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*Original Research Article*

# Effects of Cadmium and Cadmium-Chitosan Mixture on Some Blood Parameters of *Oreochromis niloticus*

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## ABSTRACT

**Effects of 1 mgL$^{-1}$ cadmium (Cd) and 1 mgL$^{-1}$ Cd-10 mgL$^{-1}$ chitosan(CT) mixture on hematocrit levels, erythrocyte numbers and on erythrocyte and erythrocyte nucleus areas were determined after exposing the animals to Cd and Cd-CT mixture over 7, 15 and 30 days. Microhematocrit and microscopic methods were used in determining the above blood parameters. No mortality was observed during the experiments. Hematocrit levels and erythrocyte numbers decreased whereas erythrocyte and erythrocyte nucleus areas increased in fish exposed to Cd alone compared with the control. No difference was observed, however, in the parameters studied in Cd-CT exposed fish compared with the control, except the erythrocyte nucleus area (P<0.05). When exposed to Cd alone erythrocyte and erythrocyte nucleus areas increased and hematocrit levels and erythrocyte numbers decreased whereas when exposed to Cd-CT mixture there was no difference in hematocrit levels, an increase in erythrocyte numbers and a decrease in erythrocyte and erythrocyte nucleus areas with increasing exposure periods. Blood parameters of *O. niloticus* were not affected when exposed to Cd together with chitosan, while exposing fish to Cd alone resulted changes in these parameters.**

**Key Words: Chitosan, cadmium, fish, hematocrit, erythrocyte morphology**

# Kadmiyum ve Kadmiyum Kitosan Karışımının *Oreochromis niloticus*'un Bazı Hematolojik Parametreleri Üzerine Etkileri

## ÖZET

**Araştırmada, 1 mgL$^{-1}$ kadmiyum (Cd) ile 1 mgL$^{-1}$ Cd - 10 mgL$^{-1}$ kitosan (CT) karışımının 7, 15 ve 30 gün süre ile etkisinde *Oreohromis niloticus*'un hematokrit düzeyi, eritrosit sayısı, eritrosit ve nükleus alanları üzerine etkisinin belirlenmesi amaçlanmıştır. Belirlenen derişim ve süreler etkisinde incelenen hematolojik parametrelerin analizinde mikrohematokrit ve mikroskobik yöntemler kullanılmıştır. Araştırma süresince balıklarda mortalite gözlenmemiştir. Belirli bir sürede Cd'un tek başına etkisi hematokrit düzeyi ile eritrosit sayısını kontrole göre azaltırken, eritrosit ve eritrosit nükleus alanını kontrole göre arttırmıştır. Cd - CT karışımının etkisi ise nükleus alanı dışında incelenen parametrelerde kontrole oranla herhangi bir değişim saptanmamıştır (P<0.05). Etkide kalma süresindeki artış, Cd etkisinde balıkların eritrosit ve nükleus alanında artış, hematokrit düzeyi ile eritrosit sayısında azalma, karışımın etkisinde ise hematokrit düzeyi değişmezken, eritrosit sayısında artış, eritrosit ve nükleus alanlarında azalma ile sonuçlanmıştır (P<0.05).*O. niloticus*'da Cd'un CT ile etkileşiminde incelenen hematolojik parametrelerde değişim saptanmazken, Cd'un tek başına etkisi değişime neden olmuştur.**

**Anahtar Kelimeler: Kitosan, kadmiyum, balık, hematokrit, eritrosit morfolojisi**

## INTRODUCTION

Increasing environmental pollution, due to uncontrolled growth of human population and technological development, has become a threat to living organisms. Organic and inorganic pollutants resulting mainly from anthropogenic activities are carried to aquatic ecosystems through hydrological cycle. Increased levels of heavy metals, an important group of inorganic pollutants, are carried to higher trophic levels through food chain where they constitute important environmental and health problems (Heath 1995, Bailey *et al.* 1999).

Cadmium has no biological function in living organisms. It widely used in automotive, dye, fertilizer, plastics and synthetic fiber industries and battery production (ATSDR 1998, Mendez-Armenta and Rios 2007).

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Even small amounts of this metal entered into aquatic systems, results in accumulation in various tissues, changes in metabolic, physiologic and biochemical parameters and death in sensitive species.

Chitin, a structural protein containing glicoseamine, takes part in the formation of exoskeleton together with proteins, Mg, $CaCO_3$ and carotenoids. Chitosan, which is a chitin derivative, cannot be found singly in nature and is obtained by deacetylation of chitin under alkali conditions. Despite chitin, chitosan contains amine rather than acetyl amine (Khor 2001).

Chitosan is a strong adsorbent especially for heavy metals. Its adsorbent characteristics are due to nitrogen atoms within its amine groups which have free double electrons that get in reactions with heavy metals (Qin *et al.* 2003).

Survival of aquatic organisms under infection, pollution and extreme environmental conditions depends upon reimplementation of homeostasis by various metabolic and physiologic arrangements (Heath 1995). Hematological parameters such as levels of hemoglobin and hematocrit and erythrocyte numbers in fish reflect oxygen carrying capacity of blood. Since these parameters change rather rapidly under stress, pollution and infectious conditions as in hot blooded animals, they can be used as indicators to determine the physiological status of the animals (Shah and Altındağ, 2004).

*O. niloticus* is a widely cultured species in tropic and subtrophic regions as a protein source since its feeding and reproduction is easy and development rapid. Due to their resistance against pollutants and diseases, the species is being used extensively as a study material in both laboratory and field studies (Sağlamtimur *et al.* 2004).

The use of chelating agents such as EDTA (Ethylenediaminetetraaceticacid) NTA (Nitrylotriaceticacid) and DTPA (Diethylenetriaminepentaaceticacid) have become widespread in recent years to remove heavy metals from waste waters (Kargın 1996, Sun *et al.* 2001, Aslanyavrusu 2010). A number of studies have shown that EDTA prevent heavy metal uptake in various fish species (Muramoto 1980, Muramoto, 1983, Holwerda *et al.* 1988). However the above chelating agents are synthetic whereas CT is a natural adsorbent (Shahidi *et al.* 1999). Cadmium is known to have adverse effects on hematological parameters in fish. Present study deals with the effect of chitosan on these adverse effects of Cd on blood parameters of *O. niloticus*. Hence fish were exposed to 1.0 $mgL^{-1}$ Cd and 1.0 $mgL^{-1}$ Cd+10 $mgL^{-1}$ CT over 7, 15 and 30 days and changes in hematocrit levels, erythrocyte numbers, erythrocyte area and erythrocyte nucleus areas were determined.

## MATERIALS AND METHODS

Study material *O. niloticus* was obtained from the culture pools of Çukurova University. The mean length and weight of the fish were 10.70 ± 0.50 cm and 20.64 ± 2.67 g respectively. Experiments were carried out at Mersin University, Basic Sciences Laboratory under controlled conditions (24±1$^0$C and 12 h light-dark photoperiod). Fish were placed in 6 aquaria, 40x100x40 cm in height, and adapted to laboratory conditions for 30 days.

Experiments were run in three aquaria of the same size. The first aquarium was filled with 120 L of 1 $mgL^{-1}$ Cd, the second with 120 L of 1 $mgL^{-1}$ Cd-10 $mgL^{-1}$ CT and the third the same amount of Cd free tap water and used as control. Fifteen fish were placed in each aquarium and 5 fish were removed from each aquarium taking the 7, 15 and 30 days of experiments into account and changes in their hematocrit levels, erythrocyte numbers, erythrocyte areas and their erythrocyte nucleus areas were determined.

Central aeration system was used for aeration. Fish were fed once a day with readymade fish feed (Pınar, pellet no:2) at amounts 2% of the total biomass. Changes in water characteristics was shown to effect blood parameters and hence some physical and chemical parameters of the experimental water are given in Table 1.

**Table 1.** Some physical and chemical parameters of the experimental water.

| | |
|---|---|
| Temperature (ºC) | 22 ± 1 |
| Dissolved Oxygen ($mgL^{-1}$) | 7,38 ± 0,59 |
| pH | 8,01 ± 0,02 |
| Total Hardness ($mgL^{-1}$ $CaCO_3$) | 241,7 ± 2,74 |
| Total Alkalinity ($mgL^{-1}$ $CaCO_3$) | 402,01 ± 0,28 |

$CdCl_2.H_2O$ salt of cadmium and CT obtained from the shells of *Callinectes sapidus* under laboratory condition were used in the preparation of experimental solutions. Shells of *C. sapidus* were washed with water to remove water soluble materials for the purification of CT, dried at $70^0$C for 24 hours and were then grinded using a blender.

Deproteinisation (DP): Grinded shells were left in 2% NaOH solution (w/v) at $100^0$C for 24 hours, washed thoroughly with water and were dried at $70^0$C for 24 hours.

Demineralisation (DM): Shell powders were left in 4 M HCl 1:15 (w/v) solution at $30^0$C for 48 hours, washed with water and were dried at $70^0$C for 24 hours.

Decoloration (DC): Samples were left under sunlight for decoloration which were named as "Chitin" after this procedure.

Deasetylation (DA): Chitin samples were first left in 40% NaOH solution at 100-$130^0$C for 24 hours and then washed with water and dried at $70^0$C for 24 hours (Sun-Ok 2004).

The required concentration of chitosan extracted by this method was prepared by dissolving it in 1% acetic acid.

Experimental solutions were changed once in two days due to possible changes in the concentrations of experimental solutions as a result of adsorption, evaporation and precipitation.

Fish were anesthetized with Ethylene Glycol Phenyl Esther ($C_8H_{10}O_2$; Merck) at the end of each experimental for possible changes in hematological parameters caused by stress. They were then washed to remove metal residues on their surface and dried with drying paper. Blood samples were obtained by cutting vertically their caudal peduncle. Giemsa method was used in the preparation of spread plates (Grimstone and Skaer 1972).

Length and width of minimum 150 erythrocytes and erythrocyte nucleus were measures under a microscope (Nikon, H550-L) and their areas were calculated using the following formulas (Gregory 2003).

$$\text{Erythrocyte area} = \pi x \frac{\text{Cell long diameter} x \text{cell short diameter}}{2} \mu m^2$$

$$\text{Erythrocyte nucleus area} = \pi x \frac{\text{Nucleus long diameter} x \text{nucleus short diameter}}{2} \mu m^2$$

Hematocrit levels of blood samples were measured microhematocrit method and erythrocyte numbers were counted under a light microscope (Leica, CME) using Thoma slide (Wedemeyer and Yasutake 1977). The numbers of erythrocytes were determined using the following formula (Konuk 1981, Gürgün and Halkman 1988).

$$\text{Erytrocyte number} = \frac{\text{Erytrocyte cell number} x \text{Diluation rate} x 100}{\text{Small square count}} (10^6 \text{cellmm}^{-3})$$

Student Newman Keul's (SNK) procedure was applied for statistical analysis of the data using SPSS package program. Arcsine transformation was applied to hematocrit level data before statistical analysis.

## RESULTS

There was no mortality of fish during the experiments. Cadmium decreased, whereas Cd-CT mixture increased the erythrocyte numbers in *O. niloticus* (P<0.05). Erythrocyte numbers of experimental group decreased in Cd exposed fish and increased in Cd-CT mixture exposed fish with increasing exposure periods (Table 2).

**Table 2.** Effects of Cd and Cd-CT mixture on erythrocyte numbers ($10^6$cellmm$^{-3}$) of *O. niloticus* at the concentrations and exposure periods tested.

| **Concentration ($\mu gL^{-1}$)** | **Exposure Period (Days)** | | |
|---|---|---|---|
| | **7** | **15** | **30** |
| | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * |
| **0.0** | 0,90 ± 0,007 as | 0,86 ± 0,006 as | 0,94 ± 0,005 as |
| **1 $\mu gL^{-1}$ Cd** | 0,57 ± 0,004 at | 0,42 ± 0,001 abt | 0,33 ± 0,005 bt |
| **1 $\mu gL^{-1}$ Cd-10 $\mu gL^{-1}$ CT** | 1,04 ± 0,002 as | 1,20 ± 0,001 bx | 1,29 ± 0,002 cx |

*=SNK; Letters a, b, c and s, t x show differences among exposure periods and concentrations respectively. Data shown with different letters are significant at the P<0.05 level

$\overline{X} \pm S\overline{x}$ = Mean ± Standard error

Hematocrit levels of *O. niloticus* decreased under the effect of Cd (P<0.05) whereas it remained unchanged when exposed to Cd-CT mixture compared with control at the exposure periods tested. Increasing exposure periods decreased hematocrit levels in cadmium exposed fish and had no effect in Cd-CT exposed fish (Table 3).

**Table 3.** Effects of Cd and Cd-CT mixture on hematocrit levels (%) of *O. niloticus* at the concentrations and exposure periods tested.

| **Concentration ($\mu gL^{-1}$)** | **Exposure Period (Days)** | | |
|---|---|---|---|
| | **7** | **15** | **30** |
| | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * |
| **0.0** | 41,5 ± 0,5 as | 40,5 ± 1,5 as | 41,0 ± 1,0 as |
| **1 $\mu gL^{-1}$ Cd** | 35,5 ± 0,5 at | 31,0 ± 1,0 bt | 28,0 ± 1,0 bt |
| **1 $\mu gL^{-1}$ Cd-10 $\mu gL^{-1}$ CT** | 39,5 ± 0,5 as | 41,0 ± 1,0 as | 39,5 ± 0,5 as |

*=SNK; Letters a, b and s, t show differences among exposure periods and concentrations respectively. Data shown with different letters are significant at the P<0.05 level

$\overline{X} \pm S\overline{x}$ = Mean ± Standard error

Erythrocyte and erythrocyte nucleus areas of *O. niloticus* exposed to 1 $\mu gL^{-1}$ Cd increased whereas they both decreased when exposed to Cd-CT mixture with increasing exposure periods. Erythrocyte and erythrocyte nucleus areas increased compared with control at a given period (P<0.05). Erythrocyte nucleus area decreased and erythrocyte area remained the same compared with control at each experimental period (Table 4, 5).

**Table 4.** Effects of Cd and Cd-CT mixture on erythrocyte area (μm$^2$) of *O. niloticus* at the concentrations and exposure periods tested.

| **Concentration ($\mu gL^{-1}$)** | **Exposure Period (Days)** | | |
|---|---|---|---|
| | **7** | **15** | **30** |
| | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * |
| **0.0** | 0,59 ± 0,0002 as | 0,60 ± 0,0001 bs | 0,60 ± 0,0001 bs |
| **1 $\mu gL^{-1}$ Cd** | 0,62 ± 0,0002 at | 0,64 ± 0,0003 bt | 0,65 ± 0,0004 ct |
| **1 $\mu gL^{-1}$ Cd-10 $\mu gL^{-1}$CT** | 0,60 ± 0,0001 as | 0,59 ± 0,0003 bs | 0,58 ± 0,0005 cs |

*=SNK; Letters a, b, c and s, t show differences among exposure periods and concentrations respectively. Data shown with different letters are significant at the P<0.05 level

$\overline{X} \pm S\overline{x}$ = Mean ± Standard error

**Table 5.** Effects of Cd and Cd-CT mixture on erythrocyte nucleus Area (μm$^2$) of *O. niloticus* at the concentrations and exposure periods tested.

| **Concentration ($\mu gL^{-1}$)** | **Exposure Period (Days)** | | |
|---|---|---|---|
| | **7** | **15** | **30** |
| | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * | $\overline{X} \pm S\overline{x}$ * |
| **0.0** | 0,10 ± 0,0003 as | 0,10 ± 0,0005 as | 0,11 ± 0,0002 bs |
| **1 $\mu gL^{-1}$ Cd** | 0,11 ± 0,0001 at | 0,12 ± 0,0003 bt | 0,13 ± 0,0007 ct |
| **1 $\mu gL^{-1}$ Cd-10 $\mu gL^{-1}$CT** | 0,10 ± 0,0006 as | 0,09± 0,0003 bx | 0,08 ± 0,0004 cx |

*=SNK; Letters a, b, c and s, t, x show differences among exposure periods and concentrations respectively. Data shown with different letters are significant at the P<0.05 level

$\overline{X} \pm S\overline{x}$ = Mean ± Standard error

## DISCUSSION

The effects of heavy metals on mortality depend not only on concentration, exposure period, interaction with other metals and physical and chemical properties of water but also on species under concern and its developmental stage. Increasing concentrations of copper in *Prochilodus scorpha* (Taneshka 2001) and *Tilapia nilotica* (Cicik and Erdem 1992, Erdem and Kargın 1992, Arslan *et al.* 2006) and lead in *Pimephales promelas* (Vosyliene 1999) were shown to cause mortality. No mortality was observed in *O. niloticus* exposed to 1 $\mu gL^{-1}$ Cd and 1 $\mu gL^{-1}$ Cd-10 $\mu gL^{-1}$ CT mixture during the 30 days of exposure period which might be due to stimulation of detoxification mechanisms.

Heavy metals cause various behavioral changes in fish (Cicik and Erdem 1992, Khunyakari *et al.* 2001, Martinez *et al.* 2004, Karaytuğ *et al.* 2007). Similar behavioral changes such as movement toward surface, increase in operculum movement, disorders in swimming coordination and rejection to take feed, were also observed in *O. niloticus* exposed to Cd but not in fish those exposed to Cd-CT mixture. These differences in behavior turn to normal on prolonged exposures, which might be due to adaption to new environment.

Morphological and numerical changes occur in blood cells of fish since heavy metals are taken into blood through gills. Studies carried out with various fish species had shown that metals cause anemia, swelling in erythrocytes, vacuole formation and anomalies in cytoplasm (Haux and Larsson 1982, Hilmy *et al.* 1982, Tewari *et al.* 1987, Gill and Epple 1993, Vosyliene 1996, Witeska and Baka 2002, Witeska 2004).

Metal uptake was shown to decrease significantly by complex forming agents (Muramoto 1983, Hansten *et al.* 1996, Tao *et al.* 2002). Copper uptake decreased by EDTA in *O. niloticus* (Aslanyavrusu 2010).

CT had an inhibitory effect on Cd intake in *Oncorhynchus mykiss* fed with Cd and Cd-CT mixed food (Ren *et al.* 2006) Copper and CT complex decreased oxidative stress in *C. carpio* (Dautremepuits *et al.* 2004).
The decrease in erythrocyte numbers and hematocrit levels might be due to hemodilution as a result of impairment in osmoregulation or osmotic hemolysis resulted from the increase in erythrocyte membrane permeability.

The increase in the erythrocyte and erythrocyte nucleus areas could be a result of uncontrolled material transference by the damage on the structural integrity of membrane under the effect of Cd.

There was no difference in nucleus numbers, hematocrit levels, erythrocyte and erythrocyte nucleus areas in Cd-CT exposed fish compared with the control fish which might be due to inhibition of metal uptake by chitosan which forms a chelate with the metal.

In conclusion CT is a natural adsorbent which can be used in removing metals from metal contaminated waters and its extensive usage is important as far as environment and human health is concerned.

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