Haemato-immunological responses to diazinon chronic toxicity in *Barbus sharpeyi*

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Abstract

The present research aimed to determine acute toxicity and evaluate the effect of sub-lethal concentrations of diazinon on some haematological and immunological parameters of *Barbus sharpeyi* by the Organization for Economic Cooperation and Development methods. Juvenile *B. sharpeyi* were exposed to 0, 5%, 10% and 25% 96h LC_{50} concentrations of diazinon for 21 days. Blood samples were taken after 7, 14 and 21 days of exposure to diazinon. Haematological and biochemical parameters including: RBC, Hb, PCV and WBC, as well as serum enzymes were evaluated. Immunological indices and resistance against bacterial infection, were determined. Results showed that 96h LC50 of diazinon in *B. sharpeyi* was estimated at 3.987 mg/L. Diazinon toxicity at a level of 1 and 0.4 mg/L decreased RBC, Hb, and Hematocrite, in almost all sampling periods compared. WBC and globular index decreased significantly in fish exposed to 1 mg/L diazinon on days 14 and 21(p<0.05). Dose dependent increase in serum enzymes were seen in fish exposed to diazinon. Diazinon toxicity showed no effect on serum LDH level (p>0.05). Dose dependent decrease in serum total protein and globulin were indicated in diazinon exposed fish. Serum lysozyme and bactericidal activity decreased in T3 and T4 at days 14 and 21. Mortality following challenge to *A.hydrophila* increased in fish exposed to 1 mg/l diazinon. It can be concluded that diazinon is toxic to *B. sharpeyi*. Therefore, the strict biosecurity should be taken into consideration when this pesticide is used in agricultural fields surrounding freshwater sources of fish cultivation.

Keywords: Diazinon, Toxicity, *Barbus sharpeyi*, Immunological parameters, Hematology

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Introduction

The aquatic environment is continuously affected by toxins and pollutants, which could alter the haematological parameters and immune response of fishes and induce alterations in host resistance against various pathogens: (Miller *et al*., 2002; Galloway *et al.,* 2003). Contamination of water by pesticides, especially organaphosphorous pesticides, is mainly due to intensive agriculture combined with surface runoff and subsurface drainage (Nouri *et al.*, 2000). Most of pesticides ultimately find their way into rivers, ponds, lakes and natural water sources (Bagheri *et al*., 2000; Talebi, 1998) and have been found to be highly toxic to non-target organisms.

Diazinon [O,O-diethyl O-(2-isopropyl-4-methyl-6- pyrimiinyl) phosphorothioate] is one of the most important and moderately persistent organophosphorus pesticide largely used in agriculture: Larkin and Jeerdema (2000). The toxicity of this pesticide is mainly due to the inhibition of acetylcholinesterase (AChE) activity, the enzyme that degrades the neurotransmitter acetylcholine in cholinergic synapses. The inhibition of AChE lead to an accumulation of acetylcholine at the nerve synapses and disruption of the nerve function (Varo *et al*., 2002; Miron *et al.,* 2005; Bretaud *et al*., 2000; Cong *et al*., 2009). Diazinon is widely used in agriculture fields in Iran (Honarpajouh., 2003; Shayeghi *et al*., 2006) Based on reports of Bulletin of Agriculture Ministry, the annual consumption of diazinon in Iran is estimated to be 3775 ton (Annual Report 2007-2010). It is mostly used in the paddy fields of north of Iran as well as 100.000 hectares of sugar cane farms in Khuzestan \rovince (Banaee *et al*., 2011). Several studies reported that some of the surface waters and the surrounding environments in Iran were contaminated with organophosphate pesticides such as diazinon and its derivates (Rahiminejhhad *et al*., 2009; Arjmandi *et al*., 2010). In recent years, incidences of fish mortality due to pesticides, industrial effluents and sewage pollution in Iran have been reported (Talebi., 1998; Banaee *et al*., 2011).

B. sharpeyi which is called “Benni“ by the locals, belongs to the class of cyprinidae existing in the Shadegan Wetland (Hashemi *et al*., 2011). Forat, Karoun Rivers (Coad, 1979). This species, indigenous (endemic) to Khouzestan Province, has a high economic value and proper resistance against environmental stressors. Its artificial propagation (more than 20 million larvae per year) and cultivation in cyprinid earthen ponds has been accomplished in the last decade (Hashemi *et al*., 2011).

To date, little is known about the effects and mechanisms of organophosphorous pesticides on the teleost immune response. Besides, knowledge about the effects of diazinon on other relevant immunological parameters is lacking. Some researchers have correlated levels of pollution with immune dysfunction and an increased

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incidence of disease amongst wildlife populations (Luebke *et al*., 1997), but very little work is aimed to find the effect of organophosphorous on the immune response of reared fish. Hence, this study is designed to investigate the effect of sublethal concentrations of diazinon on some haematological and immunological parameters in the *B. sharpeyi.*

Materials and methods

Fish

The experiment was done in two phases; phase one: determination of acute toxicity (LC50 96h), and phase 2: aimed at the determination of chronic toxicity tests. Two hundred and eighty apparently healthy, artificially propagated and pond reared *B. sharpeyi*, weighing 27±2.5 g were used in the acute toxicity tests. Two hundred and forty *B. sharpeyi* weighing 121±9.5 g were used for the chronic toxicity test. Fish were kindly donated by the "Native fish propagation and rearing center", Susangerd, Khouzestan, Iran. Fish were transferred under standard conditions to the aquarium room of Shahid Chamran University, Ahvaz, Iran.

Experimental water quality

During the acute and chronic toxicity test experiment, water in each aquarium was aerated and had the same conditions as follows: dissolved oxygen 7.8±0.5 mg/L, temperature 25±1ºC, pH 7.8±0.2, water total hardness 340 mg/L as CaCO3, NH3 and NO2<0.1 mg mL^{-1}.

Acute toxicity test of diazinon

The acute toxicity test was conducted following the Organization for Economic Cooperation and Development (OECD) Guideline No. 203 under static-renewal test conditions. Test solutions of diazinon were prepared from a commercial diazinon, Basudin 60 EM brand, with the active molecule diazinon [O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimiinyl) phosphorothioate], purity 60% dissolved in 40% acetone solution. Nominal concentrations of active ingredients tested were 0 (control), 1, 3, 6, 9, 12 and 15 mg/L and each concentration was prepared in triplicate aquaria. 10 fish were introduced into each aquarium. Fish were considered dead when gill opercula and body movement ceased; and when these characteristics occurred fish were immediately gathered by dip net. LC_{50} values were calculated by the Probit Analysis test (Aydin and Kuprucu, 2005).

Sub-lethal toxicity experiments

Fish were randomly distributed in 4 groups (each in three replicates) in 12 similar 150 L aquarium (20 fish in each replicate) to perform the 21 day period sub-lethal toxicity tests.

According to the acute toxicity test (LC50, 96h) 3 concentration of diazinon prepared: $^1/_5$ (0.2 mg l^{-1}), $^1/_{10}$ (0.4^{-1}) and $^1/_{20}$ (1 mg L^{-1}) of 96h LC_{50} of diazinon and T2, T3 and T4 exposed to these diazinon concentrations, respectively. The control group (T1) was exposed to diazinon free water. The water was changed every other day

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to reduce the build-up of metabolic wastes and to keep concentrations of diazinon near the nominal level. The fish were exposed to sub lethal concentrations for three weeks.

Blood and mucus sampling

Blood samples were taken from 9 fish in each treatment after anesthetization with 100 ppm clove essence (PI222, Baridj essence Co, Iran) after 0, 7 14 and 21 days of exposure to diazinon. Fish were bled from the caudal vein into two separate groups of microtubes, one group containing heparin and the second group without heparin. Heparinised blood was used for haematological analysis and non-heparinized blood was centrifuged for 10 min at 4000 rpm and the serum was recovered. Haematological parameters were studied immediately after bleeding. Serum samples were saved at -70ºC until use.

Mucus samples were taken from the bleeding anesthetized fish. The fish were placed with the ventral side of the body facing downward and cutaneous mucus from the dorsal side of the fish was collected by a cell-scraper and transferred to a 0.5 mL microtube. Mucus samples were kept on ice during transportation to the lab and kept frozen to avoid bacterial growth and degradation at –80 ºC until used. The mucus centrifuges (3000 rpm for 10 minutes) and supernatant were filtered by 0.2 µL millipore filters and used for lysozyme and bactericidal activity of mucus (Thompson *et al*., 1995).

Haematological parameters

Blood samples were immediately analysed for the estimation of numbers of erythrocytes (RBC), hemoglobin (Hb), hematocrit (MCV), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC). Numbers of erythrocytes count were determined by the hemocytometer method; haematocrit was determined by the microhematocrit method (Jain, 1993), and hemoglobin was determined by the cianometa-haemoglobin method. MCV, MCH and MCHC were calculated by using the following formulae (Giddings *et al.,* 1996).

MCV (picogram $cell^{-1}$) = (Packed cell volume as percentage/RBC in millions cell mm^3)×10

MCH (pg $cell^{-1}$) = (Hb in g 100 mL^{-1}/ RBC in millions cell mm^3) ×10

MCHC (g dL^{-1}) = (Hb in g100 mL^{-1}/ packed cell volume as percentage) ×100

White blood cell count (WBC) and WBC Differential count were conducted as described by Schaperclaus *et al*. (1991).

Serum biochemical analysis

Serum total protein, albumin and globulin were determined in plasma by standard procedures used in clinical biochemistry laboratories based on manual biochemical kits (Zist chimi Co., Iran). Serum enzymes activity was assayed too. Lactate dehydrogenase (LDH) activity determination is based on measuring the conversion of pyruvate to L-lactate by monitoring the

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oxidation of NADH. Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. In alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α-ketoglutarate to form glutamate and pyruvate. Pyruvate is converted by LDH to make lactate and NAD+. All these activities were monitored by measuring the change in absorbance at 340 nm. Alkaline phosphatase (ALP) assay is based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm.

Immunological parameters

Mucus and serum lysozyme activity

Serum and mucus lysozyme activity was measured as described by Ellis (1990). Briefly, 10 µL of serum/mucus was mixed with 200 µL of a *Micrococcus lisodeichticus* (Sigma) suspension at 0.2 mg mL^{-1} in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27°C, and its OD was detected after 1 and 6 min at 530 nm using an ELISA (enzyme-linked immunosorbent assay) plate reader. One unit of lysozyme activity was defined as the amount of enzyme that produced a decrease in absorbance of 0.001 min mL^{-1} serum. Lysozyme concentrations were calculated using a standard curve of lysozyme from chicken egg white (Sigma) concentrations.

Mucus and serum bactericidal activity

The method used for serum bactericidal activity followed a modified version of that adopted by Kajita *et al*. (1990). The serum samples were diluted three times with 0.1% gelatin-veronal buffer (GVB; pH 7.5, containing 0.5mMml Mg and 0.15mMmL Ca). Mucus samples were used without dilution. *Aeromanas hydrophila* (live washed cells) were suspended in the same buffer to make a concentration of 1×10^5 cfu mL^{-1}. The diluted sera and bacteria were mixed at a ratio of 1:1 and incubated for 90 min at 25°C and continuously agitated. The number of viable bacteria was then calculated by counting the resultant colonies from the incubated mixture on TSA (tryptic soy agar) plates after incubation for 24 h in duplicate.

Disease resistance

A. hydrophila (AH04 isolated from morality of *Cyprinus carpio*) was inoculated in tryptone soy broth and was incubated at 30°C. The broth was centrifuged at 800 × g for 15 min. Packed cells were washed and demand concentration was prepared in phosphate buffered saline (PBS). At the end of treatment, remaining fish in each experimental treatment was injected intraperitoneally with 0.5 mL of LD_{50} suspension of *A. hyrophila* (1.6×10^7cfu per fish) in PBS. Mortality of treated fish was recorded daily for 10 days. The cause of death was ascertained by re-isolating the infecting organism from kidney and liver of dead fish according to Divyagnaneswari *et al*. (2007).

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Statistical analysis

Statistical analyses were performed using SPSS (version 16) software. Data are presented as Mean±SD. LC_{50} value indicates using probit assay. Data were tested for normality (Kolmogorov–Smirnov test) and analyzed using two-way analysis of variances (ANOVA). The significant means were compared by Tukey's test and a $p<0.05$ was considered statistically significant.

Results

Lethal concentration of diazinon in B. sharpeyi

Acute toxicity of diazinon was determined in *B. sharpeyi* after 24, 48, 72 and 96 hours of exposure. 96 hours LC50 value (Median lethal concentration) calculated at 3.987 mg/L in *B. sharpeyi* (Table1). LC50 values significantly decreased in accordance with the exposure time from 9.84 mg/L at 24 h to 1.17 mg/L at 96 h.

Blood parameters of *B. sharpeyi* were affected with the various concentration of diazinon compared to the control specimens (Table 2). Hematocrite and RBC decreased significantly in all sampling periods in T4, whereas on days 14 and 21 in T3. Hemoglubin levels decreased just in T3 and T4 on days 14 and 21 ($p<0.05$). MCV and MCH decreased in T4 at all sampling points and in T3 on day 21($p<0.05$). Chronic toxicity with different concentration of diazinon did not affected MCHC in sampling points ($p>0.05$).

WBC and Heterophiles rates were reduced significantly almost in all diazinon treated groups and different sampling points ($p<0.05$).

Chronic toxicity of diazinon *in B. sharpeyi* changed plasma enzymes activity (Table 3) so that AST, ALT, LDH, and ALP were presented in. AST, ALP and ALT activity were significantly higher than control group at all sampling points in T4 and at days 7 and 14 in T3 ($p<0.05$). No significant differences were evidenced in LDH activity among the groups ($p>0.05$).

Plasma protein and globulin levels were significantly decreased in groups exposed to 1 and 0.4 mg/L diazinon compared with the control group ($p<0.05$) (Table 3). No significant differences were observed in the level of plasma albumin among the groups at different sampling times ($p>0.05$). 0.2 mg/L diazinon didn't impact on biochemical parameters of *B. sharpeyi*.

Serum lysozyme activity decreased at all sampling times in T4 and on day 7 in T3 (Table 4). Besides mucus lysozyme activity decreased at all sampling times just in T4.

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Table 1: Median lethal concentrations of diazinon dependant on time in *Barbus sharpeyi*.

	24h	48 h	72 h	96 h
LC_{10}	6.344	2.764	2.337	1.515
LC_{20}	6.9	3.265	2.710	1.805
LC_{50}	9.843	6.601	5.064	3.987
LC_{90}	15.273	15.766	10.973	9.791

Table 2: Changes in some haematological parameters in *Barbus sharpeyi* exposed to different concentrations of diazinon. Results are expressed as Mean ± SE. Values in rows with different small letters significantly differ ($p<0.05$) and values in rows with different capital letters significantly differ ($p<0.05$). T1: Fish exposed to diazinon free water (control). T2: Fish exposed to 0.2 mg L^{-1} diazinon concentration, T3: Fish exposed to 0.4 mg L^{-1} diazinon concentration. T4: Fish exposed to 1 mg L^{-1} diazinon concentration.

Parameters	Treatments	Day zero	days 7	days 14	days 21
	T4	40.2 ± 3.8^{Bb}	33.2 ± 2.46^{aA}	32.2 ± 5.49^{aA}	30.40 ± 7.40^{aA}
Hematocrite	T3	41.4 ± 2.77^{Bb}	38.8 ± 5.07^{abB}	32.8 ± 4.079^{aA}	31.6 ± 6.50^{aA}
(%)	T2	40.2 ± 2.5^{Bb}	39.4 ± 5.50^{bB}	40.2 ± 6.5^{bB}	33.4 ± 5.46^{abA}
	T1	39.8 ± 2.27^{Bb}	40.6 ± 4.45^{Ba}	40.4 ± 7.45^{Bb}	37.8 ± 4.71^{Bb}
	T4	8.23 ± 0.76^{Bb}	7.43 ± 0.82^{Bb}	7.27 ± 1.01^{Aa}	7.102 ± 0.66^{Aa}
	T3	9.05 ± 0.76^{Bb}	8.398 ± 0.98^{Bb}	6.86 ± 0.48^{Aa}	7.314 ± 0.69^{Aa}
Hemogolobine	T2	8.7 ± 0.76^{Bb}	7.77 ± 0.71^{Bb}	8.24 ± 0.91^{Bb}	7.46 ± 0.86^{Bab}
(g/dL)	T1	8.67 ± 0.76^{Bb}	8.19 ± 0.45^{Bb}	8.21 ± 0.85^{Bb}	8.13 ± 0.81^{Bb}
	T4	1.49 ± 0.27^{Bb}	1.31 ± 0.14^{ABa}	1.18 ± 0.20^{Aa}	1.20 ± 0.20^{Aa}
RBC	T3	1.44 ± 0.32^{Bb}	1.41 ± 0.20^{Bb}	1.24 ± 0.14^{Aa}	1.23 ± 0.21^{Aa}
(mm^{-3})	T2	1.52 ± 0.34^{Bb}	1.45 ± 0.23^{Bb}	1.43 ± 0.23^{Bb}	1.26 ± 0.16^{Aa}
	T1	1.4 ± 0.25^{Bb}	1.54 ± 0.15^{Bb}	1.44 ± 0.21^{Bb}	1.48 ± 0.14^{Bb}
	T4	281 ± 34.3^{Bb}	256.6 ± 34.4^{ABa}	252.2 ± 30.9^{Aa}	251.5 ± 26.9^{Aa}
MCV	T3	269 ± 36.4^{Bb}	281.0 ± 43.8^{Bb}	261.5 ± 14.3^{ABab}	256.6 ± 18.2^{Aa}
(Fl)	T2	274 ± 35.2^{Bb}	279.2 ± 39.4^{Bb}	281.8 ± 7.8^{Bb}	276.0 ± 24.4^{Bb}
	T1	270 ± 30.7^{Bb}	280.1 ± 14.8^{Bb}	280.0 ± 25.2^{Bb}	274.2 ± 20.7^{Bb}
	T4	56.8 ± 4.75^{Bb}	49.8 ± 5.41^{Aa}	50.3 ± 5.18^{Aa}	52.8 ± 6.36^{ABa}
MCH	T3	55.7 ± 4.7^{Bb}	55.3 ± 9.60^{Bb}	52.7 ± 5.31^{Ba}	58.6 ± 7.88^{Bb}
(pg)	T2	56.3 ± 4.52^{Bb}	55.0 ± 5.60^{Bb}	58.3 ± 3.63^{Bb}	59.1 ± 2.85^{Bb}
	T1	54.8 ± 4.2^{Bb}	55.0 ± 4.14^{Bb}	57.9 ± 4.76^{Bb}	54.6 ± 3.81^{Bb}
	T4	20.٧ ± 1.44^{Bb}	22.5 ± 3.04^{Bb}	22.8 ± 2.21^{Bb}	24.2 ± 4.54^{Bb}
MCHE	T3	19.9 ± 1.٣٢^{Bb}	21.5 ± 4.48^{Bb}	21.2 ± 2.45^{Bb}	23.7 ± 3.40^{Bb}
(%)	T2	20.1 ± 1.14^{Bb}	19.9 ± 1.71^{Bb}	20.7 ± 1.33^{Bb}	22.3 ± 1.39^{Bb}
	T1	20.37 ± 1.23^{Bb}	19.7 ± 2.12^{Bb}	20.2 ± 2.54^{Bb}	20.0 ± 1.30^{Bb}
	T4	7640 ± 979^{Bb}	6544 ± 1020^{Aa}	6432 ± 1053^{Aa}	6704 ± 1030^{ABa}
	T3	7612 ± 979^{Bb}	7530 ± 1511^{Bb}	7636 ± 936^{Bb}	7642 ± 668^{Bb}
WBC	T2	7592 ± 979^{Bb}	7664 ± 897^{Bb}	7672 ± 690^{Bb}	7720 ± 1267^{Bb}
(mm^{-3})	T1	7560 ± 979^{Bb}	7942 ± 832^{Bb}	8188 ± 1670^{Bb}	7700 ± 1315^{Bb}
	T4	83.33 ± 3.79^{Bb}	75.4 ± 6.77^{Aa}	78.00 ± 6.78^{Aa}	75.4 ± 3.71^{Aa}
Lymphocyte	T3	82.56 ± 3.79^{Bb}	79.20 ± 6.76^{ABa}	76.00 ± 1.73^{Aab}	77.00 ± 2.65^{Aa}
(%)	T2	85.65 ± 3.79^{Bb}	82.0 ± 3.61^{Bb}	82.80 ± 1.64^{Bb}	80.67 ± 3.79^{Bb}
	T1	83.45 ± 3.79^{Bb}	82.5 ± 3.51^{Bb}	84.20 ± 1.41^{Bb}	85.33 ± 3.51^{Bb}
	T4	15.33 ± 3.21^{Bb}	23.20 ± 6.38^{Aa}	20.25 ± 3.77^{Aa}	20.20 ± 3.70^{Aa}
Heterophile	T3	15.33 ± 3.21^{Bb}	16.00 ± 7.81^{Bab}	18.00 ± 2.00^{Aa}	21.00 ± 1.73^{Aa}
(%)	T2	15.33 ± 3.21^{Bb}	16.00 ± 3.61^{Bb}	16.40 ± 2.41^{Bb}	16.33 ± 1.53^{Bb}
	T1	15.33 ± 3.21^{Bb}	15.50 ± 2.65^{Bb}	13.50 ± 2.12^{Bb}	14.67 ± 3.51^{Bb}
	T4	1.00 ± 0.33^{Bb}	1.00 ± 0.82^{Bb}	1.57 ± 1.06^{Bb}	1.60 ± 0.55^{Bb}
	T3	1.00 ± 0.33^{Bb}	1.00 ± 0.5^{Bb}	1.33 ± 1.53^{Bb}	1.33 ± 1.53^{Bb}
monocyte	T2	1.00 ± 0.33^{Bb}	1.66 ± 2.00^{Bb}	0.57 ± 0.55^{Bb}	1.67 ± 2.08^{Bb}
(%)	T1	1.00 ± 0.33^{Bb}	1.50 ± 2.00^{Bb}	1.78 ± 0.71^{Bb}	0.77 ± 0.77^{Bb}
	T4	0.33 ± 0.58^{Bb}	0.75 ± 0.96^{Bb}	1.00 ± 1.41^{Bb}	1.15 ± 1.14^{Bb}
Eosinophil	T3	0.33 ± 0.58^{Bb}	0.67 ± 1.15^{Bb}	0.50 ± 0.72^{Bb}	0.33 ± 0.87^{Bb}
(%)	T2	0.33 ± 0.58^{Bb}	0.75 ± 0.76^{Bb}	0.74 ± 0.55^{Bb}	0.67 ± 1.15^{Bb}
	T1	0.33 ± 0.58^{Bb}	0.50 ± 0.58^{Bb}	0.33 ± 0.76^{Bb}	0.67 ± 1.33^{Bb}

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Table 3: Changes in serum biochemical parameters of *Barbus sharpeyi* exposed to different concentrations of diazinon. Results are expressed as mean ± SE. Values in rows with different small letters significantly differ (p<0.05) and values in rows with different capital letters significantly differ (Two-way ANOVA, p<0.05). Legends are the same as Table 2.

Parameters	Treatments	Day zero	days 7	days 14	days 21
ALTU mL^{-1}	T4	41.2±9/7 ^{Ab}	48.9±9 ^{ABb}	63.9±13.8 ^{Bb}	62.6±13.8 ^{Bb}
	T3	40.1±9.2 ^{Ab}	51.1±8.9^{Bb}	50.9±9.5 ^{Bb}	65.6±9.5 ^{Bb}
	T2	39.7±9.6 ^{Ab}	42.3±7.5 ^{Ab}	47.3±6.2 ^{Ab}	44.2±6.2 ^{Ab}
	T1	42.1±11.2 ^{Ab}	38.1±16.5 ^{Ab}	64.4±8 ^{Bb}	46.9±8 ^{Bb}
ASTU mL^{-1}	T4	162.4±33 ^{Ab}	208.4±16.2 ^{Bb}	299.6±38.3 ^{Bb}	198.4±59.8 ^{Bb}
	T3	167.2±29.2 ^{Ab}	191.4±21.1 ^{Bb}	202.2±54 ^{Bb}	158.44±38.8 ^{Bb}
	T2	168.4±32 ^{Ab}	151.6±19.8 ^{Ab}	169.6±15.4 ^{Ab}	155±28.2 ^{Ab}
	T1	159.9±29.9 ^{Ab}	152.4±27.2 ^{Ab}	157±24.2 ^{Ab}	166.4±24.9 ^{Ab}
ALPU mLL^{1}	T4	40.3±5.2 ^{Ab}	55±13.1 ^{Bb}	60.6±10.4 ^{Bb}	57.8±11.9 ^{Bb}
	T3	42.7±4.8 ^{Ab}	56.2±7.9 ^{Bb}	42±7.0 ^{Ab}	48.4±11.3 ^{Bb}
	T2	43.7±4.5 ^{Ab}	41.8±5.2	40.8±5.5	44.2±10.4
	T1	42.2±5.5 ^{Ab}	40.1±6.4 ^{Ab}	41.2±10.3 ^{Ab}	40.8±9.6 ^{Ab}
LDHU mL^{-1}	T4	189±21.6 ^{Ab}	197.6±12.9 ^{Ab}	209.8±25.4 ^{Ab}	203.2±51.3 ^{Ab}
	T3	180±20.4 ^{Ab}	202.4±20.9 ^{Ab}	206.4±51.3 ^{Ab}	193.4±42.8 ^{Ab}
	T2	191±22.5 ^{Ab}	187.8±29.4 ^{Ab}	201.6±10.5 ^{Ab}	191.2±38.5 ^{Ab}
	T1	189±19.5 ^{Ab}	195.8±15.5 ^{Ab}	192.4±29.7 ^{Ab}	192.6±40.5 ^{Ab}
Total protein g dLl^{-1}	T4	3.21±0.20 ^{Ab}	3.23±0.11 ^{Ab}	3.13±0.15 ^{Bb}	3.10±0.10 ^{Bb}
	T3	3.37±0.15 ^{Ab}	3.30±0.16 ^{Ab}	3.27±0.25 ^{Bb}	3.18±0.15 ^{Bb}
	T2	3.32±0.22 ^{Ab}	3.27±0.06 ^{Ab}	3.40±0.17 ^{Ab}	3.33±0.15 ^{Ab}
	T1	3.25±0.11 ^{Ab}	3.33±0.23 ^{Ab}	3.35±0.19 ^{Ab}	3.35±0.15 ^{Ab}
Albumin g dl^{-1}	T4	2.02±0.14 ^{Ab}	2.10±0.04 ^{Ab}	2.07±0.14 ^{Ab}	2.05±0.08 ^{Ab}
	T3	2.14±0.17 ^{Ab}	2.17±0.12 ^{Ab}	2.11±0.28 ^{Ab}	2.10±0.04 ^{Ab}
	T2	2.10±0.10 ^{Ab}	2.11±0.09 ^{Ab}	2.15±0.34 ^{Ab}	1.56±1.04 ^{Ab}
	T1	2.06±0.09 ^{Ab}	2.07±0.15 ^{Ab}	2.20±0.28 ^{Ab}	2.15±1.04 ^{Ab}
Globulin g dl^{-1}	T4	1.20±0.08 ^{Ab}	1.13±0.11 ^{ABb}	1.06±0.13 ^{Bb}	1.05±0.04 ^{Bb}
	T3	1.23±0.12 ^{Ab}	1.13±0.06 ^{ABb}	1.15±0.06 ^{ABb}	1.08±0.14 ^{Bb}
	T2	1.22±0.11 ^{Ab}	1.15±0.06 ^{ABb}	1.25±0.17 ^{Ab}	1.26±0.15 ^{Ab}
	T1	1.24±0.10 ^{Ab}	1.26±0.16 ^{Ab}	1.15±0.13 ^{Ab}	1.21±0.15 ^{Ab}

Serum and mucus bactericidal activity decreased in T4. No significant changes were evidenced in serum and mucus lysozyme and bactericidal activity in fish exposed to 0.2 mg/L diazinon (p>0.05).

Disease resistance

Mortality rate following the challenge with *A. hydrophila* significantly increased in fish exposed to atrezine (Fig. 1).

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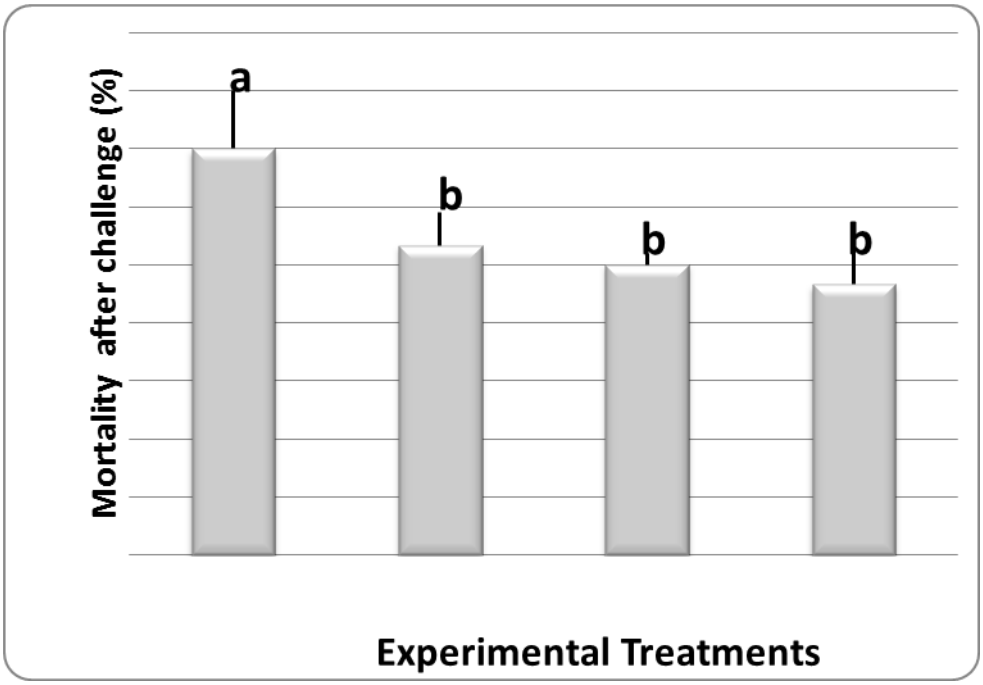

Figure 1: Percentage mortality of *Barbus sharpeyi* exposed to different diazinon concentrations, 10 days after challenge with *Aeromonas hydrophila* . Bars represent the standard deviation of the mean for each treatment. Different letters over the bars indicate significant differences among treatments (p<0.05). Legends are the same as Table 2.

Mortality rate in T2, T3 and T4 were 50%±2%, 53%±5.77% and 70%±10%, respectively, whereas mortality in the control group was 47%± 5.77%. The mortality rate significantly increased in T4 compared to that in the control (p<0.05).

Discussion

The present results showed that the toxicity of diazinon on *B. sharpeyi* increased either with increasing concentration or exposure period of diazinon. In addition, the 24, 48, 72 and 96 h LC_{50} values of diazinon in *B. sharpeyi* were found to be 9.84, 6.60, 5.06 and 3.987 mg/L, respectively. Environmental Protection Agency of America (USEPA) reported 96 h LC_{50} values for diazinon in different species such as bluegill, (0.46 mg/L), rainbow trout (0.9–1.65 mg/L), fathead minnow (7.80 mg/L), cutthroat trout (2.15 mg/L) and *Cyprinodon variegates* (1.4 mg/L) : Shrma (1990). The present results are in accordance with what reported by USEPA for cyprinid fish being the 96 h LC_{50} included in their range.

Acute toxicity tests of fish exposed to diazinon have shown that 96 h sublethal values vary by several orders of magnitude between species (Keizer *et al*.,1991; Oh *et al*., 1991), so that the 96 h LC50 values of diazinon for guppy (*Poecilia reticulate*) was found to be 0.8 mgL^{-1} but for zebra fish (*Brachydanio rerio*) it was found to be 8 mgL^{-1} : Keizer (1991). The selective toxicity of diazinon for various fish species depend on different inhibition

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of acetyl-cholinesterase, different detoxification, and absorption (Oh *et al*., 1991).

Chronic toxicity with $^1/_4$, $^1/_{10}$ and $^1/_{20}$ of 96h LC_{50} concentration of diazinon (1, 0.4 and 0.2 mg/L), induced different effects on hematological and immunological parameters in *B. sharpeyi*. Among the haematological parameters, RBC, PCV and Hb value in fish exposed to 1 and 0.4 mg/L had significantly decreased in almost all sampling times; however exposure to 0.2 mg/L diazinon just decreased RBC value on day 21 after exposure. MCV and MCH value decreased in fish exposed to 1 mg/L diazinon in all sampling periods, as well as in 0.4 mg/l just in 14 days after exposure. WBC value and heterophiles ratio in differential count of WBC had decreased in fish exposed to 0.4 and 1 mg/L diazinon (p<0.05). Other parameters showed no significant changes among treatments. The toxicity effect of diazinon on hematological parameters seems to be dose dependent. Effects of diazinon on hematological parameters have been investigated in several fish species including Catfish (Koprucu *et al*., 2006), *Clarias batrachus* (Benarji and Rajendranath, 1990), *Oncorhynchus mykiss (*Banaee *et al.*, 2011), and European catfish (Koprosu *et al*., 2006). Most of the studies on the effects of organophosphorous pesticids are confined to reporting the biochemical and physiological changes, but little attention has been paid to the hematological modulation induced by diazinon : Svoboda (2001).

Decreased RBC, WBC value and hemoglobin content in *C. carpio* after exposure to diazinon were also reported by Svoboda *et al*. (2001). Other effective substances of organophosphorous pesticides also induce changes which give evidence for decreased hemotopoiesis followed by anaemia induction in fish. It regards, e.g., changes in erythrocyte profile induced by acute effect of dichlorvos in *C. batrachus (*Benarji and Rajendranath, 1990), and trichlorphon in *Piaractus mesopotamicus (*Tavares *et al*., 1999). The decrease in erythrocyte, leukocytes and hemoglobin content observed in this study may be due to the disruptive action of the organophosphorous on the haemotopoietic tissue as a result of which the viability of the cells might be affected (Morgan *et al*., 1980). Reduction in life time of fish blood cells exposed to diazinon can be an important cause of haematological changes. Reduction of heterophils rate showed suppressed nonspecific immune response in fish. Heterophils take in, digest and present the foreign antigens to functional immune cells, therefore, their reduction suppresses these process.

This study is one of the few works that has examined the effect of diazinon on immunological parameters in fish. Our results showed significant decrease in serum lysozyme activity, serum total protein, immunoglobulin, and serum bactericidal activity in fish exposed to 1

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and 0.4 mg/L diazinon compared to the control (p<0.05). Besides, decrease in mucus lysozyme and bactericidal activity were seen in fish exposed to 1 mg/L diazinon (p<0.05). To the best of our knowledge, this is the first study reporting the negative effects of diazinon on mucus and serum lysozyme and bactericidal activity in fish. Lysozyme plays an important role in the innate immune system, effectively protecting against gram-positive bacterial infections (Shailesh, 2008). Decreased lysozyme and bactericidal activity can be related to the suppressing effects of diazinon on non-specific immune response, particularly production and differentiation of leukocytes. Giron-Perez *et al.* (2009) reported that serum lysozyme activity wasn't affected by diazinon in tilapia.

Total protein, albumin and globulin tests are used to monitor the course of diseases in immune disorders, liver dysfunction and impaired kidney activity (Mochida *et al*., 1994). According to the test results, levels of total protein, and globulin had decreased in fish exposed to1 and 0.4 mg/L diazinon. Serum total proteins and IgM are the indicators of immune statues of fish (Mochida *et al*., 1994). Reduced total proteins were probably induced by decreased immune related proteins such as lysozyme, complement component, antibacterial peptides and so on. Other authors also found that the levels of total protein and globulin decreased in the fish exposed to different pollutants and pesticides: (Velisek *et al*., 2008; Banaee *et al*., 2011). Decreased globulin levels after exposure to pesticides have been reported in rainbow trout (Banaee *et al*., 2011), tilapia and beluga sturgeon (*Huso huso*) (Khoshbavar *et al*., 2006). Diazinon-induced tissue destruction and hepatocyte apoptosis might be the most important reasons responsible for reducing the synthesis of total protein, and immunoglobulin by the liver (Gokcimen *et al*., 2007). Contrarily, increased IgM level was reported in mouse and tilapia exposed to diazinon (Garg *et al*., 2004; Giron-Perez *et al*., 2009). These contradictory results can be related to dose and duration of diazinon in chronic toxicity as well as to differences in fish species.

Fish resistance to bacterial infection depends basically on the immune response. It is important to estimate the resistance against bacterial infection in fish exposed to a toxin to determine its practical effects on immune responses. Mortality after experimental challenge with *Aeromonas hydrophila* increased significantly (p<0.05) in fish exposed to 1 mg/L diazinon compared to that in the control. This result indicates that exposure to 1 mg/L diazinon for 3 weeks suppressed the immune responses and resistance to bacterial infection in *B. sharpeyi*.

Although AST, ALT and ALP levels increased significantly in *B. sharpeyi* exposed to 1 and 0.4 mg/L diazinon almost in all sampling points, no remarkable change in these enzymes level were observed in fish exposed to 0.2 mg/L diazinon. AST, ALT and ALP are synthesized and found mostly in the

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liver (Srivastava *et al*., 2004), heart, skeletal muscle (Petrovic *et al*., 1996), kidney, pancreas, spleen, erythrocyte, brain and gills (Battacharya *et al*., 2008). When diseases or injuries affect the liver, the cells are destroyed and these enzymes are released into the plasma. Keizer *et al*. (1995) showed that during diazinon metabolism in the liver, reactive oxygen species (ROS) are generated. The increase in intracellular levels of ROS may lead to lipid peroxidation resulting in an increased permeability of liver cell membrane. As a result, liver enzymes including AST and ALT are released into plasma (Srivastava *et al*., 2004; Rao, 2006).

Lactate dehydrogenase (LDH) is an enzyme found in almost all body tissues (Hasnain, 2005). LDH measurement is used to detect tissue disorders and as an aid in the diagnosis of tissue damage (Hasnain, 2005; Rao, 2006). No significant changes were observed in LDH content of plasma in fish exposed to different levels of diazinon. Our data are in accordance with several reports that revealed few effects of pesticide on LDH activity in different fishes such as rainbow trout (Banaee *et al*., 2011), *O. Mossambicus* (Rao, 2006), and *C. punctatus* (Agrahari *et al*., 2007). Diazinon is able to cause inhibition of LDH activity. However, our results in LDH activity indicated that diazinon concentrations used in the present study were not enough to produce significant LDH alterations.

In conclusion, the haematological and biochemical parameters and immunological indices of *B. sharpeyi* measured in the present study were useful for monitoring the long-term effects of diazinon in wild and cultured fish. It can be concluded that exposure to chronic sub-lethal concentrations of diazinon resulted in significant biochemical changes. These changes may be potentially disruptive and immunosuppressive for the survivability of *B. sharpeyi* in wild environments and aquaculture farms. Therefore, strict biosecurity should be taken into consideration when this pesticide is used in agriculture fields surrounding freshwater sources of fish cultivation.

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