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Chemiluminescent response of grass carp (*Ctenopharyngodon idella*) following exposure to sublethal concentrations of diazinon

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Abstract: Chemiluminescent response of grass carp (*Ctenopharyngodon idella*) was assessed to determine the effect of various concentrations of diazinon on the phagocytosis of grass carp in order to evaluate the fish immunity after exposure to this toxic chemical. One hundred and twenty specimens with individual weight of 850±155g were exposed to various concentrations of diazinon at 1, 2 and 4mg/L provided as a bath for 12 hours at 20±2°C in Institute of the Caspian Sea Ecology, Sari in 2003. Maximal values of spontaneous chemiluminescent responses (SCL) were found in all experimental fish only on days 1 and 7 post-exposing to the toxicant compared to control one ($P<0.05$). Also, such values on days 15, 30 and 45

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post-exposure to toxicant were insignificantly higher than control group ($P>0.05$). Generally, the level of chemiluminescent response activated by zymosan (CRAZ) at all concentrations of toxicant were significantly higher than control group after 1, 7, 15, 30 and 45 days post-exposure to the toxicant ($P<0.05$).

Keywords: Diazinon, Chemiluminescence, Grass carp

Introduction

The phagocytic cells of an organism (monocytes, macrophages and polymorphonuclear leukocytes) constitute a first line of protection against invading microorganisms and potential pathogens. These cells utilize a wide range of defense mechanisms against their targets, including phagocytosis and the production of reactive oxygen intermediates (ROIs) (Roszell & Anderson, 1994). Phagocytosis in fish is the main mechanism of the nonspecific immune response to pathogenic factors such as bacteria, viruses and parasites; lysozyme is also an element of the non-specific humoral response that is present in mucus, serum and phagocytic cells of many fish species. It is a process of several stages: chemotaxis, opsonization, adhesion, absorption, intracellular destruction and digestion (Fletcher & White, 1973 ; Siwicki & Studnicka, 1987). Fish macrophages comprise an important part of the cellular immune system and function to protect the host by phagocytosing invading microorganisms as they enter the tissues. During phgocytosis, fish macrophages show a burst of oxygen consumption associated with the formation of microbiocidal active oxygen species (Warinner *et al.*, 1988 ; Secombes & Fletcher, 1992 ; Steiro *et al.*, 1998).

Following phagocytosis, or upon contact with a variety of soluble or particulate stimuli, phagocytes undergo a respiratory burst. One of the most phylogenetically conserved features of macrophages and granulocytes is the respiratory burst (Rice, 2001). Production of antimicrobial oxygen radicals by macrophages is often measured by the chemiluminescence (CL) assay. Allen *et al.* (1972) first described this assay using human polymorphonuclear (PMN) cells, and since then the CL assay has been used to measure phagocytosis by cells for many species (Stave *et al.*, 1984). CL measures the respiratory burst activity of phagocytic cells in which

oxygen is converted into reactive oxygen intermediates (ROIs). The ROIs can activate probes such as luminol, triggering the emission of photons which can then be measured photometrically. It had been found that a reduced pyridine nucleotide was required, reducing O_2 only partially to H_2O_2, rather than completely to H_2O as it is in the mitochondrial respiratory chain. One of the reasons that it is easy to observe this respiratory burst in phagocytic cells is that they have few mitochondria, the cells relying on glycogen and glycolysis for their supply of ATP. Most of the respiration of phagocytic cells is required for the two, non-ATP generating pathways, microbial killing and eicosanoid production. Ultraweak chemiluminescence occures in a varity of phagocytic cells, including neutrophils, eosinophils, monocytes, microphages, Kupffer cells, when exposed to phagocytic stimulus. Chemotactic peptides and a range of artificial and pathological stimuli can also provoke the response in neutrophils and other phagocytes. As with whole organ, the light emission is very weak, although it can sometimes be as high as 10^{-2} photons per second per individual cell. Thus all phagocytes appear to be able to generate ultraweak chemiluminescence. The production of ROIs as measured by CL is important in host defense since killing of micro-organisms is at least partially dependent on the production of ROIs such as H_2O_2 and O_2^- in fish (Eliss, 1977 ; Badwey & Karovsky, 1980 ; Baboir, 1984 ; Smith *et al.*, 1991 ; Secombes & Fletcher, 1992 ; Roszell & Anderson, 1994 ; Steiro *et al.*, 1998). The CL responses assay was used for *in vitro* and *in vivo* studies of the effects of aquatic environmental contaminants on the phagocytic activity of PMN and mononuclear (MN) cells in fish (Siwicki *et al.*, 1998). Several early laboratory studies indicate that a wide variety of organic and inorganic environmental pollutants can suppress phagocytosis. Most studies have shown suppression at high doses, while others have shown slight activation at lower doses (Elsasser *et al.*, 1986; Cossarini-Dunier *et al.*, 1988 ; Ghanmi *et al.*, 1989 ; Wishkovsky *et al.*, 1989 ; Kangarot & Tripathi, 1991 ; Anderson & Brubacher, 1992 ; Zelikoff *et al.*, 1995).

The CL assay provides valuables information regarding the health of aquatic organisms such as fish especially as good indicator to determine fish health measurements. *In vitro* and *in vivo* exposure of fish to environmental pollutants

have been demonstrated to be dose-dependent modification in reactive oxygen intermediates formation as measured by the CL response of fish phagocytes (e.g. Anderson, 1990 ; Siwicki *et al.*, 1990 ; Anderson, 1993 ; Anderson & Zeeman, 1995). Diazinon is one of major herbicidal/insecticidal chemicals currently used in both north and south of Iran, where grass carp is cultivated on large scale.

Materials and methods

1. Fish and maintenance:

One hundred and twenty grass carp, *Ctenopharyngodon idella,* with individual weight of 850±155g from Mazandaran province, northern Iran, were used in the experiment. Fish were held in 1200 L tanks receiving constant supply of fresh water. The experiments were initiated after 4 days of acclimatization. Twelve tanks were stocked with 10 fish each (3 treatments, one control and 3 replicates). Fish were fed with fresh vegetables consisting of clover, lettuce and lucerne grass. Water temperature, dissolved oxygen and pH were maintained at 18-22°C, 7.7mg/L and 7.5, respectively.

2. Diazinon exposure:

Diazinon at concentrations of 1, 2 and 4mg/L (treatments) were added to the designated 9 tanks at 20-22°C for 12 hours, providing 3 control tanks with no diazinon added. Fish were then transferred to clean water and were kept for 45 days before blood sampling. Control groups were kept in clean water separately.

3. Collection and processing of samples:

Samples were collected after 1, 7, 15, 30 and 45 days post exposure to toxicant. Five fish per treatment were collected at each sampling time. Blood was obtained by cutting fish tail after removing the scales and scraping the tail with alcohol. The blood samples (3 drops=100µl) were collected in heparinized Eppendorf tubes for chemiluminescent assay.

4. Chemiluminescent assay:

The activity of leucocytes of the blood was measured by chemiluminescent assay with (Chemiluminescent Response of Phagocytes Activated by Zymozan = CLRPAZ) and without stimuli (Spontaneous Chemiluminescent = SCL) as described by Mathews *et al.* (1990) with some modifications by Pico-lite luminometer (Lucifer-B, MIR-Dialogue, Germany 1999).

Fish serum-opsonised zymozan was used as the stimulator of oxidative burst and luminol as the substrate. Light emission was measured on an automated system for chemiluminescent analysis. Briefly, 3 drops (100µl) of blood was added to eppendorf containing 20µl of heparin and 100 µl of heparinized blood was diluted with 900µl of Hanks. Then 200µl of diluted blood was added to tube containing 200µl of luminal (Sigma) and the light emission was measured after 30 minutes. 50µl of zymozan (Sigma) was then added to tube and the light emission was again measured after 30 minutes.

Results

Chemiluminescent assay:

Spontaneous (SCL) and activated chemiluminescent (CLRPAZ) responses of fish leucocytes to diazinon are shown in Table 1 and Figures 1-2. The level of SLC response of all of diazinon concentrations at days 1 and 7 post exposure were significantly ($P<0.05$) and at days 15, 30 and 45 post exposure to toxicant were insignificantly ($P>0.05$) higher than control groups. The level of CLRPAZ response of all diazinon concentrations were significantly ($P<0.05$) higher than control group (Table 1 and Fig. 2) at days 1 and 7 post exposure to toxicant. The highest values of CLRPAZ and SCL response were observed at one day post exposure to diazinon. However, at days 7 post exposure the values of CL response (CLRPAZ and SCL) gradually fall back to levels similar to those recorded in control fish.

Table 1: Intensity of spontaneous and activated chemiluminescent response (impulse/second) of leucocytes of grass carp exposed to various concentrations of diazinon at 20-22°C (n=5).

Days post exposure	Control		Diazinon concentrations					
			1ppm		2ppm		4ppm	
	CLRPAZ	SCL	CLRPAZ	SCL	CLRPAZ	SCL	CLRPAZ	SCL
1	180.25±0.44	171.12±0.25	250.5±0.42*	205±1.1*	200.16± 0.79*	199± 0.57*	190 ± 0.54*	185 ± 0.36*
7	175±0.57	172.3±0.42	200±0.57*	185±0.57*	190±0.57*	180±0.36*	187±0.57*	184±0.47*
15	174.87±0.08	171.94±0.17	174±0.21*	172.5±0.78	180.1±0.35*	172.3±0.15	186.9±0.35*	171.7±0.15
30	174.75±0.16	170.25±0.16	172.37±0.18*	170.2±0.13	174.5±0.18*	171.5±0.16	180.5±0.32*	170.72±0.17
45	162.75±0.84	160.5±0.79	171.06±0.19*	167.75±0.16	172.18±0.1*	161.3±0.13	175±0.31*	161.6±0.24

*** = Significant at P< 0.05**

CLRPAZ =Chemiluminescent Response of Phagocytes Activated by Zymosan

SCL = Spontaneous Chemiluminscent

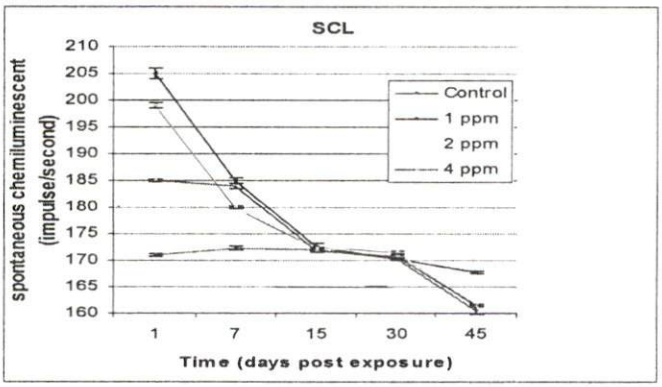

Figure 1: Intensity of spontaneous chemiluminescent response (impulse/second) of leucocytes

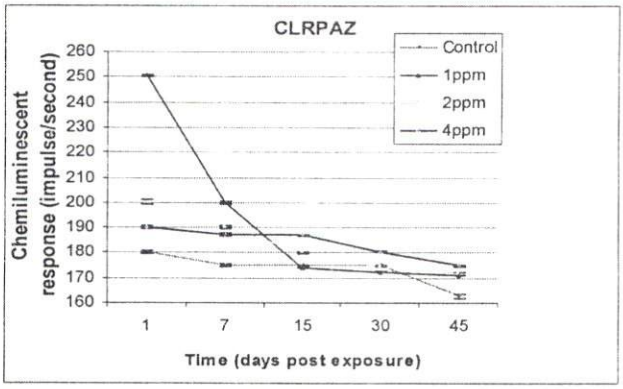

Figure 2: Intensity of activated chemiluminescent response (impulse/second) of leucocytes

Discussion

The analysis of data on chemiluminscent response of leucocytes (Table 1 and Figures 1-2) indicated that cellular factors of fish immunity under influence of diazinon, at 1, 2 and 4mg/L, responded by changes in their functional activities, with maximal intensity of CL response observed for fish exposed to minimal concentration of toxicant. However, such toxicant effect was lower at 15 days post-exposure as compared to control group ($P>0.05$). Moreover stimulation by zymozan presents a high CL response at all three concentrations of diazinon, with more stimulating effect recorded at lower concentration of 1mg/L (Table 1). Thus, diazinon probably indicates some impacts on grass carp immune system in especial reaction of englobing cells, that finds the reflection in change of normal functioning of immunocytes as particularly, presented at low concentration of 1mg/L. Kelly and Weeks (1994) suggested that macrophages of *F. heteroclitus* may be rendered fully active due to the effects of increased lymphokines which themselves have been stimulated by constant exposure to contaminated river water. Rice and Weeks (1990) demonstrated that the increased CL activity was the result of calcium influx into the macrophage and the resultant increased formation of reactive oxygen intermediates. Siwicki *et al.* (1990) found a decrease in the

phagocytic ability of neutrophils in carp exposed *in vivo* to high doses of the organophosphorus insecticide trichlorphon. They observed the cytotoxic effect of trichlorphon on the immunocompetent cells in blood and found the immunosuppressive effect in correlation with disease conditions in the carp. *In vitro* exposure of toadfish cells to environmental contaminants was found to suppress CL (Wishkovsky *et al.*, 1989). Kelly and Weeks (1994) hypothesized that CL response of macrophages of *Fundulus heteroclitus* living in a polluted river would have decreased as compared to the response of macrophages from the ones living in a cleaner river

In conclusion, low concentration of diazinon probably enhances respiratory burst by grass carp leucocytes. Such positive effect will improve fish immunity to resist potential pathogenic microorganisms. However, more work is required to assess the effects of concentrations of diazinon on cellular structures of immunocompetent cells in particular macrophages and neutrophils.

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