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Effect of sub-lethal Diazinon Concentrations on Blood Plasma Biochemistry

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ABSTRACT: Diazinon is commonly used for pest control in the agricultural fields surrounding freshwater reservoirs. This study was conducted to determine the chronic toxicity of organophosphorous pesticide and its effects on some hematological parameters and biochemical blood plasma profiles of common carp, *Cyprinus carpio*. Diazinon was applied at concentrations of 60 and 120µg/L preparations in 10, 20 and 30 days since the experiments were initiated. The experimental groups showed significantly lower values ($p < 0.05$) of erythrocyte count, haemoglobin content, haematocrit, leucocytes, Lymphocyte and monocyte, as well as in alkaline phosphatases and significantly higher ($p<0.05$) values of plasma glucose, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, neutrophile and eosinophile compared to the control group. There was not significant difference in value of total protein ($p< 0.05$) in the experimental groups and control group. Values of MCV, MCH and MCHC of experimental species were compared to the control groups. The results of examinations of the biochemical blood plasma profile indicate a marked neurotoxic effect of diazinon in fishes. Changes in values of both erythrocyte and leukocyte profile after exposure to diazinon-based preparation may be referred to disruption of haematopoiesis as well as to a decrease on non-specific immunity of the fish.

Key word: Diazinon, Common carp, Hematological parametrs, Biochemical, Blood

INTRODUCTION

Organophosphorous pesticides have fully replaced the persistent chlorinated pesticides in the 1970's and on the beginning of 1980's. The main advantage of the organophosphorous pesticides was their low cumulative ability and short-term persistence in the environment (Svoboda, *et al.,* 2001). Diazinon [0, 0-diethyl-0-(2-isopropyl-6-methylpyrimidin-4yl) phosphorothioate] is a contact organophosphate pesticide and extensively used, both in agriculture and households to control insects in soil, plants, fruit, and vegetable crops. After its application on crops and plants, diazinon is easily washed into surface waters and enters the ground water. Eventually, it enters the aquatic environment in large quantities (Kuivila and Foe, 1995). Diazinon degrades rapidly, but under conditions of low temperature, low moisture, high alkalinity, and lack of suitable microbiological degraders, it may remain biologically active in soils for six months or longer. Because of its aquatic distribution, diazinon affects a wide range of non-target organisms, like invertebrates, mammals, birds, and fishes, especially those inhabiting aquatic environment (Burkepile, *et al.,* 2000) Due to its chemical properties, widespread use, and application, diazinon is frequently found in point sources (wastewater treatment plant effluent) and non-point sources (storm water runoff) in urban and agricultural areas. Diazinon is known to be extremely toxic to birds and aquatic life (USEPA, 2005). Diazinon is transported into rivers largely via storm water runoff, with rain events producing pesticide pulses in rivers and streams (Ferrari, *et al*., 1997).

Teleost fish may be good indicators of contamination by pollutants because their

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biochemical responses are quite similar to those found in mammals. The response of some aquatic organisms to pollutants has been studied through the measurement of hematological and physiological parameters (Begum, 2004). The major reason for carrying out toxicity tests with fish and other aquatic organism is to determine which concentrations of substances are harmful to the organisms and which have no apparent effect. A second objective toxicity tests is to monitor the toxicity of effluents or evaluate the quality of surface waters. Using fish to assess the quality of and meaningful procedure, especially if many waste substances are present or if it is not known exactly what is present.

Hematological and physiological research, along with histopathology, is the major means to learn a toxicants mode of action. Hematological and clinical chemistry parameters can be detected rapidly and hence can be used for prediction and diagnosis of pesticide toxicity. Alterations in these parameters show toxic stress in the treated animals especially on blood and blood-forming organs (Rahman and Siddiqui, 2006). The purposes of this study were to evaluate the chronic toxicity of Diazinon on anemia by determining hematological and biochemical indices, and to establish a possible relationship among alterations in hematological indices, anemia, plasma biochemical profile and changes in behaviors and mortality of common carp.

MATERIALS & METHODS

Healthy common carp (mean body weight 265.0 ± 22.6 g) were purchased from a fish farm affiliated to Veterinary Department, Tehran University in Iran, and were transported to the laboratory of Fishery Department. The experiment was conducted with a water temperature of 25 ± 1ºC and a dissolved oxygen concentration between 6.0 to 7 mg/L by continuous aerating. Fish were allowed to acclimate for 14 days prior to experimentation in 200 L fiberglass tank containing dechlorinated tap water and were fed with commercial common carp food at a rate of 2.0% of body weight per day.

Ten acclimated fish without administration were expressed as 0 h and sampled 48h prior to experiment. A total of acclimated common carp (N=60) were exposed to Diazinon. Fish in the low dose group (N=30) and high dose group (N=30) were exposed to Diazinon at 60 µg/L and 120µg/L, respectively. The 30 fish in each dose group and the control were respectively divided equally into 9 tanks. Three sampling points were set during a period of 30 days in the experiment (10, 20 and 30 days post treatment). At each sampling point, 10 fish (from 3 tanks) for each dose group and the control were anaesthetized with whit carnation powder at a dilution 1:5000. Blood samples were taken by caudal puncture with heparinized syringes. Blood was centrifuged at 3000 rpm for 15 min at -4 ºC and plasma was stored at -70 ºC until analysis.

Hematological and biochemical parameters were measured in triplicates, and averaged for statistical use. Red blood cell counts (RBC) and white blood cell counts (WBC) were determined by hemocytometer method (Stevens, 1997). Hematocrit (Ht v/v ratio or %) was determined by microhematocrit method (Goldenfarb, *et al.,* 1971) and hemoglobin concentrations (Hb g/L) were determined by cyanometahemoglobin method (lee, *et al.,* 1998). Red cell indices, mean corpuscular volume (MCV: μm^3/cell), mean corpuscular hemoglobin (MCH: pg/cell), and mean corpuscular hemoglobin concentration (MCHC: g l^{-1}) were calculated from RBC, Ht, and Hb according to Lee et al. (1998) as follows:
MCV [µm3 cell-1] = Ht [v/v ratio] ×1000/RBC [106 cell µl-1], MCH [pg cell-1] = Hb [g l-1] / RBC [106celll-1] and MCHC [g l-1] = Hb [g l-1] ×10/Ht [v/v ration].

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were estimated according to Yatzidis (1960). The enzyme activity was expressed as µmol of product formed/mg protein/h. The products formed by enzyme action are glutamate and oxaloacetate for AST and glutamate and pyruvate for ALT. Oxaloacetate formed in the AST is unstable and immediately converted into pyruvate. Hence, pyruvate standard was used in both enzyme estimations. Lactate dehydrogenase (LDH) activity was determined by the method of McQueen (1975), with NADH oxidation recorded at 340 nm. The results were expressed as µmol formazan formed/mg protein/h. alkaline phosphatase (ALP) was estimated with sodium β-glycerophosphate as substrate and measuring

phenol was spectrophotometrically done following Moss, *et al.* (1971). Enzyme activity was expressed as µmol of phenol formed/mg protein/ h. Glucose was estimated by the enzymatic method of Man Company recipe. Total protein was determined by the Modi-biuret method.

Statistical significance in each experiment was determined using a one-way analysis of variance (ANOVA) and a Tukey test ($\alpha = 0.05$) A value of $P < 0.05$ was considered significant.

RESULTS & DISCUSSION

However, in both dose groups, fish exhibited uneasiness and frantic swimming behavior during exposed to diazinon, and then showed sluggish swimming. Treated fish also lost swimming coordination and buoyancy control with elevation of opercular beat rate, which increased with time. The depression and lethargy became more pronounced in the high dose group with increasing duration of the experiment. But, no mortality was found in both the experimental groups and the control.

The mean hematological indices of the common carp in experimental groups and control are shown in Table 1 and 2. Compared to the control specimens, those after the sub-lethal exposure to diazinon had significantly lower ($p < 0.05$) erythrocyte count, haemoglobin content and haematocrit, but a parallel increase in MCV and MCH values were recorded in experimental groups. There were no significant changes in MCHC between the treatments and the control. It was evident that the sub-lethal exposure to diazinon resulted in lower leukocyte count ($p < 0.05$), as well as lymphocyte and monocyte count ($p < 0.05$). In contrary, there was an increase in neutrophile granulocytes.

Results of biochemical blood plasma profile of the control and experimental groups under study are given in Figs. 1 to 3. Fish exhibited significantly ($p < 0.05$) higher AST, ALT and LDH activities in plasma during exposure to diazinon. Whereas, a significant reduction in the ALP levels in plasma was observed during exposure tenure. No significant differences in the total protein levels in plasma were observed between the experimental groups and control group. Glucose content was increased in plasma following 10, 20 and 30 days of exposure to diazinon in comparison to controls.

Table 1.Changes in erythrocyte profile of common carp exposed to different concentrations of diazinon (0, 60 and 120 µg/L) in 10, 20 and 30 days

Treatment	Factor	RBC (10^6cells)	Hb (g/100ml)	Hct (%)	MCH (10^{-5}pg)	MCV ($10^{-4}mm^3$)	MCHC (%)
10^{th} days	1	2.0244 ±0.1623[a]	7.8667 ±0.4924[a]	36.000 ±1.225[a]	3.91 ±0.417[a]	2.39 ±1.22[a]	21.777 ±1.428[a]
	2	1.5556 ±0.1236[b]	7.0111 ±0.3140[b]	33.333 ±1.000[b]	4.53 ±0.358[b]	2.16 ±0.195[a]	21.458 ±2.114[a]
	3	1.3622 ±0.0750[c]	6.9944 ±0.1304[b]	32.111 ±1.167[b]	5.15 ±0.273[c]	2.36 ±0.155[a]	21.803 ±0.761[a]
20^{th} days	1	1.9956 ±0.1200[a]	7.9222 ±0.3667[a]	36.000 ±0.707[a]	3.98 ±0.292[a]	1.81 ±0.116[a]	21.901 ±1.201[a]
	2	1.4233 ±0.1356[b]	6.8633 ±0.2238[b]	33.000 ±1.118[b]	4.86 ±0.455[b]	2.34 ±0.240[b]	20.814 ±0.841[a]
	3	1.2267 ±0.0931[c]	6.7644 ±0.1786[b]	31.667 ±1.225[c]	5.54 ±0.447[c]	2.59 ±0.214[c]	21.382 ±0.792[a]
30^{th} days	1	2.0344 ±0.1181[a]	7.9222 ±0.3032[a]	36.333 ±0.707[a]	3.90 ±0.185[a]	1.79 ±0.124[a]	21.819 ±1.109[a]
	2	1.4211 ±0.1700[b]	6.6244 ±0.3254[b]	32.778 ±1.641[b]	4.73 ±0.710[b]	2.34 ±0.321[b]	20.263 ±1.516[a]
	3	1.1822 ±0.1011[c]	6.3289 ±0.6562[b]	29.667 ±1.581[c]	5.38 ±0.680[b]	2.54 ±0.321[b]	21.439 ±2.905[a]

Blood parameter values in rows with different letters significantly differ ($p < 0.05$). Each value is a means ± standard error of 9 individual observations

Table 2. Changes in leukocyte profile of common carp exposed to different concentrations of diazinon (0, 60 and 120 µg/l) in 10, 20 and 30 days

Factor		WBC	Lymphocyte	Monocyte	Eosinophil	Neutrophile	Basophile
Treatment							
10th days	1	14.089 ±0.706[a]	89.333 ±0.707[a]	2.7778 ±0.8333[a]	2.4444 ±0.7265[a]	2.778 ±0.833[a]	2.6667 ±1.0000[a]
	2	11.861 ±0.628[b]	86.667 ±1.414[b]	2.3333 ±0.5000[ab]	3.3333 ±0.7071[b]	5.667 ±1.323[b]	1.7778 ±0.6667[ab]
	3	10.721 ±0.757[c]	84.111 ±1.054[c]	1.6667 ±0.8660[b]	4.1111 ±0.7817[b]	8.444 ±0.882[c]	1.6667 ±0.7071[b]
20th days	1	13.944 ±0.826[a]	88.889 ±1.054[a]	3.1111 ±0.7817[a]	2.5556 ±0.7265[a]	2.778 ±0.833[a]	2.6667 ±1.0000[a]
	2	11.674 ±0.720[b]	87.444 ±1.236[b]	2.2222 ±0.4410[b]	3.2222 ±0.6667[ab]	5.444 ±0.882[b]	1.7778 ±0.6667[ab]
	3	10.697 ±0.597[c]	84.111 ±1.364[c]	1.3333 ±0.5000[c]	3.8889 ±0.7817[b]	9.222 ±1.394[c]	1.6667 ±0.7071[b]
30th days	1	13.994 ±1.180[a]	89.222 ±0.972[a]	2.6667 ±0.7071[a]	2.4444 ±0.7265[a]	2.778 ±0.833[a]	2.6667 ±1.0000[a]
	2	1.366 ±0.141[b]	86.889 ±1.833[b]	1.7778 ±0.4410[b]	3.1111 ±0.6009[ab]	6.444 ±1.424[b]	1.7778 ±0.6667[ab]
	3	10.709 ±0.803[c]	85.667 ±2.121[b]	1.3333 ±0.5000[b]	3.3333 ±0.7071[b]	8.333 ±1.581[c]	1.6667 ±0.7071[b]

Blood parameter values in rows with different letters significantly differ ($p < 0.05$). Each value is a means ± standard error of 9 individual observations

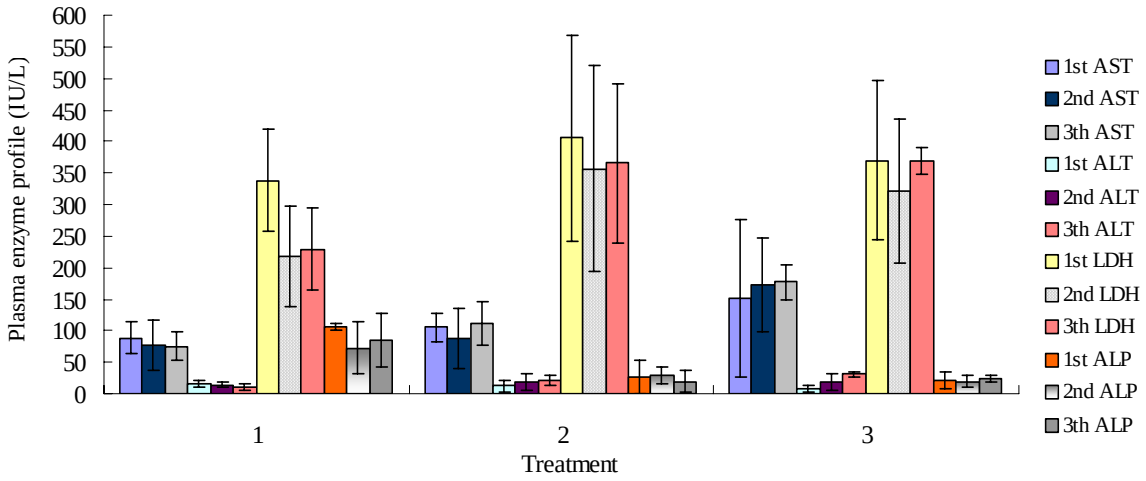

Fig. 1. Changes in AST, ALT, LDH and ALP (IU/L) activities of common carp during sub-lethal exposed to 0.0, 60 and 120 µg/L of diazinon in 10, 20 and 30 days . Values are means ± SD (n = 9). Different letters show significant differences among exposure concentrations (P<0.05).

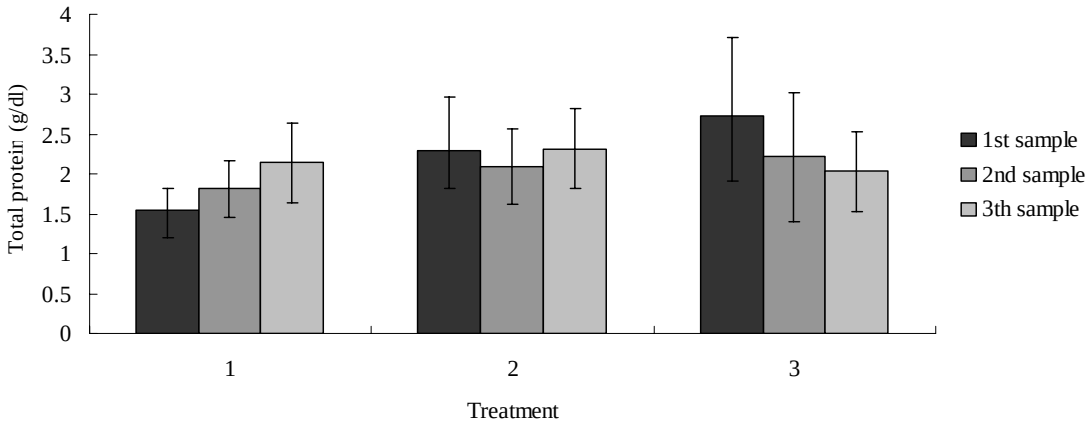

Fig. 2. Changes in levels of total protein (g/dl) in plasma of common carp during sub-lethal exposed to 0.0, 60 and 120 µg/L of diazinon in 10, 20 and 30 days. Values are means ± SD (n = 9). Different letters show significant differences among exposure concentrations (P<0.05)

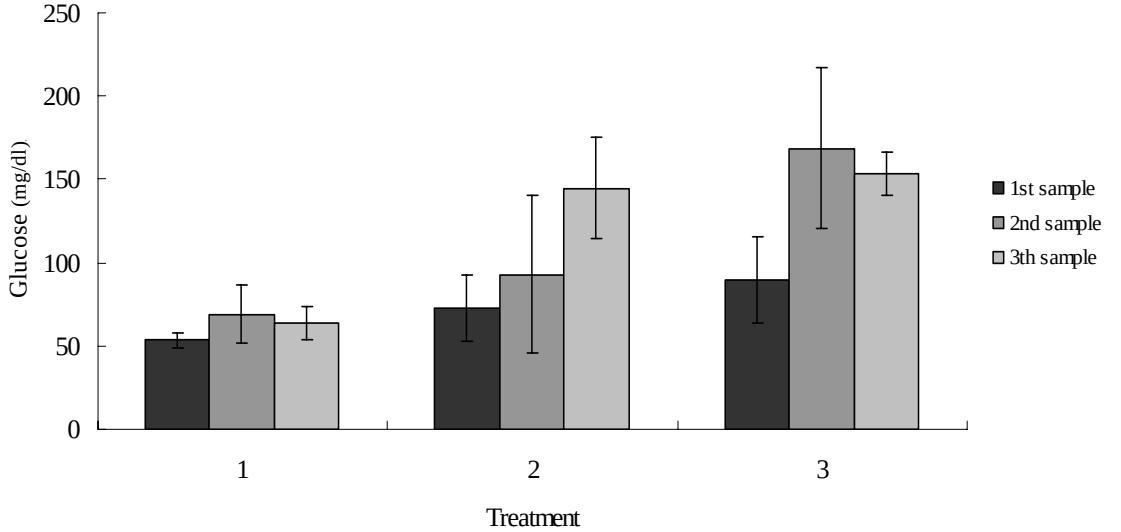

Fig. 3. Changes in levels of glucose (mg/dl) in plasma of common carp during sub-lethal exposed to 0.0, 60 and 120 µg/L of diazinon in 10, 20 and 30 days. Values are means ± SD (n = 9). Different letters show significant differences among exposure concentrations (P<0.05)

Pesticides applied to the land may be washed into surface waters and may kill or at least adversely influence the life of aquatic organisms (Hill, 1989; Hayes and Laws, 1991). Fish are extremely sensitive to the neurotoxic effects of these pesticides.The effect of exposure to a sub-lethal concentration of diazinon (60 and 120 µg/L) on enzyme activities in plasma and Hematological parameters of the freshwater teleost fish, *Cyprinus carpio*, was studied during 10, 20 and 30 days of exposure. The fish under pesticide stress showed symptoms of dullness, loss of equilibrium, loss of feeding and erratic swimming.

The main hematological response of common carp to chronic exposure to diazinon based

organophosphorous pesticide in 60 and 120 µg/L concentrations was a significant decrease ($p < 0.05$) of erythrocyte count, hematocrit value and hemoglobin content compared to the control group. A low red cell or hemoglobin count indicates anemia, or severe bleeding. Low hemoglobin usually means the animal has anemia. Anemia results from conditions that decrease the number or size of red cells, such as excessive bleeding, a dietary deficiency, destruction of cells because of a transfusion reaction or mechanical heart valve, or abnormality formed hemoglobin (Hisa and Connie, 1998). Decreases in the number or size of red cells also decrease the amount of space they occupy, resulting in a lower hematocrit. A low hematocit, combined with abnormal blood tests, confirms the diagnosis.

Decreased erythrocyte count and haemoglobin content in freshwater fish *Channa punctatus*, (Anees, 1978) and *Cyprinus carpio* (Svoboda, *et al.,* 2001) after acute exposure to diazinon. Another type of hematological response to the effect of organophosphrous compounds was a significant increment of mean corpuscular volume (MCV) associated with increase of hematocrit value and drop of MCHC. MCV is the index most often used. It measures the average volume of red blood cell by dividing the hematocrit by RBC. The MCV categorizes red blood cells by size. Under a microscope, stained red blood cells with a high MCV appear larger than cells with a normal or low MCV. Mean corpuscular hemoglobin (MCH) measures the average amount of hemoglobin within a red cell. A similar measurement, mean corpuscular hemoglobin concentration (MCHC), expresses the average concentration of hemoglobin in the red blood cells. In contrary, values of MCV, MCH and MCHC registered in during exposure to diazinon based pesticide in 60 and 120 µg/L concentrations to common carp were comparable with the control group. Alteration in values of MCV, MCH and MCHC in *Cyprinus carpio* (Svoboda *et al*., 2001) was reported.

The white blood cell (WBC) count determines the total number of white cells (leukocytes) in blood sample. Fewer in number than the red cells, WBC are the body's primary means of fighting infection. There are five main types of white cells (lymphocytes, monocytes, neutrophile, eosinophile and basophiles), each of which plays a different role in responding to presence of foreign organisms in the body. A differential white cell count is done by staining a smear of the fish blood with a Wrigh's stain, allowing the different types of white cells to be clearly seen under the microscope.

The number of white blood cells may increase or decrease significantly in certain diseases. We observed significant decrease ($p<0.05$) of leukocyte count of common carp in during exposure to sub-lethal concentration of diazinon. A low white blood cell count may mean dysfunction in hematological tissues (spleen and kidney) or certain infectious diseases. Lower than normal levels of lymphocytes (lymphopenia) can be an indicator of immune system deficiency. Poisonous substances treatments can also deplete the body's supply of lymphocytes, as can exposure to diazinon. Lymphopenia as a consequence of methyparathion based pesticide was reported by Nat and Banerjee (1996) in *heteropneustes fossilis* and also by Siwicki, *et al.,* (1990) in common carp after an acute effect of trichlorfon. Decreased in lymphocyte and monocyte percentage in smear were showed in *Cyprinus carpio* (Svoboda, *et al.,* 2001). In contrary, we observed significant increment ($p < 0.05$) of neutrophile percentage in smear of common carp in during exposure to 60 and 120 µg/L concentration of diazinon. The most common and important cause of neutrophilia is infection, and most infections cause neutrophilia. The degree of elevation often indicates the severity of the infection. Tissue damage from other causes raises the neutrophile for similar reasons. Poisonings, and severe disease, like kidney failure all cause neutrophilia (Holland, *et al.,* 1997). Ghosh and Banerjee (1993) reported lymphopenia and increased in both neutrophile and eosinophile in *heteropneustes fossilis*, after an effect of dimethoate in 96h LC_{50} concentration.

AST, ALT and LDH are found in heart, liver, skeletal muscle, kidney, pancreas, spleen, lung (gill), red blood cells, and brain tissue. When disease or injury affects theses tissues and the cells are destroyed, especially liver, AST and ALT are released into the bloodstream. The amount of AST is directly related to the number of cells affected by the disease or injury (Pagana and Kathleen Deska, 1998; Abdolahi and Gazi

Khonsari, 1380). Also, the LDH test is used to detect tissue alterations and as an aid in the diagnosis of anemia, gill and liver disease (Khazraiinia, *et al.,* 1379).

Fish exhibited higher AST and ALT activities in plasma during exposure to diazinon than control group ($p < 0.05$). The increase in the activity of aminotransferases in plasma may be due to liver damage, which results in the liberation of these intercellular enzymes and raise plasma aminotransferase levels (Venkateswara Rao, 2006). A briefly elevated AST and ALT that reveled in treatment groups may indicated the cells of liver, spleen, kidney and others tissue are damaged. Serum AST and ALT of eel increase by 20% as the animals were exposed to deltametrin (Balint, *et al.,*1997). Experiments with *C. carpio* exposed to 2,4-Diamin showed an inhibition of ALT and AST activities in the serum after 30 days (Oruc and Üner, 1999). Poleksý´ c and Karan (1999) observed an increase in activities of this enzyme in the liver and serum of C. carpio exposed to 0.02 mg/L of trifluralin herbicide. Vel´ý¡sek, *et al.,* (2006) observed a significant increase ($p < 0.05$) in AST and ALT levels in carp after acute exposure to deltamethrin in concentration of 3.25-g/L. Also, alterations in ALT and AST activities in plasma of silver catfish *R.quelen* during exposure to clomazone are reported by Lazzari, *et al.,* (2006).

Also, chronic exposure of rats and mice to OPs led to increased levels of serum ALT and AST (Gomes, *et al.,* 1999). Kossmann, *et al.,* (1997) observed an elevation of AST and ALT in workers engaged in the production of chlorphenvifos, an OP compound. Likewise, a positive association between occupational exposure to pesticides and increased AST, ALT levels was found with OPs (Zarei, *et al.,* 1376). Our data also pinpoint a role for pesticides on LDH, because higher enzyme levels ($p < 0.05$) were observed in both experimental groups than control. On the other hand, the observed significance of lower LDH activity at the control group compared with treatment to exposure to diazinon. The LDH is also elevated in disruption of the liver, in certain types of anemia, and in cases of excessive destruction of cells, as in liver damage, and shock (Pagana and Kathleen Deska, 1998). Some pesticides, such as OPs, organochlorines and pyrethroids are able to cause inhibition of LDH (El-Demerdash, *et al.,* 2001).

On the contrary, most authors report increased plasma LDH concentration in various fishes e.g. *A.anguilla* (Ceron, *et al.,* 1997 and Sancho et al., 1997), *Puntius conchonius* (Gille, *et al.,* 1990), *Heteropneustes fossilis* (Singh and Srivastava, 1982) and *Channa striatus* (Natarajan, 1989) following acute effects of organophosphorous pesticides including diazinon. The levels of LDH were increased with cypermethrin according to (Philip, *et al.,* 1995) and (Das and Mukherjee, 2003) in *Labeo rohita.*

The alkaline phosphatase of the liver is produced by the cells lining the small bile ducts in liver. Alkaline phosphatase is an enzyme found throughout the body (Pagana and Kathleen Deska, 1998). According to our results, the significantly decreased activity of this enzyme in the blood plasma of our carp exposed to diazinon ($p < 0.05$). On the other hand, the activities alkaline phosphatases in blood plasma were almost identical in the control and experimental groups of carp. On the other hand, Dobsikova, *et al.,* (2006) found decreased ALP levels in blood plasma of common carp (*Cyprinus carpio* L.) exposed to cypermetrhin (96 h LC_{50}).

Increased total protein levels are seen in dehydration, in some cases of chronic liver disease. Decreased protein levels may be seen in starvation, and malabsorption or malnutrition (Pagana and Kathleen Deska, 1998). There are not significant differences in total protein levels between the control and treatment groups. Organophosphates are known to methylate and phosphorylate cellular proteins directly (Wild, 1975). Shanmugam (1977) suggested that tissue proteins were broken down to maintain plasma proteins in a condition of protein deficiency.

The significant differences in glucose concentrations in plasma ($p < 0.05$) between the control and treatment fish, following the action of diazinon, which may be considered to be the manifestation of stress. In agreement with our results, Ceron, *et al.,* (1997) report significant glucose increase in common eel (*Anguilla anguilla*) following a 96 h action of sub-lethal concentrations of diazinon. Bhatia, *et al.,* (1972) and Weiss, *et al.,* (1984) reported a pronounced

increase in blood sugar level which was going parallel to the inhibition of the cholinesterase and the appearance of manifestation of cholinergic stimulation as a result of parathion intoxication. Glucose increase is a general response of fish to acute pollutant effects, including organophosphates (Svobodova, 1971; Srivastava, 1981; Singh and Srivastava, 1982; Mishra and Srivastava, 1983; Natarajan, 1989; Gill, *et al.,* 1990; Balint, *et al.,* 1995; Sancho, *et al.,* 1997). Plasma glucose was elevated in treated silver catfish *Rhamdia quelen* after all periods of clomazone exposure (Lazzari, *et al.,* 2006).

CONCLUSION

The changes in levels of erythrocyte, red blood index and lymphocytes (lymphopenia) can be an indicator of anemia and immune system deficiency, respectively. Poisonous substances treatments can also deplete the body's supply of lymphocytes, as can expose to diazinon. The present biochemical estimations in carp, sub-lethal intoxicated with diazinon suggests that the treated fish are faced with a serious metabolic crisis. The results revealed that diazinon affects the intermediary metabolism of *C.carpio* at many levels and the increase of biomarker enzymes in plasma, might be indicative of liver necrosis. The other hand, our results showed that anemia and alteration in the plasma enzymes profile caused by kidney, spleen and liver impairment. The above results clearly indicate that the usage of these pesticides has brought out great concern in the scientific community on the possible toxic effects of pesticide contaminations to both aquatic flora and fauna as well as to humans.

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