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Effects of Low Concentration of Cadmium on the Level of Lysozyme in Serum, Leukocyte Count and Phagocytic Index in *Cyprinus carpio* under the Wintering Conditions

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Abstract: In order to determine the effects of low concentration of cadmium in some parameters that involved in fish innate immune system at the low temperature seasons, lysozyme activity, total white blood cell count and phagocytic activity in common carp (*Cyprinus carpio*) were estimated after 15 and 30 days exposure to low concentration of cadmium ($30 \mu\text{g L}^{-1}$). Forty eight common carp were obtained from a local fish farm in Iran, randomly distributed in two 1000 L fiberglass tanks. The fish were kept in condition similar to wintering pond conditions. All tanks were supplied with well water (hardness 320.6 mg L^{-1} as CaCO_3 , dissolved oxygen 8 mg L^{-1} , pH 7, temperature $9^\circ\text{C} \pm 3$), with continuous aeration and without water flow. The fish were starved during the whole trial. Fish in group I were kept in cadmium free water while group II exposed to $30 \mu\text{g L}^{-1}$ (30 ppb) cadmium as cadmium chloride for 30 days. Water quality was measured two times weekly throughout the exposure. At the days 15 and 30 post exposure, the immune response was monitored using haematological and serological assays. The results revealed that low concentration of cadmium significantly ($p \leq 0.05$) decreased the serum lysozyme content in common carp serum after 30 days. However, 15 days following exposure, the level of lysozyme in group II was similar to those recorded in control fish. At the day 15th, a significant ($p \leq 0.05$) decrease in total leukocyte count and phagocytic index with considerable increase in monocyte took place in compare with control. Thirty days post exposure white blood cell count in group II was significantly lower than it was in the day 15th ($p \leq 0.05$). It seems decrease in total leukocyte count is depending on exposure time.

Key words: *Cyprinus carpio*, cadmium, lysozyme, leukocyte count, phagocytic index

INTRODUCTION

In the last few decades, a possible influence of environmental pollution on the aquatic environment has gained considerable interest. Fish have become a favorable subject for

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research in this area, because temperature changes, habitat and water quality deterioration as well as aquatic pollution adversely affect fish health, which may result in mortalities and population decline. Among various biochemical, cellular and physiological systems, certain innate immune responses are considered as suitable biomarkers for monitoring biological effects of pollution (Bols *et al.*, 2001). Fish are frequently exposed to many pollutants in their aquatic environment, their nonspecific defense mechanism and specific immune response may also compromise with environmental contaminant such as cadmium compound. Cadmium is a heavy metal commonly used in environmental studies because it is highly toxic (Faroon *et al.*, 1994) widely distributed in the environment (Camusso *et al.*, 1995; Cinier *et al.*, 1999).

Impairment of immune functions, which protect fish against invading pathogens, can lead to harmful consequences at the individual level, such as disease outbreak followed by death and at the ecosystem level, as population reductions are followed by changes in the entire ecosystem. One important humoral component in the innate immune system is lysozyme, which involved in defense against Gram positive and negative bacteria, parasites and viruses (Ingram, 1980; Alexander and Ingram, 1992).

Lysozyme is present in mucus, lymphoid tissue, serum and other body fluid of most fish species. Fish lysozyme is generally believed to be leukocyte origin (Lie *et al.*, 1989; Ceccheni *et al.*, 2000). Lysozyme is localized in the lysosomes of neutrophils and macrophages and is released into the blood from these cells Murray and Fletcher (1976). From previous studies, it is known that lysozyme activity in fish blood is sensitive to environmental contaminants (Bols *et al.*, 2001). The Data obtained by various researcher (reviewed by Jezierska and Witeska, 2001) show that intoxication of fish with heavy metals may some times cause symptoms similar to the stress reaction also heavy metal intoxication almost always reduces count of white blood cells, particularly lymphocytes. According to Donaldson and Dye (1975), heavy metal exposure causes an increase in cortisol level in fish which is responsible for a decrease in WBC, particularly in count of lymphocytes and their activity. In the present study, experimentally, the effects of cadmium at a low concentration on some selected blood and innate immune parameters of common carp, the most important cultured fish species in Iran, under wintering conditions have been investigated. In one hand decreasing in water temperature causes decreasing in fish immunoactivities and in other hand results decreasing in cadmium uptake.

MATERIALS AND METHODS

Common carp were obtained from a local fish farm in Iran, acclimated in holding tank for 1 week, then 48 apparently healthy fish, mean weight 700 g were randomly divided in to two groups (I, II); 1000 L indoor fiberglass tanks supplied with well water (hardness 320.6 mg L⁻¹ as CaCO₃, dissolved oxygen 8 mg L⁻¹, pH 7, temperature 9°C±3) with continuous aeration and without water flow. Group I were kept in cadmium free water as a control group while group II were exposed to 30 ppb cadmium for 30 days. The fish were starved during the whole examinations period. This study was conducted from December 2006 to March 2007.

Application of Cadmium

The experiment was conducted in 1000 L fiberglass tanks. Thirty ppb cadmium as CdCl₂ (Merck) was added to tank water, respectively for 30 days.

Collection of Blood Samples

At the days 15 and 30 post exposure blood samples were collected from the caudal vein of 12 fish from each tank. Half of blood samples was used for serum separation and remaining was used for hematological examinations after adding an aqueous solution of heparin as anticoagulants.

Lysozyme Assay

Lysozyme activity of common carp plasma was determined by means of a turbidimetric assay according to Ellis (1990) with some modifications. The 1.75 mL of a suspension of 0.375 g L^{-1} *Micrococcus lysodeikticus* (Sigma, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 250 μL common carp serum and the optical density was read in a spectrophotometer at 670 nm, immediately after mixing and then 30 sec and 3 min later, at room temperature. The decrease in absorbance was used to calculate lysozyme activity. Hen egg white lysozyme (Sigma-Germany) was used as an external standard. Lysozyme activity of experimental samples was calculated from standard curve prepared from hen egg white.

Blood Parameters

At the days 15 and 30 post exposure, the white blood cell count ($\text{WBC}: 10^4 \text{ mL}^{-1}$) were determined from a 1:200 dilution of the blood sample in Turke's solution with a Neubaure hemocytometer. Also, blood smears were prepared and stained in Giemsa for leukocyte differential count under light microscope (Klontz, 1994).

Phagocytosis Assay

Blood samples were obtained from 5 fish of each group using puncture of caudal vein at day 15 and 30 post exposure with heparinized syringe, $\frac{1}{4}$ inch needle. These fish were discarded after use. The phagocytic activity was evaluated using a microscopic counting technique described by Eline *et al.* (1990), method with some modifications. In the present study, yeast was used instead of formalin-killed *E. coli*. The number of yeast cells engulfed, were determined and a phagocytic index was calculated as number of yeast cell engulfed/number of phagocytes counted (Fig. 1).

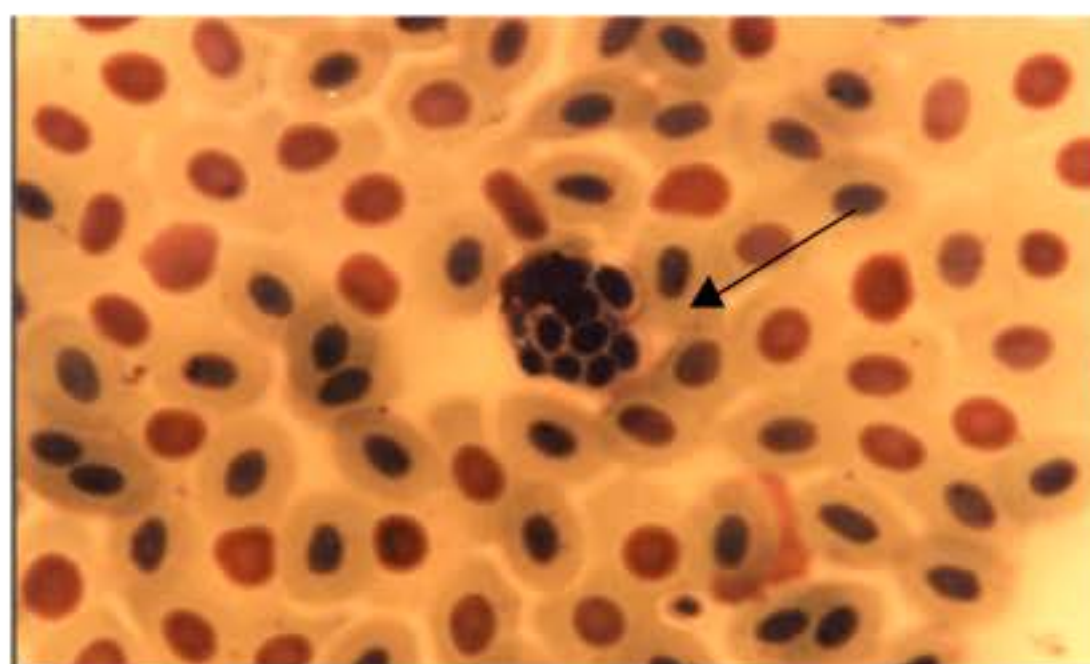


Fig. 1: *Cyprinus carpio*, the yeast cells engulfed with phagocytic cell

Statistical Analysis

The data of control and exposure fish were, analyzed using student's t test and data were significant as $p \leq 0.05$.

RESULTS

Lysozyme

Cadmium in concentration 30 ppb was not lethal for common carp over the 30 days experimental period. Fish in cadmium exposure group demonstrated a significant decrease in plasma lysozyme level from 0.7163 ± 0.1840 (mg mL^{-1}) in control to 0.3392 ± 0.2142 (mg mL^{-1}) 30 days post exposure (Table 1, Fig. 1) however, after 15 days there was no significant differences between lysozyme activity in control and treated fish.

WBC Count

In this study, there was a significant ($p \leq 0.05$) decline in white blood cell count in treatment group from 3.9750 ± 1.3226 in control to 2.5200 ± 0.3033 in exposed group (II) at the day 15. In treated fish white blood cell count significantly ($p \leq 0.05$) decreased to 1.7000 ± 0.3317 in the day 30th when compared with the day 15th (Table 1).

Differential Count

As shown in Table 1, the level of monocyte in fish exposed to 30 ppb cadmium is significantly ($p \leq 0.05$) higher than control at the day 15 and after 30 days monocyte and lymphocyte were shown a decrease in population when compared with control but these difference were not significant ($p \leq 0.05$).

Phagocytic Index

The results revealed that, after 15 days phagocytic index significantly ($p \leq 0.05$) decreased from 2.9110 ± 0.4968 in control to 2.5720 ± 0.5133 in exposed group also significant ($p \leq 0.05$) difference between phagocytic index in control and exposed fish at the 30th day post exposure with 30 ppb cadmium so that phagocytic index increased from 2.7000 ± 0.1581 in control to 2.9700 ± 0.1691 in treated group.

Table 1: *Cyprinus carpio*, Leukocyte profile, lysozyme content and phagocytic index 15 and 30 days post exposure to 30 ppb ($30 \mu\text{g L}^{-1}$) cadmium chloride at $9^\circ\text{C} \pm 3$ (Mean $\pm$ SD)

Sampling time (day)	15	30
Lysozyme (mg mL^{-1})		
Group I	0.6620 ± 0.2902	0.7163 ± 0.1840
Group II	0.4736 ± 0.1912	0.3392 ± 0.2142
WBC (10^4 mL^{-1})		
Group I	3.9750 ± 1.3226	3.1000 ± 0.3606
Group II	2.5200 ± 0.3033	1.7000 ± 0.3317
Lymphocyte (%)		
Group I	85.5000 ± 3.4157	80.6667 ± 5.0332
Group II	86.8000 ± 10.7331	76.0000 ± 4.6904
Monocyte (%)		
Group I	2.0000 ± 0.0000	3.3333 ± 2.3094
Group II	4.0000 ± 0.0000	2.6667 ± 1.1547
Neutrophil (%)		
Group I	13.5000 ± 3.4157	18.0000 ± 0.0000
Group II	10.8000 ± 6.0314	22.4000 ± 6.5422
Phagocytic index		
Group I	2.9110 ± 0.4068	2.7000 ± 0.1581
Group II	2.5720 ± 0.5133	2.9700 ± 0.1691

DISCUSSION

Lysozyme

The results revealed that low concentration of cadmium significantly ($p \leq 0.05$) decreased the lysozyme content in common carp serum from 0.7163 ± 0.1840 (mg mL^{-1}) in control to 0.3392 ± 0.2142 (mg mL^{-1}) in exposed fish after 30 days, while 15 days following exposure the decrease in level of lysozyme in group II was not prominent and was nearly similar to those recorded in control fish.

Leukocyte such as monocyte, macrophage and polymorphonuclear granulocytes are known to synthesize and secrete lysozyme in fish Murray and Fletcher (1976).

The present data, indicated that duration of exposure to cadmium has an important role in lysozyme plasma activity. Lysozyme is an effective bacteriolytic agent against both Gram-positive and Gram-negative fish pathogen (Grinde, 1989; Yousif *et al.*, 1994). Demers and Bayne (1997) reported an immediate positive response in lysozyme activity to stress in trout, suggesting an early enhancement of innate immunity. In addition to differences between species, environmental factors (e.g., water temperature) and physiological state of fish affect the level of lysozyme (Schrock *et al.*, 2001; Langston *et al.*, 2002). Present findings indicate lysozyme activity in common carp serum is decreased following exposure to cadmium that suggest cadmium be able to enhance susceptibility of common carp to disease due to decrease innate immune response.

Lysozyme is an important bacteriolytic agent found in a variety of freshwater and marine fish species (Lie *et al.*, 1989). Lysozyme isolated from fish has been found to be effective as a bacteriolytic agent against both Gram-positive and Gram-negative fish pathogens (Grinde, 1989; Yousif *et al.*, 1994).

WBC Count, Differential Count

Fish serum lysozyme is generally believed to be of leucocyte origin (Ceccheni *et al.*, 2000). Other authors Tort *et al.* (1988) determined a decrease in the leucocytes concentration in the blood of dog fish (*Scyliorhinus canicula*) after 24-96 h exposure to $0.25 \mu\text{g L}^{-1}$ of cadmium. It has been determined that a decrease in the leukocyte count in blood of sturgeon (*Acipenser baeri*) depends on the concentration of cadmium and time of exposure Mikryakov and Lapirova (1997). A decrease in the leucocytes count at the expense of small lymphocytes and an increase in amount of neutrophils was reported in tilapia (*Oreochromis mossambicus* Peters) exposed to sub-lethal $0.1-10.0 \mu\text{g L}^{-1}$ concentration by Ruparella *et al.* (1990). In the white cell system a decrease in cell count, especially of lymphocytes usually occurs in fish subjected to stress Ellsaesser and Clem (1986). Changes in leukocytes count in the blood of carp were also mostly dependent on the duration of exposure: after initial increase the count of leucocytes (lymphocyte and monocyte) decreased after 11 day exposure (Palackova *et al.*, 1994). The results shows cadmium is able to affect leucocytes count in a time dependent manner. Decreasing in white blood cell count in cadmium exposed fish suggesting an immunological suppression. It is possible, significant decline in WBC count can affect lysozyme level in serum and phagocytic index. In the present study the significant decrease ($p \leq 0.05$) in phagocytic activity in exposed fish at the day 15th was followed by an increase in phagocytic activity in at the day 30th. The significant ($p \leq 0.05$) decrease in phagocytic activity after 15 days in exposed fish is very similar to stress reaction. It is known that cortisol secreted during stress reaction. According to Donaldson and Dye (1975), heavy metals exposure cause in fish increase in cortisol level. It is possible that decrease in phagocytic activity at the day 15th is due to cortisol effects and increasing in phagocytic

activity after 30 days reflect a general adaptation to stress. Furthermore, other workers (Witeska and Wakuleska, 2007) were found that in common carp, phagocytic activity was less sensitive to heavy metals than was lymphocyte viability. It was significantly reduced following exposure to 50 and 100 μ M cadmium and 100 μ M zinc, but no effects were observed with either lead or copper. It seems that cadmium can induce different effects on the immune system in variety of fish species (Witeska and Wakuleska, 2007). It can be concluded that a number of heavy metal pollutants shown to be immunotoxic in mammalian systems, including cadmium, chromium, copper, lead, manganese, nickel and zinc (Zelikoff, 1993). The cadmium accumulates in high concentration in fish tissues, is found in polluted aquatic environments, so immunotoxic effects of cadmium on human who consume the fish find in polluted water need to be approached with caution.

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