minute in fishes exposed to diazinon. The fish exposed to sublethal concentrations of the pesticide exhibited alterations in various blood parameters. A significant dose and time dependent decrease ($p < 0.05$) in haematocrit (PCV), haemoglobin (Hb), RBC's, lymphocytes and monocytes was recorded. An increasing trend was, however, noticed in counts of white blood cells (WBC). The study results highlight the importance of haematological indices as a valuable tool for monitoring diazinon-induced toxicity in aquatic organisms, particularly in fish.

***IndexTerms* - Diazinon, Channa striatus, sublethal, depigmentation**

I. Introduction

Diazinon is a widely used toxicant in a number of organophosphorous pesticides (Roberts and Hutson 1998). Although the aquatic environment is not the target one for the use of such pesticides the results of a number of monitoring studies have evidenced the presence of diazinon and its metabolite, diazoxon, in surface waters (Mansingh and Wilson 1996; Tsuda et al. 1996; Van der Geest et al. 1997; Bailey et al. 2000; de Vlaming et al. 2000). That is why great attention has been paid to the effect of diazinon on fish organism.

Blood and tissues of living organisms are very sensitive to changes and are widely used in Ichthyology research (Adedeji et al., 2000). Many studies conducted in recent years have shown the harmful effects on fishes and the aquatic ecosystem of pesticides. Haematological parameters in fish living in the different environmental conditions are very important for toxicological studies (Schlenk, 2005; Banaee et al., 2008, 2011). Haematological indices are of different sensitivity to various environmental factors and chemicals (Vosyliene, 1999). Haematology and clinical chemistry analysis, although not often used in fish medicine, can provide substantial diagnostic information. On the basis of haematological studies, it would be possible to predict the physiological state of fish in natural water bodies. Hematological parameters like MCH, MCV, MCHC, Hct, Hb, RBC, WBC values are widely utilized to assess the toxic stress of environmental contaminants (Kavitha et al., 2010).

The main haematological response of common carp to the acute exposure to diazinon based organophosphorous pesticide in 32.5 mg.l-1 concentration was a significant decrease ($p < 0.01$) of erythrocyte count, haematocrit value and haemoglobin content compared to the control group. Decreased erythrocyte count and haemoglobin content in freshwater fish Channa punctatus after acute exposure to diazinon was also reported by Anees (1978). Other effective substances of organophosphorous pesticides also induce changes which give evidence for decreased haematopoiesis followed by anemia induction in fish. It regards e.g. changes in erythrocyte profile induced by acute effect of dichlorvos in Clarias batrachus (Benarji and Rajendranath, 1990), Ekolux organophosphorous preparation in Oreochromis mossambicus (Sampath et al., 1993), formothion in Heteropneustes fossilis (Singh and Srivastava, 1994), malathion in Cyprinion wabsoni (Khattak and Hafeez, 1996), and of trichlorphon in Piaractus mesopotamicus (Tavares et al. 1999).

Another type of haematological response to the effect of organophosphorous compounds is a significant increment of mean corpuscular volume (MCV) associated with increment of haematocrit value and drop of MCHC. This response was registered in common carp after acute effect of phenitrothion, imidan and dichlorvos (Svobodova 1971, 1975). In contrary, values of MCV, MCH and MCHC registered after 96 h exposure to diazinon-based pesticide in 32.5 mg.l-1 concentration to common carp were comparable with the control group. Significant decrease of leukocyte count and significant relative and absolute lymphopenia and granulocytosis characterize the leukocyte profile of common carp after the acute exposure to diazinon-based pesticide. Lymphopenia as a consequence of methyl parathion based pesticide was reported by Siwicki et al. (1990) in common carp and Nath and Banerjee (1996) in Heteropneustes fossilis after an acute effect of trichlorfon. Ghosh and Banerjee (1993) report lymphopenia and both neutrophile and eosinophile granulocytosis in Heteropneustes fossilis after an effect of dimethoate in 96h LC50 concentration. A decreased non-specific immunity in fish can be expected after acute exposure to organophosphorous pesticides due to decreased leukocyte count, lymphopenia and granulocytosis. Numerous authors report lymphopenia and granulocytosis after exposure to many pollutants (Wlasow 1985; Murad and Houston 1988; Svobodova et al. 1996). These changes in differential leukocyte count also give evidence for decreased level of nonspecific immunity in fish after acute exposure to toxic substances. The present study was undertaken to have a basic understanding of the acute toxicity of Diazinon 60 EC on the experimental fish Channa striatus species through haematological investigation.

II. MATERIALS AND METHODS

Collection and maintenance of fish:

Fish of particular size (22-24 cm) and weight (25-30 g) range were used irrespective of their sex for the experiments. Only healthy individuals collected from the same body of water were employed in all tests. The test solutions were renewed every 24 hr to maintain the optimum dissolved oxygen level. While conducting the experiments, care was taken not to deviate from the modified main principles of bioassay techniques outlined by Sprague (1973) and recommended by APHA (1989). Fish were fed with 2% of body weight fish feed once a day. Before attempting, the fish divided into groups were kept in 1 x 1.2m (wide-deep) fiber-glass tanks one week for adaptation. Aeration was allowed during the trial. The quality parameters of water were measured as pH=7.3-7.5, Temperature=27 ± 1 º C, DO: 7.2-7.4 ppm, salinity=0.42-0.49 ppt, alkalinity=260 mg/L as $CaCO_3$, hardness=323 mg /L $CaCO_3$.

Acute Toxicity:

To evaluate the acute toxicity studies the static renewal toxicity test were conducted according to the methods recommended by American Public Health Association (1960). In the present investigation the median lethal concentration (LC50) of diazinon for 24, 48, 72 and 96 hr were analyzed. The screening test was conducted to avoid delay and to save time and effort. The objective of this test is to obtain approximate indication of the concentration of a substance likely to be hazardous to the test fish and fishes in general in their natural environment (Alabaster and Lloyd, 1982). The toxicant concentration used in the present series of tests were approximately the wide range of concentrations viz., Control, LC10, LC20, LC30, LC40, LC50, LC60, LC70, LC80, LC90, LC99 aqueous solutions were prepared. In each group twenty one fishes were introduced with three replications. The tests were conducted in the plastic troughs. The troughs were cleaned well and dried before conducting experiments. Then the tests were conducted by allowing twenty one fishes of Channa striatus in each plastic trough containing 10 liters of water with particular concentration of the diazinon. The screening tests was continued to assess the concentration at which all fishes survived for 24 hr and likewise the concentration at which most of the fishes died simultaneously (Bansal et al., 1980)

Statistical analysis:

The variations through the medium of (±SEM) between groups were evaluated using Independent Samples-t test. 95% confidence limits were considered important. SPSS 15.0 software was used for data analysis.

III. RESULT

For investigation into the effect of Diazinon on the hematological indices, thirteen blood parameters were studied, which include PCV (%); hemoglobin (g/dL); RBC (X 106/μL); MCV (fL); MCH (pg); MCHC (g/dL); WBC (X 103/μL); lymphocytes (X 103/μL); monocytes (X 103/μL); The haematology is the best indicator to reflect any changes disastrous to health in terms of the changes in the blood profile. The findings during the present research tenure could form a baseline for environmentalists to assess the quantum of change in the health status of fish.

Hematocrit levels also serve as an indicator of health conditions. Thus, tests on hematocrit levels are often carried out in the process of diagnosis of conditions of stress because of environmental conditions. The mean±SD value of PCV (Pack Cell Volume) expressed in per cent (%) was 37.25±0.57 during R1 (N = 21). The PCV showed a decrease after the exposure with a mean±SD of 25.00±1.00 (V = 1.01; $p>0.1$). In R2, PCV was 39.34±0.43 (N = 21), which showed a decrease (28.3±0.40) (V = 0.16; $p>0.1$). Significant ($P<0.05$) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance of 5.445 for 96 h LC50. The Haemoglobin (Hb) expressed in g/dl, was 10.30±0.15 during R1 (N = 21), which showed a decrease to 6.32±0.06 after the exposure (V = 0.004; $p>0.1$). In R2, Hb was 9.88±0.07, which showed a decrease to 7.2±0.2 (V = 0.04; $p>0.1$). Significant ($P<0.05$) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance of 151.38 for 96 h LC50.

Red blood cells (RBCs), also called erythrocytes, are the most common type of blood cell and the vertebrate organism's principal means of delivering oxygen (O_2) to the body tissues—via blood flow through the circulatory system. The mean±SD value of red blood corpuscles (RBC) expressed in x106/µl, was 2.45±0.06 during R1 (N = 21), which showed a decrease after the exposure to 2.10±0.11 (V=0.01; $p<0.01$). In R2, RBC showed a decrease to 2.10±0.1 (V=0.01; $p>0.01$). Significant ($P<0.05$) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance of 262.205 for 96 h LC50. An MCV blood test is one component of the CBC blood test that measures the number and the different types of cells in the blood. Among these parameters, MCV is the most useful value used to classify the type of anemia based on red cell morphology. The mean±SD value of Mean Cell corpuscles (MCV) expressed in fl, was 152.04±3.98 during R1 (N = 21), which showed a decline to 119.04±2.48 (V=6.15; $p>0.01$). In R2, MCV was 154.27±0.49, which showed a decline after the exposure to 134.76±0.62 (V=0.38; $p>0.01$).

Mean corpuscular hemoglobin (MCH) reflect the average hemoglobin content of red blood cells. The mean±SD value of MCH expressed in pg, was 42.04±1.38 during R1 (N = 21), which showed a decline post exposure to 30.09±1.23 (V=1.52; $p>0.01$). In R2, MCH was 38.74±0.17, which showed a decline after the exposure, to 34.28±0.47 (V=0.22; $p>0.01$). MCHC compares the amount of the hemoglobin compared to the size of the cell per red blood cell. MCHC expresses the average weight of hemoglobin per unit volume of red blood cell. During present experimental period, the mean±SD value of MCHC expressed in g/dL, was 27.65±0.32 during R1 (N = 21), which showed a decrease after the exposure to 25.28±0.8 (V=0.64; $p>0.01$). In R2, MCHC was 25.11±0.14, which showed an increase to 25.44±0.02 (V=0.06; $p>0.01$).

A WBC count is a test to measure the number of white blood cells (WBCs) in the blood. The white blood cell count (WBC) is used as part of a full complete blood count (CBC) to monitor the body's response to various treatments. The mean±SD value of red blood corpuscles (WBC) expressed in x103/µl, was 4.02±0.92 during R1 (N = 21), which showed an increase to 5.02±0.8 (V=0.64; p>0.01). In R2, WBC was 3.89±0.16, which showed an increase to 4.7±0.65 (V=0.42; p>0.01). Significant (P<0.05) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance of 206.045 for 96 h LC50.

A lymphocyte is one of the three subtypes of white blood cell in a vertebrate's immune system. White blood cells help protect the body against diseases and fight infections. The lymphocyte count has a tendency to increase when the body is stressed to act as a defense line. A lymphocyte count is usually part of a peripheral complete blood cell count and is expressed as the percentage of lymphocytes to the total number of white blood cells counted. The mean±SD value of lymphocyte expressed in x103/µl, was 45.33±0.61 during R1 (N = 21), which showed a decrease to 19.36±0.43 (V=0.64; p>0.01). In R2, the lymphocyte showed a decrease to 20.02±0.64 (V=0.40; p>0.01). Significant (P<0.05) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance 12.4002 for 96 h LC50. Monocytes are a type of white blood cells (leukocytes). They are the largest of all leukocytes. They are part of the innate immune system of vertebrates including fish. The mean±SD value of monocytes expressed in x103/µl, was 0.33±0.01 during R1 (N = 21), which showed a decrease to 0.23±0.01 (V=0.00; p>0.01). Significant (P<0.05) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance of 305.79 for 96 h LC50. Significant (P<0.05) differences were observed among the mean, with F crit value of 2.85 (df = 21) and variance of 305.79 for 96 h LC50.

IV. DISCUSSION

Hematological parameters like MCH, MCV and MCHC and Hematological indices such as Hct, Hb, RBC, WBC values and biochemical indicators like glucose are widely utilized to assess the toxic stress of environmental contaminants (Kavitha et al., 2010). Hematological and biochemical changes induced by sub-lethal concentrations of different organophosphate pesticides have previously been investigated in several fish species. Such as, dichlorvos in Clarias batrachus (Benarji and Rajendranath 1990), formothion in Heteropneustes fossilis (Singh and Srivastava 1994), diazinon in Silurus glanis (Koprucu et al., 2006), Cyprinus carpio (Ahmad, 2011), and malathion in Clarias gariepinus (Ahmad, 2012). Diazinon is commonly used in agriculture and previous studies have reported contamination of fresh water reservoirs in Pakistan with diazinon and its derivatives (Mastoi et al.2008; Iram et al. 2009).

The acute toxicity test lasting 96 h was performed semistatically on common carp juveniles by Svoboda et al. (2001) who worked on the effect of Diazinon on haematological indices of Common carp (Cyprinus carpio L.). The effect was assessed based on results of acute toxicity tests and on a comparison of results of haematological examination of a control and an experimental group exposed to Basudin 600 EW (32.5 mg l-1) pesticide preparation (active substance 600 g.l-1 of diazinon). The authors documented that the experimental group of one- to two-year-old common carp showed significantly lower values (p < 0.01) of erythrocyte count (RBC), haemoglobin content (Hb) and haematocrit (PCV) compared to the control group, which lends complete support to our findings which also showed lower values (P<0.05) for haemoglobin (Hb), RBC and PCV values. In contrary, authors reported a significant decrease in leukocyte count (Leuko) (p < 0.01), as well as in both the relative and absolute lymphocyte count (p < 0.01). The findings by the authors supports our findings, which showed decrease in leukocyte count, monocytes, and other non specific cells as compared to the control (untreated) group.

In order to assess the effect of diazinon on cultured and wild African catfish (Clarias gariepinus), Adedeji et al. (2008) compared the results of haematological examination of a control and an experimental group exposed to DiazintolR pesticide preparation (active substance 162mg.l-1of diazinon). The authors reported that after 96 hr of exposure to DiazintolR the African catfish showed significantly lower values (p < 0.05) of erythrocyte count (RBC), haemoglobin content (Hb) and haematocrit (PCV) compared to the control group. Values of MCV, MCH and MCHC were comparable in both groups during the study. In contrary, there was a significant decrease in leucocyte count (p < 0.05), relative and absolute lymphocyte count (p < 0.05) and a significant increase in both the relative and absolute count of developmental forms of neutrophile granulocytes: myelocytes (p < 0.05) and metamyelocytes (p < 0.05) in the experimental group. The findings of the above authors lends complete support to our findings.

Banaee et al. (2008) determined the chronic toxicity of Diazinon and its effects on some hematological parameters and biochemical blood plasma profiles of common carp, Cyprinus carpio. The authors reported that the experimental groups showed significantly lower values (p < 0.05) of erythrocyte count, haemoglobin content, haematocrit, leucocytes, lymphocyte and monocytes count, showing coherence with the present findings. The common carp (Cyprinus carpio) was exposed to sub lethal concentration of diazinon by Zubair (2011). Fishes exposed to sub-lethal concentrations (0.5, 1.0 and 2.0 mg/l) showed increase in Red blood cell (RBC) counts, haemoglobin concentration and haematocrit values and decreased in white blood cell (WBC) count. The findings of the author are contrary to our observations and also contradict with the general fish physiology.

In yet another trial, fishes were exposed to six different concentrations of diazinon (0, 5, 10, 20, 40, 80 ppm) for 96 h by Khalid (2013). The results showed that the number of leukocytes (WBC), erythrocytes (RBC), haematocrit (PCV) and hemoglobin (Hb) was significantly decreased (P<0.05), but the amount of MCV and MCH was increased significantly by 50 % and decreased again. Lymphocyte decreased significantly by 60 % and then increased by 80 %. There was a significant increase in neutrophils count by 55 % and then decrease by 70 %. Resulted changes in erythrocyte and leukocytes after exposing to Diazinon are due to malfunction in hemopoiesis and decrease in non-specific immune system. The authors concluded that long-term exposure to diazinon at sub-lethal concentrations induced alterations in haematological indices in common carp and offers a simple tool to evaluate toxicity derived alterations.

A semistatic experiment was conducted to find out the acute toxicity of diazinon as an aquatic pollutant on some hematological parameters of Indian carp (Cirrhinus mrigala) by Rauf and Arain (2013). The results after acute exposure to 8.15 mg/L of diazinon for 96 h showed that compared to the control fish, the total erythrocytes, leukocytes, hemoglobin, and hematocrit values decreased significantly ($P < 0.05$) in exposed fish. The mean corpuscular volume and mean corpuscular hemoglobin increased significantly ($P < 0.05$), while the mean corpuscular hemoglobin concentration of both groups remained unchanged during the study. Kamal et al. (2014) documented the same results while working on effects of long-term diazinon exposure on some immunological and haematological parameters in rainbow trout Oncorhynchus mykiss (Walbaum, 1792). The findings during the present research work gets complete support from the observations of the above authors.

To investigate the effect of cypermethrin and diazinon on haematology of Labeo rohita (H.), Ayesha et al. (2014) exposed twelve batches of fish to two concentrations of cypermethrin (0.15 and 0.30 µl/L) and diazinon (0.002 and 0.004 ml/L) for 96 hours and three batches of fish were treated as control. The authors reported a decrease in WBC and RBC count, Hb and PCV values ($P<0.05$). On the other hand, WBC count and MCHC value were increased with increasing concentration of diazinon exposure whereas RBC count, Hb, PCV, MCV and MCH values were increased in both concentrations of diazinon as compared to the control fish ($P<0.05$). In another attempt, Indian carp (Cirrhinus mrigala) exposed to two sub-lethal concentrations (0.815 mg/L and 1.63 mg/L) of diazinon for 30 days showed a significant decrease in erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, as documented by Haider and Rauf (2014). Compared to the controlled fish, leukocyte count decreased significantly in fish exposed to both sub-lethal concentrations after 20 and 30 days in fish exposed to 0.815 mg/L of diazinon.

Aliakbar Hedayati and Niazie (2015) evaluated the effect on the hematological changes of silver carp (Hypophthalmichthys molitrix) in response to half-lethal concentration (LC50) of Diazinon pesticide, after 0, 24, 48 and 96 h. The results showed that the values of leukocytes (WBC), haematocrit (Ht), hemoglobin (Hb), MCHC, lymphocyte, were significantly increased ($P < 0.05$). The amount of MCV and MCH were significantly increased at 48 h and then decreased at 96 h ($P < 0.05$). Moreover, there was a significant increase in neutrophil count at 48 h, and then a significant decrease at 96 h ($P < 0.05$). There were no significant differences in RBC, monocyte and eosinophile counts among treatment groups at different sampling intervals. The work of the above authors lends partial support to our findings. A significant decrease in PCV ($y = -2.633x + 43.51$), Hb ($y = -2.47\ln(x) + 11.35$), RBC count ($y = -0.31\ln(x) + 2.655$), lymphocyte count ($y = -14.2\ln(x) + 47.02$), and monocytes ($y = -0.10\ln(x) + 0.414$) was recorded post Diazinon exposure to Channa striatus. While as significant ($P<0.05$) increase in WBC ($y = 0.749\ln(x) + 3.532$) was observed during the present study. Our findings are supported and established by the work of above authors, who documented changes in the blood components due to stress of the toxic pesticide.

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Table 1: Probit analysis for first replica of mean Haematological parameters of *Channa striatus* (Bloch) affected by sublethal concentration of Diazinon

Indices	Units	Groups	N	Means	SD	Variance	Probability	Sig. 1	Sig. 2	95% Confidence Interval	
										Lower Bound	**Upper Bound**
PCV	%	Control Treated	10 21	37.25 25.00	0.57 1.00	0.32 1.01	0.98	0.59	0.62	-16.96	19.07
Hemoglobin	g/dL	Control Treated	10 21	10.30 6.32	0.15 0.06	0.02 0.004	0.99	3.12E-16	3.58E-11	0.433	0.433
RBC	X $10^6/\mu L$	Control Treated	10 21	2.45 2.10	0.06 0.11	0.003 0.01	0.75	1.36E-5	1.92E-6	1.77	1.79
MCV	fL	Control Treated	10 21	152.04 119.04	3.98 2.48	15.84 6.15	0.96	2.36E-15	2.55E-15	0.62	0.68
MCH	pg	Control Treated	10 21	42.04 30.09	1.38 1.23	1.90 1.52	0.95	3.97E-16	4.01E-15	0.89	0.96
MCHC	g/dL	Control Treated	10 21	27.65 25.28	0.32 0.8	0.10 0.64	0.73	3.97E-16	3.22E-14	2.5	3.0
WBC	X $10^3/\mu L$	Control Treated	10 21	4.02 5.02	0.92 0.8	0.85 0.64	0.97	3.67E-17	3.56E-11	0.86	1.01

Lymphocytes	X $10^3/\mu L$	Control Treated	10 21	45.33 19.36	0.61 0.43	0.37 0.18	0.99	2.91E-17	1.98E-15	0.69	0.78
Monocytes	X $10^3/\mu L$	Control Treated	10 21	0.33 0.23	0.01 0.01	0.00 0.00	0.93	7.03E-16	6.68E-12	1.5	1.9

Table 2: Probit analysis for second replica of mean Haematological parameters of *Channa striatus* (Bloch) affected by sublethal concentration of Diazinon

Indices	Units	Groups	N	Means	SD	Variance	Probability	Sig. 1	Sig. 2	95% Confidence Interval	
										Lower Bound	**Upper Bound**
PCV	%	Control Treated	10 21	39.34 28.3	0.43 0.40	0.18 0.16	0.99	1.66E-15	1.32E-14	0.93	1.25
Hemoglobin	g/dL	Control Treated	10 21	9.88 7.2	0.07 0.2	0.004 0.04	0.98	5.47E-15	3.99E-14	2.85	3.98
RBC	X $10^6/\mu L$	Control Treated	10 21	2.55 2.10	0.02 0.1	0.004 0.01	0.90	8.12E-16	6.89E-15	5.0	7.5
MCV	fL	Control Treated	10 21	154.27 134.76	0.49 0.62	0.24 0.38	0.99	1.06E-14	1.09E-15	1.25	4.36
MCH	pg	Control Treated	10 21	38.74 34.28	0.17 0.47	0.03 0.22	0.97	7.47E-15	7.26E-15	2.68	6.38
MCHC	g/dL	Control Treated	10 21	25.11 25.44	0.14 0.02	0.01 0.06	0.65	3.12E-17	2.95E-15	0.17	0.56
WBC	X $10^3/\mu L$	Control Treated	10 21	3.89 4.7	0.16 0.65	0.27 0.42	0.99	2.25E-15	1.95E-12	3.93	6.32
Lymphocytes	X $10^3/\mu L$	Control Treated	10 21	43.98 20.02	0.51 0.64	0.26 0.40	0.99	2.58E-15	1.79E-15	1.25	2.99
Monocytes	X $10^3/\mu L$	Control Treated	10 21	0.40 0.27	0.02 0.01	0.006 0.002	0.92	2.6E-16	6.68E-12	0.6	0.93