Some hematological and biochemical changes in blood serum of Grass carp (*Ctenopharyngodon idella*) vaccinated with *Aeromonas hydrophila* following exposure to sublethal concentration of diazinon

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Abstract

Diazinon is commonly used for pest control in the agricultural fields in north of Iran. This study was conducted to determine the chronic toxicity of organophosphorous pesticide (Diazinon) in vaccinated fish and its effects on some hematological parameters and biochemical blood plasma profiles of Grass Carp (*Ctenopharyngodon idella*).This experiment was carried out in three groups. The first group was vaccinated and exposed to diazinon (group A) while the second group was vaccinated and bathed with PBS bath (group B). The remaining fish were used as unvaccinated fish and were kept in clean water separately (group C). Diazinon was applied at concentrations of 2 mg/L for 12 hours since the experiments were initiated. The experimental groups(A and B) showed significantly lower values ($p < 0.05$) of erythrocyte count, haemoglobin content, haematocrit, leucocytes, Lymphocyte, myelocyte and monocyte, as well as in alkaline phosphatases, alanine aminotransferase, aspartate aminotransferase and, lactate dehydrogenase compared to the control group(C). Values of MCV, MCH and MCHC of experimental species (A and B) were compared to the control group(C). 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.

Keywords: Diazinon, Organophosphorous pesticide, Grass Carp, Hematological parameters

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Introduction

Pesticide use is known to cause serious environmental problems, especially in the dry season, because during this period the dilution capacity of the water systems is low, thus increasing the risk of high concentrations of toxic chemicals. Moreover, the dry season is often the critical period for many animals, especially fish and birds. Fish stocks suffer from natural mortality and high fishing pressure at the end of the dry season. Contamination of water by pesticides either directly or indirectly can lead to fish kills, reduced fish productivity or elevated concentrations of undesirable chemicals in edible fish tissue which can affect the health of humans eating these fishes (Adedeji et al., 2009).

Teleost fishes have proved to be good models to evaluate the toxicity and effects of contaminants on animals, since their biochemical responses are similar to those of mammals and of other vertebrates (Sancho et al., 2000; Kathya et al., 2010).

Hematological parameters such as haematocrit, haemoglobin, number of erythrocytes and white blood cells are indicators of toxicity with a wide potential for application in environmental monitoring and toxicity studies in aquatic animals (Sancho et al., 2000; Barcellos et al., 2003; Kathya et al., 2010). Some biochemical parameters also represent fine tools for evaluating the effects of contaminants and for environmental monitoring (Ahmad et al., 2004; Kathya et al., 2010).

Aeromonas hydrophila is the causative agent of the disease known as "haemorrhagic septicaemia", "ulcer disease" or "red-sore disease" (Guz and Kozinska, 2004). *A. hydrophila* is generally found in the gastrointestinal tract of fish and is considered an opportunistic pathogen. Most of bacteria, which are termed "opportunistic" usually, do not cause disease unless other factors are involved. *A. hydrophila* is always capable of producing disease if given the chance. Outbreaks of the disease are usually associated with a change in environmental conditions, such as stress, overcrowding, a sudden change of temperature, transfer of fish, mishandling, poor water quality, high nitrite and carbon dioxide levels (Adanir and Turutoglu, 2007).

The pathogenesis of *A. hydrophila* is multifactor. A variety of factors may contribute to the overall virulence of this bacterium. Extracellular products (ECPs) including toxins, autolysins, proteases and acetylcholinestrases (Ljungh and Wadstrom, 1982; Leung and Stevenson, 1988; Nieto et al., 1991; Inglis et al., 1994; Angka et al., 1995; Fang et al., 1998) appear to contribute to the establishment of *A. hydrophila* infection in fish. Other virulent determinants such as the presence of the S-layer and serum resistance are also implicated in helping the bacteria resist attack by the host's non-specific immunity such as serum and macrophages killing (Leung et al., 1996).

The objective of this study was to evaluate some hematological and plasma biochemical parameters of grass carp vaccinated with formalin inactivated *A. hydrophila* following exposure to organophosphorous pesticide diazinon.

Materials and methods

Fish

Ninety grass carps, *Ctenopharyngodon idella* weighing 250± 55 g from Mazandaran province fish farms (north of Iran) were used in this experiment. Fish were held in nine 1200 L tanks receiving a constant supply of fresh water. The experiments were initiated after 4 days of acclimatization. Fish were fed with fresh vegetables consisting of Lucerne grass. Water quality parameters consisting of water temperature, dissolved oxygen and pH were maintained at optimum condition i.e. 18-20°C, 7.7 mg/L and 7.5, respectively.

Fish vaccination

Bacteria were suspended in phosphate buffer saline (PBS) and stored at 4º C until used. The tests were conducted in six 1200 L fiberglass tanks containing 15 fish in each tank. 60 fish were intraperitoneally vaccinated with formalin inactivated whole cell of *A. hydrophila* antigens (1×10^8 cells of *A. hydrophila*).

Application of diazinon

Two weeks later the vaccinated fish were divided in two groups. The first group was exposed to diazinon at 2 mg/L for 12 h (group A) while the second group was bathed with PBS bath as a positive control (group B). The remaining 30 fish were used as unvaccinated fish and were kept in clean water separately (group C).

Sample collection and assays

Samples were collected after 1, 7, 14, 21 and 30 days post exposure (dpe) to toxicant. Five fish per treatment were used each sampling time. Blood was sampled by tail vein puncture and sampled blood was stabilized with 50 IU EDTA per 1 ml blood. At the same time, blood smears were also made. The smears were first air dried fixed in 96% ethanol for 30 minutes, stained by Giemsa staining for 30 minutes and were examined for leukocyte differential count under compound microscope (Klontz, 1994). The hematological parameters consisting of erythrocyte count (RBC), haematocrit (PCV), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (WBC) and differential leukocyte count (Klonts, 1994). The non-fixed blood samples were centrifuged for 15 minutes at 400 g and separated sera were used to determine the levels of the following factors: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) using an automatic analyzer machine (Eppendorf, Epos 5060 and optimized tests of Bochring Manaheim GmBH by spectrophotometer way).

Statistical analysis

Statistical parameters including mean, standard deviation and standard error for test and control groups were calculated (SPSS Ver.9) and results were processed statistically by means of the analysis of variance (ANOVA) at $p < 0.05$.

Results

Hematological and biochemical studies

Results of hematological studies are shown in Tables 1 and 2. After one day post-exposure the levels of lymphocytes, Hb, MCV, MCH, and MCHC in test group were lower than C ($p<0.05$). However, PMN and LDH level in test group were higher than C ($p<0.05$), and levels of PMN and LDH were also higher than B ($p<0.05$), while level of lymphocytes and RBC counts in A were lower than B ($p<0.05$). After one week post-exposure the levels of HCT, MCV, lymphocytes, myelocytes and WBC count in A were lower than C ($p<0.05$). However, PMN rate was higher than C ($p<0.05$). Levels of HCT, lymphocytes and myelocytes in A were lower than B ($p<0.05$). However, PMN rate and ALP level in A were higher than B ($p<0.05$). After 2 weeks post-exposure the levels of HCT, Hb, MCV, lymphocytes, myelocytes, WBC, and RBC count, in test group(A) were lower than negative control(C) ($p<0.05$). However, level of PMN in test fish was higher than C ($p<0.05$). Levels of HCT, HB, WBC, lymphocytes and myelocytes in test group (A) were lower than positive control (B) ($p<0.05$).While, PMN rate in A was higher than C ($p<0.05$). After 3 weeks post-exposure the levels of MCH, WBC, lymphocytes, ALT, AST, ALP, and LDH in A were lower than C ($p<0.05$). However, level of PMN in A was higher than C ($p<0.05$). Levels of WBC, lymphocytes, ALT, AST, ALP, and LDH in A were lower than B ($p<0.05$). However, level of PMN in test fish (A) was higher than positive control (B) ($p<0.05$). After 4 weeks post-exposure the levels of PCV, MCV, MCH, WBC, RBC, ALT, AST, lymphocytes and myelocytes in A were lower than C ($p<0.05$), while, the level of MCHC in test fish (A) was higher than C ($p<0.05$). Levels of MCV, MCH, WBC, ALT, and LDH in A were lower than B ($p<0.05$). However, levels of PMN and AST test fish (A) were higher than positive control (B) ($p<0.05$).

Table 1: Effects of 2 mg/L of diazinon exposure on hematological indices of grass carp (n = 90)

	Time post-sampling (day)				
Trial	1	7	14	21	30
WBC (/mm^3)					
A	2670 ± 170.7	2060 ± 100.4 [a]	2000 ± 139.6 [aь]	1270 ± 81.5 [aь]	1858 ± 30.2 [a ь]
B	1950 ± 236.6 [a]	2420 ± 75.1 [a]	2490 ± 55.6	3060 ± 180.5	2750 ± 143.1
C	2974 ± 31.5	2841.7 ± 24.7	2599 ± 35.8	2513 ± 32.5	2412.5 ± 65.7
Lymphocyte (%)					
A	69.8 ± 0.8 [aь]	65.2 ± 0.8 [aь]	61.4 ± 0.5 [aь]	64.2 ± 0.5 [aь]	67.2 ± 0.9 [a]
B	76.6 ± 0.6	71.4 ± 0.7	74.0 ± 1.0	75.2 ± 0.8	68.0 ± 1.7[a]
C	74.2 ± 0.7	72.0 ± 0.7	74.0 ± 0.7	74.9 ± 0.4	74.0 ± 0.6
Myelocyte (%)					
A	2.2 ± 0.4	0 [a]	0 [aь]	2.0 ± 0.2 [a]	0.4 ± 0.2 [a]
B	1.4 ± 0.6	1.2 ± 0.3 [a]	1.6 ± 0.4	1.4 ± 0.2 [a]	0.4 ± 0.2 [a]
C	1.5 ± 0.2	3.3 ± 0.2	3.3 ± 0.4	3.9 ± 0.2	3.0 ± 0.4
Monocyte (%)					
A	0	0	0	2 ± 0.4	0
B	0.4 ± 0.4	0.6 ± 0.4	0.6 ± 0.4	0	0
C	0	0	0	0	0
PMN (%)					
A	28.0 ± 1.3[aь]	34.8 ± 1.3 [aь]	38.6 ± 0.8 [aь]	31.8 ± 0.9 [aь]	32.4 ± 2.3[a]
B	21.6 ± 1.1	26.8 ± 1.1	23.8 ± 1.0	23.4 ± 0.9	31.6 ± 1.6 [a]
C	24.2 ± 1.0	24.7 ± 0.8	22.7 ± 0.8	21.2 ± 0.8	23.0 ± 2.1
RBC (10^6 /mm^3)					
A	1.5 ± 0.0 [ь]	1.6 ± 0.1[a]	1.6 ± 0.1[a]	1.5 ± 0.0 [a]	1.4 ± 0.1[a]
B	1.8 ± 0.0 [a]	2.2 ± 0.1	2.2 ± 0.1	1.5 ± 0.1 [a]	1.7 ± 0.1
C	1.6 ± 0.0	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	1.8 ± 0.1
PCV (%)					
A	22.6 ± 1.3	14.6 ± 0.5 [aь]	14.0 ± 0.4 [aь]	20.0 ± 0.4	14.0 ± 1.0 [a]
B	21.2 ± 1.4	19.4 ± 0.5	18.6 ± 0.5 [a]	19.4 ± 0.5 [a]	17.2 ± 0.6
C	19.7 ± 0.2	19.7 ± 0.6	22.2 ± 0.4	21.7 ± 0.4	17.7 ± 0.5
Hb (g/100ml)					
A	5.0 ± 0.1[a]	4.1 ± 0.2	3.3 ± 0.1 [aь]	4.4 ± 0.2	4.4 ± 0.2
B	5.4 ± 0.2	4.8 ± 0.2	4.6 ± 0.1	5.0 ± 0.1	4.6 ± 0.4
C	6.5 ± 0.2	5.0 ± 0.4	4.6 ± 0.2	5.0 ± 0.4	5.5 ± 0.3
MCV (m)					
A	$1x10^{-5}$± $6x10^{-6a}$	$9x10^{-5}$± $5x10^{-6a}$	$9x10^{-5}$± $5x10^{-6a}$	$1x10^{-4}$ ± $4x10^{-6}$	$9x10^{-5}$± $2x10^{-6a ь}$
B	$1x10^{-4}$± $8x10^{-6}$	$1x10^{-4}$± $4x10^{-6}$	$1x10^{-4}$± $4x10^{-6a}$	$1x10^{-4}$ ± $2x10^{-6}$	$1x10^{-4}$± $3x10^{-6}$
C	$1x10^{-4}$± $2x10^{-6}$	$1x10^{-4}$± $1x10^{-6}$	$1x10^{-4}$± $1x10^{-6}$	$1x10^{-4}$ ± $1x10^{-6}$	$1x10^{-4}$± $3x10^{-6}$
MCH (pg)					
A	$2x10^{-5}$±$2x10^{-6a}$	$3x10^{-5}$± $2x10^{-6}$	$3x10^{-5}$± $1x10^{-6}$	$2x10^{-5}$ ± $2x10^{-6a}$	$3x10^{-5}$± $8x10^{-7a ь}$
B	$3x10^{-5}$± $1x10^{-6}$ [a]	$2x10^{-5}$± $3x10^{-6}$	$2x10^{-5}$± $3x10^{-6}$	$3x10^{-5}$± $8x10^{-7a}$	$4x10^{-5}$± $1x10^{-6}$
C	$4x10^{-5}$± $1x10^{-6}$	$4x10^{-5}$± $8x10^{-7}$	$3x10^{-5}$± $4x10^{-7}$	$4x10^{-5}$± $8x10^{-7}$	$4x10^{-5}$± $7x10^{-7}$
MCHC (%)					
A	23.5 ± 1.0 [a]	28.9 ± 2.0	25.4 ± 0.7	20.3 ± 0.8	27.5 ± 1.5 [a]
B	25.8 ± 0.8	25.0 ± 1.2	26.9 ± 1.9	20.5 ± 0.6 [a]	27.1 ± 0.6 [a]
C	27.5 ± 0.3	25.6 ± 0.2	25.0 ± 0.4	23.0 ± 0.4	23.7 ± 0.4

a = Significant differences ($p<0.05$) with C

b = Significant differences ($p<0.05$) with B

A = Vaccinated fish and exposed to diazinon

B =Vaccinated fish and unexposed to diazinon

C = Unvaccinated fish and unexposed to diazinon

PCV=Packed Cell Volum, Hb=Hemoglobin, MCV=Mean Corpuscular Volume, MCH=Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration, RBC=Red Blood Cell, WBC=White Blood Cell, PMN=Polymorphonuclear,.

Table 2: Effects of 2 mg/L of diazinon exposure on biochemical indices of grass carp (n = 90)

	Time post-sampling (day)				
Trial	1	7	14	21	30
ALT (µ / l)					
A	5.4 ± 1.3	4.2 ± 0.3	2.4 ± 0.5	7.6 ± 0.5 [ab]	10.0 ± 0.7 [a b]
B	6.2 ± 1.2	3.2 ± 0.8	2.4 ± 0.5	11.4 ± 0.5	13.2 ± 0.6
C	7.2 ± 0.4	3.2 ± 0.2	2.7 ± 0.2	13.0 ± 0.4	13.0 ± 0.4
AST (µ / l)					
A	44.0 ± 7.4	58.2 ± 4.3	37.2 ± 5.2	59.4 ± 1.5 [ab]	88.6 ± 3.6 [a b]
B	50.8 ± 2.3 [a]	62.8 ± 9.3	38.2 ± 4.9	96.8 ± 3.0[a]	53.2 ± 5.0[a]
C	59.5 ± 0.8	56.7 ± 5.0	41.0 ± 2.8	78.5 ± 1.5	113.7 ± 3.2
ALP (µ / l)					
A	160.8 ± 14.3	199.0 ± 10.2 [b]	132.2 ± 11.9	155.6 ± 4.9 [ab]	144.2 ± 4.2
B	156.6 ± 12.4 [a]	131.0 ± 18.1	135.6 ± 14.3	206.2 ± 7.1 [a]	127.4 ± 6.3 [a]
C	210.5 ± 11.3	170.5 ± 8.0	135.7 ± 18.9	256.7 ± 4.7	149.5 ± 2.6
LDH (µ / l)					
A	1625.4 ± 119.1[ab]	1792.4 ± 29.7	1351.2 ± 99.4 [a]	1417.6 ± 57.5 [ab]	1188 ± 27.9 [b]
B	1108.4 ± 106.2	1891.4 ± 417.2	1086.0 ± 66.8 [a]	2388.4 ± 92.9	1456 ± 50.4 [a]
C	1121.5 ± 48.5	1819.7 ± 205.7	1932.0 ± 66.5	2389.0 ± 56.3	1222 ± 40.0

a = Significant differences ($p<0.05$) with C
b = Significant differences ($p<0.05$) with B
A = Vaccinated fish and exposed to diazinon
B =Vaccinated fish and unexposed to diazinon
C = Unvaccinated fish and unexposed to diazinon
ALT=Alanine Aminotransferase, AST=Aspartate Aminotransferase, ALP=Alkaline Phosphatase, LDH=Lactate Dehydrogenase.

Discussion

The examination of hematological and biochemical parameters of grass carp, indicated that the organophosphorous insecticide, diazinon at sub-lethal level, elicited significant and insignificant changes. The significant decrease of RBC counts, WBC counts, Hb, MCV and PCV values (Table 1) after exposure to diazinon indicated that the toxicant have been caused an effect similar as anemia. Similar results have been reported by other workers. For example, Eisler (1967) reported for fishes exposed to methylparathion and by Anees (1978) for fishes exposed to three organophosphorus insecticides (diazinon, methylparathion and dimethoate). Decrease in erythrocytes has been reported in *Tilapia zilii* due to water pollution Abdelmeguid et al. (2002). Changes in blood cell profile have been reported in *Cyprinus carpio* due to the effect of diazinon by Svoboda et al (2001). Organophosphate effect on hematological indices has been reported by Chindah et al. (2004) in *Tilapia guineensis*, (Lipika and Patra, 2006).

Alterations in the hematological parameters were brought about by diazinon as an anemic condition due to decreased synthesis of red blood cells and erythrocyte in bone marrow equivalents (Sibel et al., 2006).

In addition, a significant ($p<0.05$) decrease of lymphocytes values and significant increase of PMN value have been observed (Table 1) and this is in agreement with results of other workers (Pourgholam and Saeidi, 2000; Svoboda et al., 2001; Adedeji et al., 2009). The mechanism inducing such a lymphopenia might be a decrease in delivery of lymphocytes to the circulatory system through a reduced lymphocyte production or because of a rapid destruction of cells and an increased rate of peripheral removal of lymphocytes. The most probable explanation is that there was a failure in lymphocyte production together with the dissolution of cells (Alkahem, 1994). Ellis (1981) suggested that lymphopenia in fishes was accompanied by neutrophilia, as seen in the present study. In addition, Svobodo et al. (2001) has discussed that changes in values of both the erythrocyte and leukocyte profile after exposure to diazinon, may be referred to disruption of hematopoiesis as well as to a decrease on nonspecific immunity of the fish as was mentioned by other workers (Camerson, 1970; Anees, 1978). However, it was expected that blood values after this stage would change accordingly. There was significant and insignificant increase or decrease in some blood parameters such as myelocytes counts, MCH, MCHC, AST, ALP, ALT and LDH level in different days of post exposure of diazinon. Such fluctuations indicated that fish hematopoietic tissues were in stress and were in constant struggling to maintain normal condition (Anees, 1978).

AST, ALT and LDH are found in heart, liver, skeletal muscle, kidney, pancreas, spleen, gill, red blood cells, and brain tissue. While they are damaged and destroyed, especially liver, AST and ALT are released into the bloodstream (Sharifpour et al., 2006). The amount of AST is directly related to the number of cells affected by the disease (Pagana, 1998). Also, the LDH test is used to detect

tissue alterations and as an aid in the diagnosis of anemia, gill and liver disease (Banaee et al., 2008).

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´ 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) and Banaee et al. (2008).

Some pesticides, such as OPs, organochlorines and pyrethroids are able to cause inhibition of LDH (El-Demerdash et al., 2001). *Aeromonas hydrophila* whole cell antigen stimulated both specific and non-specific immunity of great sturgeon through the enhancement of leucocyte populations, lysozyme activity, respiratory burst capacity and antibody production (Khoshbavar Rostami et al., 2007).

Kozineneko et al. (1999) reported an increase of lysozyme following immunization of fish with antigens. Therefore, in aquaculture practices the fish exposed to the toxicant become more susceptible to the secondary infections than unexposed fish resulting in lower fish production in the polluted environment.

In conclusion, immunization of grass carp with formalin–killed cells of *A. hydrophila* can cause an increase in indices of immunocompetent cells, such as total leukocyte, lymphocyte and monocyte populations. Such enhancement in immunocompetent cells coincides with the increasing in the levels of respiratory burst of phagocytic cells and activity of lysozyme. Therefore, immunization of grass carp fingerlings against motile Aeromonas septicemia, the common bacterial infection in the region, is recommended. However, such immunization may require an application of a polyvalent vaccine because of heterogeneity of *A. hydrophila* strains.

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