Iranian Journal Fisheries Sciences 5(2) 67-82 (2006)

Determination of diazinon LC_{50} in Grass carp (*Ctenopharyngodon idella*) and the effect of sublethal concentration of toxin on some hematological and biochemical indices

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Received: July 2005 Accepted: November 2005

Abstract: Toxicity of the organophosphate, diazinon was studied in grass carp (*Cetenopharyngodon idella*) weighing 5±1.0g, under static water quality condition at 16°C. Also, some hematological and biochemical variables of fish weighing 50±5.0g were studied 12 hours post-exposing fish to the toxicant at sublethal concentration of 5.6mg/L at 16°C. Values of 18.19, 17.21, 16.68, and 15.13mg/L, were obtained as LC_{50} after 24, 48, 72 and 96 hours post-exposing fish to the toxicant, respectively. Levels of red blood cells (RBC), hematocrit (PCV), hemoglobin (Hb), mean corpuscular hemoglobin concentration (MCHC), leukocyte count (WBC), lymphocyte and monocyte were significantly lower in the

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exposed fish than control fish ($p<0.05$), whilst values of polymorphonuclear cells (PMN) and myelocytes counts were higher in the test group ($p<0.05$). In addition, the levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were significantly lower in fish exposed to the toxicant ($p<0.05$), while the level of alanine aminotransferase (ALT) was higher in experimental fish ($p<0.05$) than control fish. The levels of glucose and triglyceride were higher in fish exposed to toxicant than control fish. However, such differences were only significant for glucose level ($p<0.05$). In contrast, the levels of total protein and cholesterol decreased insignificantly in fish exposed to the toxicant compared to those in control fish ($p>0.05$). The obtained results show that although diazinon can be classified as a slight toxic chemical for grass carp, the toxicant negatively affects some immunophysiological functions of the fish including immunocompent cells.

Keywords: diazinon, hematology, grass carp, acute toxicity and LC_{50}

Introduction

The increased used of toxic chemicals as pesticides and/or insecticides in agricultural industry can seriously affect aquatic animals including fresh water fish (Tsuda *et al.*, 1997). This is very critical when leaching of such chemical substances into water continuous for a substantial long period. Results of studies have shown that such toxic chemicals could affect fish health conditions at various levels including fish immune system resulting in an increase in fish susceptibility to infectious diseases (Keizer *et al.*, 1991, 1993 ; Sancho *et al.*, 1992, 1997 ; Tsuda *et al.*, 1997 ; Bailey *et al.*, 2000 ; Svoboda *et al.*, 2001 ; Luskova *et al.*, 2002). Diazinon is an organophosphorus chemical possessing a wide range of insecticidal activities, and has been used in large amounts in many countries including Iran (Eisler, 1986; Moore & Waring, 1996 ; Nasritajan, 1996 ; Aminirad, (1997) ; Talebi, 1998 ; Piri & Ordog, 1999 ; Piri *et al.*, 1999). The exhibitions of poisoning and asphyxia signs due to inhibition of acetylcholinesterase (AChE) by organophosphorous toxins such as diazinon have been well documented (Eisler, 1986 ; Gallo & Lawryk, 1991; Luskova *et al.*, 2002). Also some workers have reported the susceptibility of different fish species to various organophosphorus chemicals including diazinon (Sancho *et al.*, 1993 ; Dutta *et al.*, 1997 ; Wall, 2000; Luskova *et al.*, 2002 ; Svoboda *et al*, 2001). However, little attention has been paid to elucidate the relationship between the blood parameters and pesticide in grass

carp, particularly with reference to organophosphorus formulations. This study was undertaken to determine the acute toxicity of diazinon and to evaluate its sublethal concentration on some hematological and plasma biochemical parameters of grass carp.

Materials and Mathods

Fish and maintenance

Apparently healthy grass carp weighing 5±1.0g (200 fish) and 50±5.0g (20) were used. The fish were obtained from a fish farm in Mazandaran province, North of Iran. Fish were acclimated for four days before being used for the experiment. Water quality conditions maintained at pH 7.5-8, total alkalinity 326mg/L, acidity 40mg/L, NH_3< 0.01mg/L, NO_2<0.1mg/L, temperature 16±1.0°C, dissolved oxygen 7-9mg/L and total hardness 310-430mg/L and monitored daily (APHA, 1980). Fish were not fed during the experiments.

Toxicant

Diazinon[O-O-diethylO-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphorothioate] (Eisler, 2000) (Shimi Keshavarz Co. Maccidal EC 600) obtained from a local market was used as the test chemical. Eight concentrations consisting of 160, 80, 40, 20, 10, 5, 2.5, 1.25, and 0.0mg/L of the toxicant were used.

Acute toxicity

Determination of survival rate:

Before implementation of the study it was necessary to check the health status of the fish. The toxicity tests, which were done to determine the concentration of diazinon being lethal to 50% of the test fish which in 96h (96-h LC_{50}), were conducted under static conditions in 250-L tanks. The fish were kept in tanks with good aeration and the mortality rate was recorded for 8 days.

Determination of limited lethal concentration of toxicant:

The death limit test was undertaken using 5 groups of fish in triplicates. Each replicate of 10 fish kept in the same mentioned water quality conditions. Diazinon

was added at concentrations of 160, 80, 40, 20, 10, 5, 2.5, 1.25, and 0.0mg/L (by geometrically serial dilutions). The mortality rate was then recorded daily for 4 days.

Determination of LC_{50} 96 hours:

Five concentrations of diazinon namely 10.0, 12.5, 15.0, 17.5 and 20.0 mg/L were used in 3replicates each. The acute toxicity (LC_{501} -96 hours) level was then measured according to probity method (Wardlaw, 1985) as the standard method recommended by Organization of Economic Cooperation and Development (OECD, 1984).

Data analysis

The concentration-response relationship was determined by a probit analysis (Finney, 1971). After determination of the mortality rate of fish exposed to different concentrations of diazinon, the percentages of mortality were compared with control and concentration logarithm and recorded numbers were obtained through probity value table. The obtained data were processed using SPSS. The regression equation was then calculated for different levels of LC_{10}, LC_{50} and LC_{90} during 96 hours.

Hematological and biochemical studies

Hematological and biochemical studies were carried out using grass carps weighing 50±5.0g. A number of 20 fish were exposed to the toxicant at 5.6mg/L for 12 hours as a constant bath (Blaxhall, 1972 ; Behera *et al.*, 1990 ; Klont, 1994; Svoboda *et al.*, 2001). A control group, without diazinon bath was also included and kept separately. Blood samples were obtained by caudal vein puncture into heparinized and non-heparinized tubes (1-1.5ml/tube). At the same time, blood smears were prepared for Geimsa staining. The smears were first air dried, fixed in 96% ethanol for 30 minutes, stained by Giemsa staining for 30 minutes and were examined for leucocyte differential count. The hematological parameters consisting of erythrocyte counts (RBC), hematocrit (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean

corpuscular hemoglobin concentration (MCHC), leukocyte count (WBC) and differential leukocyte count were measured.

The non-heparinized blood samples were centrifuged for 15 minutes at 400xg and the separated sera were used to determine the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), glucose, total protein, cholestrol and triglyceride using an automatic biochemistry analyzer (Eppendorf, Epos 5060). Results of hematology and biochemistry examinations were analyzed statistically by the analysis of variance (ANOVA) (Luskova *et al.*, 2002).

Results

Acute Toxicity

No mortality was observed in any groups at the end of 8 days. Also the limited lethal concentration was calculated at 10-20mg/L (Table1).

Table 1: Limited lethal concentration of diazinon in grass carps at 16±1.0°C (n= 150)

Mortality after 96 hours (%)	Concentration of diazinon (mg/L)
100	160.0
100	80.0
100	40.0
100	**20.0**
00.0	**10.0**
00.0	5.0
00.0	2.5
00.0	1.25
00.0	00.0

The minimum concentration to cause 100% mortality and the maximum concentration causing no mortality were calculated 20 and 10mg/L, respectively. Therefore, five concentrations of 10, 12.5, 15, 17.5, and 20mg/L of toxicant were selected to determine the LC_{50} 96 hours.

The results of LC_{10}, LC_{50}, and LC_{90} measurements after 24, 48, 72, and 96 hours are shown in table 2 and figure 1. LC_{50} of diazinon after 24, 48, 72, and 96 hours were 18.19, 17.21, 16.68, and 15.13mg/L, respectively (Table2, Figure 1).

Table 2: Determination of LC_{10}, LC_{50} and LC_{90}- 96 hours of diazinon in grass carp at 16±1.0°C (n= 150).

	LC \ Time post-exposure(h)	24	48	72	96
Diazinon Concentration (mg/L)	LC_{10}	15.13	14.11	13.89	13.4
	LC_{50}	18.19	17.21	16.68	15.13
	LC_{90}	21.87	20.11	18.97	16.98

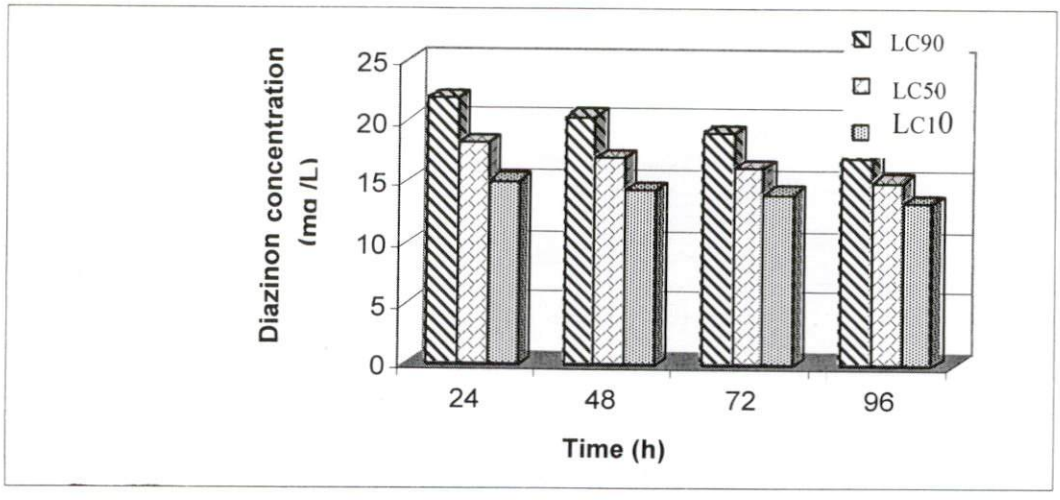

Figure 1: Acute toxicity of diazinon in grass carp at 16±1.0°C (n= 150)

Hematology

Compared to the control group, fish exposed to diazinon had lower values of RBC, PCV, Hb and MCHC ($p<0.05$)(Table 3). Also, values of WBC, lymphocyte and monocyte counts in the test group were lower ($p<0.05$) than those in the control group, while there was an increase in the level of PMN ($p<0.05$) and myelocytes counts in the test group compared to the control group (Table 4).

Table 3: Erythrocyte profile of grass carp following exposure to sublethal concentration diazinon (5.6 mg/L) at 16±1.0°C.

Indices	Treatment	N	Mean ± S.E
PCV (%)	Control	5	33.36±2.00
	Experiment	15	20.66±1.12*
Hb (g/100ml)	Control	5	8.82±2.00
	Experiment	15	4.46±1.12*
MCV(m)	Control	5	$1x10^{-4}$±2.00
	Experiment	15	$2x10^{-4}$±1.12
MCH (pg)	Control	5	$4x10^{-5}$±2.00
	Experiment	15	$4x10^{-5}$±1.12
MCHC (%)	Control	5	26.46±2.00
	Experiment	15	21.86±1.12*
RBC ($10/mm^3$)	Control	5	2.06±2.00
	Experiment	15	0.15±1.12*

PCV=Packed Cell Volum, Hb=Hemoglobin, MCV=Mean Corpuscular Volume, MCH=Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration, RBC=Red Blood Cell

*** indicative of signifiant différence at p< 0. 05**

Biochemical

The levels of AST, ALP and LDH were significantly lower in fish exposed to the toxicant compared to those in the control fish ($p<0.05$) (Table 5). However, ALT activity was higher in experimental fish than that in the control group ($p<0.05$). In addition, levels of glucose and triglyceride were higher in fish exposed to diazinon than those in the control group (Table 5). However, such differences were only significant for glucose levels ($p<0.05$). The concentration of total protein and cholesterol decreased in fish exposed to the toxicant compared to the control fish. Such differences were however, insignificant ($p> 0.05$).

Table 3: Leucocyte profile of grass carp following exposure to sublethal concentration diazinon (5.6mg/L) at 16±1.0°C.

Indices	Treatment	N	Mean ± S.E
WBC (/mm³)	Control	5	7320.00±2.00
	Experiment	15	3116.66±1.12*
Lymphocyte (%)	Control	5	87.80±2.00
	Experiment	15	82.33±1.12*
PMN (%)	Control	5	7.40±2.00
	Experiment	15	12.79±1.12*
Myelocyte (%)	Control	5	2.40±2.00
	Experiment	15	4.26±1.12*
Monocyte (%)	Control	5	2.40±2.00
	Experiment	15	0.66±1.12*

WBC= White Blood Cell, PMN= Polymorphonuclear

*** indicative of signifiant différence at p< 0. 05**

Table 4: The effect of diazinon (5.6mg/L) on enzyme activities, cholesterol, triglyceride, glucose and total protein of blood plasma of grass carp at 16±1.0°C.

Indices	Treatment	N	Mean ± S.E
AST (µ/L)	Control	5	258.40±13.04
	Experiment	15	200.66±6.89*
ALP (µ/L)	Control	5	218.40±2.78
	Experiment	15	170.53±9.16*
ALT (µ/L)	Control	5	9.00±0.31
	Experiment	15	15.60±0.86*
LDH (µ/L)	Control	5	1624.80±42.09
	Experiment	15	1324.33±60.77*
Cholesterol (mg/dl)	Control	5	196.00±91.00
	Experiment	15	162.40±12.98
Triglyceride (mg/dl)	Control	5	84.50±41.50
	Experiment	15	96.40±6.32
Glucose (mg/dl)	Control	5	38.60±0.50
	Experiment	15	51.53±3.08
Total protein (mg/dl)	Control	5	2.24±0.09
	Experiment	15	1.78±0.06*

ALT=Alanine Aminotransferase, AST=Aspartate Aminotransferase, ALP=Alkaline Phosphatase, LDH=Lactate Dehydrogenase

*** indicative of signifiant différence at p< 0. 05**

Discussion

Based on the registered 96 hour LC_{50} values (15.13mg/L) obtained in this study, diazinon could be classified as slightly toxic for grass carp. In the report by Nasritajan (1996) the 96h LC_{50} using 5% granule and 60% emulsion of diazinon on *Abramis brama* fingerlings (1-3g) was 256 and 8.1mg/L respectively.

The 96h LC_{50} of diazinon (60% emulsion) in silver carp (*Hypophthalmichthys molitrix*) (Piri & Ordog, 1999) and *Rutilus frissi kutum* (Piri *et al.*, 1999) was measured at 1.9 and 0.34mg/L, respectively. In studies by Svoboda *et al.* (2001), the 96h LC_{50} of diazinon on common carp was calculated at 16mg/L using semi static test conditions. While, the 96h LC_{50} values of 9 and 8mg/L were reported for *Carassius auratus* (Eisler, 1986) and *Brachydanio rerio* (Keizer *et al.*, 1991), respectively. The specific sensitivity of fish to diazinon might be associated with different abilities of absorption, acetylcholinestrase inhibition and detoxication. It might also depend upon the water quality parameters. Although, species, size and weight differences of fish might make it difficult to compare the acute toxicity results among the different studies (Oh *et al.*, 1991). However, it seemed that both species of grass carp weighing 5±1.0g used in this study and common carp weighing 9±3.2g in the studies by Svoboda *et al.* (2001) had the identical sensitivity to diazinon.

Significant decrease occurred in the levels of RBC, PCV, Hb, MCHC, WBC and lymphocyte counts of grass carp exposed to sublethal concentrations of 5.6mg/L of diazinon compared to those in the control fish. However, such differences were insignificant ($p>0.05$).The levels of granulocytes and myelocytes were also significantly increased in the exposed fish. Similar results on RBC, Hb and PCV were reported by Svoboda *et al.* (2001), while values of MCV, MCHC in common carp exposed to acute level of diazinon at 19-21°C were identical to control group ($p>0.05$). Results of other studies also shows that other effective substances of organophosphourus pesticides, similar to diazinon, affect hematopoiesis follow by anemia induction in fish (Anees, 1978 ; Khattak & Hafeez, 1996 ; Tavares *et al.*, 1999). In addition, significant decrease in leucoytes count and occurrence of lymphopenia and granulocytosis characterized the

leukocyte profile in grass carp after exposure to diazinon at 16°C. Similar results were observed by Svoboda *et al.* (2001) after exposing common carp to acute concentrations of diazinon at 19-21°C. Therefore, a decrease in non-specific immunity in fish could be expected after acute exposure to organophosphorus pesticides due to decreased leuckocyte count and lymphopenia. Occurrence of lymphopenia in fish exposed to various pollutants have been also reported by a number of authors (e.g. Schwaiger *et al.*, 1993 ; Thakur & Sahai, 1993 ; Alkahem, 1994 and Svobodova *et al.*, 1996).

The levels of LDH, AST and ALP were found to be significantly lower in grass carp exposed to diazinon compare to control fish. Similar results were seen in level of LDH in *Channa punctata* and common carp following exposure to acute level of diazinon for 96-hour (Sastry & Sharma, 1980 ; Luskova *et al.*, 2002). The reduction in the LDH level in the exposed fish, as compared to that in the control group, might indicate that there has been a decrease in the glycolytic process due to the lower metabolic rate. Luskova *et al.* (2002) observed similar results when common carp were exposed to acute level of diazinon. However, several authors reported an increase in blood plasma LDH levels in various fish species e.g. carp and eel (Gill *et al.*, 1990 ; Ceron *et al.*, 1997 ; Sancho *et al.*, 1997 ; Luskova *et al.*, 2002). Sastry and Sharma (1980) and Cox (1992), reported a decrease in level of ALP after 96-hour exposing fish (*Ophiocephalus punctatus*, Channa) to diazinon. A reduction in level of ALP of grass carp also supported the assumption that the liver tissues of the experimental fish was affected by diazinon as necrosis of hepatocytes was seen under microscopic examination (Sharifpour, *et al.*, 2006). In addition, a decrease in the levels of ALT and AST in the experimental fish indicated that diazinon damagea the paranchymatous tissues or probably skeletal musculature. However, ALT was seen to be increased in grass carp exposed to diazinon. Compare to control fish also, a significant increase in level of glucose of experimental fish ($p<0.05$) may be considered to be the manifestation of stress as mentioned by Ceron *et al.* (1997) and Luskova *et al.* (2002), Balint *et al.*, (1995) and Sancho *et al.*, (1997). Exposing grass carp to diazinon also caused a significant decrease in protein concentration in the blood plasma compared to the control

group. Similar findings were observed when eel, *Cyprinion watsoni* and common carp were exposed to fenitrothion, malathion and diazinon, respectively (Khattak & Hafeez, 1996 ; Sancho *et al.*, 1997 ; Luskova *et al.* 2002).

In conclusion, the leaching of long lasting of diazinon into natural waters used for culturing of grass carp may lead to serious changes in fish physiological functions resulting in suppression of fish immune system, as significant leucopenia and lymphopenia were observed in this study. The decrease of LDH activity, an activator of glucose metabolism, indicated a decrease in intensity of this process in the experimental group due to toxic effect by diazinon. This hypothesis is also supported by an increase in glucose concentration test fish compare to control group. However, based on the registered 96 hour LC_{50} values (15.13mg/L) obtained in this study, diazinon could be classified as slightly toxic for grass carp.

Acknowledgement

This work was financially supported by Iranian Fisheries Research Organization, and Research Council of University of Tehran.

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