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INFLUENCE OF WATERBORNE CADMIUM ON THE GROWTH AND IMMUNE-PHYSIOLOGICAL PARAMETERS OF NILE TILAPIA (*Oreochromis niloticus)*

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SUMMARY

Nile tilapia, Oreochromis niloticus (65.2±2.2 g) were randomly distributed into 12 glass aquaria to represent 4 treatments (3 replicates per treatment). Fish were exposed to cadmium chloride at concentrations of 0.0 (control), 0.05 (T1), 0.10 (T2) and 0.15 mg L^{-1} (T3) for 4 weeks. After the first, third and fourth week, fish were immediately anesthetized and blood was collected. Fish weight was significantly affected by Cd exposure concentrations after the fourth week. Survival rate was decreased significantly with increasing Cd concentrations at the end of the experiment. The Cd accumulation was determined in fish gills, liver and muscles of Nile tilapia,; however, which these values increased significantly by increasing Cd concentrations and exposure period concentrations after the third and fourth week of exposure. One week after exposure, IgM titer was significantly increased with increasing cadmium concentrations, while after the third and fourth week of exposure, IgM was significantly decreased with increasing Cd concentrations. RBCs and Ht values were significantly decreased by increasing Cd concentrations where their highest values were obtained at control and the lowest ones were obtained at T3. The lowest values of Hb were recorded at T1. The MCV and MCHC values were insignificantly affected by Cd exposure, while MCH values were increased significantly with increasing Cd concentrations. Serum total protein and globulin were not significantly affected by increasing Cd concentrations for the whole period, while albumin was significantly affected at T3 after the first and fourth week of exposure. This study provides significant evidence that waterborne cadmium concentrations cause anemia and suppress immunity in exposed fish. It could be concluded that Cd toxicity induced deleterious effects on fish, which were reflected on growth rate, residual of Cd in different tissues, the concentration of blood IgM and haematological parameters. Moreover, immune suppression could play an important role in predisposing for further infections conditions.

Keywords: Oreochromis niloticus, Cadmium, growth, haematology, immune response

INTRODUCTION

Fish are particularly sensitive to water pollutants, which could impair many physiological and biochemical processes assimilated by fish tissue (Durmaz *et al*., 2006). The agricultural and industrial wastes, partially treated or without treatment are being discharged into surface water (Burger *et al.*, 2007 and Forstner and Wittmann, 2007). Such metals are absorbed from polluted water through gills, skin, and digestive tract of fish by bio-concentration and bio-magnification. Heavy metals are persistent contaminants in the environment that come to the forefront of dangerous substances such as cadmium, lead, mercury, copper and zinc causing serious health hazards in humans and animals (Zaki *et al.* 2009). Moreover, heavy metals such as iron, copper, zinc, lead, cadmium, mercury, chromium and manganese when discharged into the water bodies can enter the food chain, bioaccumulate in fish and hence become a threat to a man (Ajmal *et al.*, 1985). A comparative assessment of numerous heavy metals is an important in determining the degree of pollution in environmental systems. However, interpretation of data sets comprising analyses of several metals is complicated (Bhuiyan *et al*., 2010).

Cadmium (Cd) is a non-nutrient metal that can be found in the water and sediment as a result of anthropogenic activities such as mining and industrial processes (Baldisserotto *et al*., 2004). The concentration of this element in ambient waters varies between 0.1 to 10 mg l^{-1}, while, in anthropogenically polluted waters, it can reach 50 mg l^{-1} (Linnik and Iskra, 1997) and induces C-reactive protein and metallothioneins (Paul and Mandal, 1998; De Smet *et al*., 2001). Although WHO (1989) reported that the permissible level of Cd in water and fish muscles are 0.01 ppm and 1.0 mg g^{-1}, respectively, fish can accumulate Cd to levels 10–1000 times higher than its level in ambient water. Low existence of cadmium in ambient water can produce significant accumulation of cadmium in fish muscles and its subsequent consumption by human may cause some pathophysiological disturbances in human body (Malekpouri *et al.*, 2011).

This metal suppresses immune response to parasites (Thuvander, 1992). Exposure to Cd

causes the most pronounced changes in the leucocytes ratio in peripheral blood of common carp compared to other metals in the order Cd > Pb > Cu > Hg, and neutrophils are the most sensitive type of blood cells (Serpunin and Korobeinikova, 1997). A study performed on Mozambique tilapia, *Oreochromis mosambicus* revealed not only neutrophilia but also thrombocytosis (Ruparelia *et al*., 1990). At the same time, exposure of rainbow trout, *Salmo gairdneri* to Cd did not reveal any changes in the leucocyte formula and in ration of phagocyte cells (Thuvander, 1989). The immune response can be modified by several stessors. Both defense mechanisms and non-specific activity may be affected (Anderson, 1990). However, sometimes pollutant stressors may enhance certain defense parameters, e.g. an increase in circulating antibodies in the serum of striped bass exposed to water with small amounts of cadmium (Robohm, 1986). The immune system is by many environmental pollutants, including heavy metals, even at low levels of exposure (Exon and Koller, 1986). Fish are often exposed to subtoxic concentrations of Cd, which may affect the immune system, causing a modified immune response to infectious agents.

Nile tilapia, *Oreochromis niloticus* (L.) are native to Egypt and are worldwide distributed (El-Sayed, 2006). This species has been used previously in laboratory studies and has been shown to be a suitable organism for monitoring the effects of xenobiotics.

The goal of the present work is to study changes in immune-physiological parameters in the peripheral blood and immune-competent organs in response to sublethal waterborne Cd exposure. In addition, the study was set to answer the question of if the above mentioned parameters may be used for indication of the state of fish and the environment.

MATERIALS AND METHODS

Fish:

The present study was carried out at the wet Laboratory of the Animal Production and Fish Resources Department, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. Nile tilapia, *Oreochromis niloticus* were obtained from Central Laboratory for Aquaculture Research at Abbassa, Abou Hammad, Sharqia, Egypt and acclimated for 15 days before the beginning of the experiment for the normal laboratory conditions. Fish (65.2±2.2 g) were randomly distributed into 12 glass aquaria (35 X 40 X 70 cm) at a rate of 10 fish per each to represent 4 treatments (3 replicates per treatment). De-chlorinated tap water was used throughout the study. Fish were exposed to cadmium chloride at concentrations of 0.05 (T1), 0.10 (T2) and 0.15 mg Cd L^{-1} (T3) for 4 weeks. Fish were fed the experimental diet at the rate of 3% of body weight per day, 30%-crude protein diet up to satiation twice daily six days a week. Siphoning a half of aquarium's water was done every day for excreta removing and replaced by an equal volume of water containing the same Cd concentration.

Physiological measurements:

After the first, third and fourth week of Cd exposure, fish were immediately anesthetized with MS222 (30 mg l^{-1}; Ethyl 3-aminobenzoate methanesulfonate salt; Sigma), and blood was collected with a hypodermic syringe from the caudal vein. The extracted blood was divided in two sets of Eppendorf tubes. One set contained EDTA used as an anticoagulant, for haematology (haemoglobin and red blood cell counting). The second set, without anticoagulant, was left to clot at 4 ^{o}C and centrifuged at 5000 rpm for 5 min at room temperature. The collected serum was stored at –20 ^{o}C for further assays. Hemoglobin (Hb g dl^{-1}) level was determined colorimetrically by measuring the formation of cyanomethaemoglobin according to Handy *et al*. (1999). Haematocrit (Ht %) was measured as packed cells volume (PCV %) by using a micro haematocrit method. Red blood cells (RBCs x 10^{6}/cm) were counted under the light microscope using a Neubauer haemocytometer after blood dilution with phosphate-buffered saline (pH 7.2). The red blood indices; mean corpuscular volume (MCV μm^{3}), mean corpuscular haemoglobin (MCH pg) and mean corpuscular haemoglobin concentration (MCHC g dl^{-1}) were calculated according to Brown (1980). Total protein, albumin, and globulin (Sundeman, 1964), were measured in blood serum by colorimetric methods using commercial kits.

Measurement of serum immunoglobulin M concentration:

Total Immunoglobin M (IgM) concentration in the serum was measured by enzyme-linked immunosorbent assay (ELISA) according to the method of Takemura (1993). Purified tilapia IgM, rabbit anti-tilapia IgM antibody (a-IgM) and a-IgM labeled with horseradish peroxidase (a-IgM HRP) was prepared in advance (Takemura, 1993). Each well of a 96-well microtiter plate (Becton Dickinson Labware, Franklin Lakes, NJ) was coated with 100 µl of a-IgM (6.8 µg/ml) in 0.05 M sodium carbonate buffer, pH 9.6, and incubated for 2 h at 25 ^{o}C. Residual protein binding sites were blocked by adding 200 µl of 1 % gelatin (BioRad Laboratories, Richmond, CA) dissolved in 10 mM phosphate-buffered

saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBS-Tween) to the wells for 60 min at 25 ^{o}C. After washing the wells three times with PBS-Tween using a plate washer (Immunowash 1573, BioRad), 100 µl of plasma sample (1:10 000) or standards (serial dilution of purified tilapia IgM) was added to the well and then incubated overnight at 4 ^{o}C. All the dilutions were made with PBS-Tween. After washing the wells three times with PBS-Tween, 100 µl of a-IgM HRP (diluted 1:20000 in PBS-Tween) was added to the wells and incubated for 2 h at 25 ^{o}C. After three successive washes with PBS-Tween, peroxidase activity was measured by adding 100 µl of 100 mM citrate buffer, pH 4.5, containing 0.01% o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and 0.04% H_2O_2. Following 30-min incubation at 25 ^{o}C, the enzymatic reaction was stopped by adding 25 µl of $4NH_2SO_4$. The optical density of each sample was determined at 490 nm using a microplate reader 550 (BioRad). Computer software (Microplate Manager III, version 1.57) was used for conversion from optical density to IgM concentration.

Residual measurement:

Residual heavy metal (Cd) was detected in gills, liver, and muscles according to APHA (1985). Fish tissues were ashed in a muffle furnace at 550°C for 6 h, then 2 ml HNO_3 was added and the sample was filtered to get a pure solution. After that, Cd was measured by using atomic absorption spectrophotometer (Syatem, 45AA, International Equipement Trading Ltd. Vernon Hills, Illinois, USA.

Statistical analysis

The data were statistically analyzed by completely randomize experiment (one-way ANOVA) according to the following Model 1: Yij = µ + Ti eij, where Yij = an observation, µ = the overall mean, Ti = the fixed effect of cadmium concentrations (i= 1, ..3), and eij = random error. Statistical significance was set at the 5% probability level and means were determined by Duncan's New Multiple Range test (Duncan, 1955). The software SPSS, version 15 (SPSS, Richmond, USA) was used as described by Dytham (1999).

RESULTS

Fish weight (Table 1) was significantly affected by Cd exposure for 4 weeks. The lowest fish weight was obtained at T2 and T3 (73.3 and 72.8 g, respectively). Survival rate decreased significantly (P<0.05) with increasing Cd concentrations after the fourth week of exposure. The Cd accumulation in fish gills, liver and muscles is shown in Table (2). Results showed that Cd accumulated in gills increased significantly by increasing Cd concentrations after the third and fourth week of exposure. The same results were obtained for Cd accumulated in liver and muscles.

This experiment studied the effects of various Cd concentrations on the total IgM (Figure 1) present in *O. niloticus* serum. One week after exposure, IgM titer was significantly increased with increasing Cd concentrations, while after the third and fourth week of exposure, IgM was significantly decreased with increasing Cd concentrations (P<0.05). RBCs and Ht % were significantly affected by Cd exposure concentrations, the highest RBCs and Ht values (Figure 2) were obtained at control and significantly decreased with increasing Cd concentrations after the first, third and fourth week of exposure. Control recorded the highest Hb values, whereas the lowest values of Hb were recorded at T3 (4.44, 4.15 and 3.02 g dl^{-1}) after first, third and fourth week of exposure, respectively.

The MCV and MCHC values were insignificantly (P<0.05) affected with Cd exposure concentrations, while MCH values were increased significantly with increasing Cd concentrations especially after the 1st and 3rd week of exposure (Figure 3).

Serum total protein and globulin were not significantly affected by increasing Cd concentrations for the whole period, while albumin was significantly affected (P<0.05) at T3 after the first and fourth week of exposure (Table 3).

DISCUSSION

Concerning the weight gain of Nile tilapia exposed to Cd for 4 weeks, the obtained data revealed a significant decrease in body weight gain which it could be due to the reduction in food consumption and/or the decrease in gross food conversion rate which resulted in inhibition of growth as previously reported (Abbas, 1994 and Marie *et al*., 1994). There are significant increases in Cd residues in fish gills, liver and muscles due to Cd exposure if compared with control. The Cd contents in gills is a result that fish accumulate the heavy metals from the water primarily through the gills (Zaghloul, 1997) and its uptake could be controlled by the amount of water passing through the gills. This accumulation may also due to the binding of heavy metals to metallothionein (Pratap *et al*., 1989). The previous data showed that the highest Cd concentrations were found in liver. This may be attributed to the major role of liver in the detoxification and protection from heavy metals exposure, both by producing metallothioneins and by acting as a storage site

for bound metals (Freeman *et al*., 1983 and Pratap *et al*., 1989). In the last position, muscles possessed low Cd concentrations at T3 after the first, third and fourth week of exposure. These results were in agreement with Zaghloul (1997). This means that, although Nile tilapia which is the most popular fish, lives under the previous experimental polluted conditions, yet it is still considered as a good edible fish and not harmful to man if only muscles are eaten.

The present study provides evidence of the effect of various Cd concentrations on the basal level of fish immunoglobuins were examined, in relation to the period of exposure. However, a significant decrease of IgM and total protein during the experimental period were observed due to Cd toxicity. Reduction of IgM level indicated that the cadmium chloride toxicity leads to suppression of immune system of exposed fish which become susceptible to any infective agents (Fuda *et al*., 1991). The serum IgM was determined to find out information about fish immune system which was previously investigated in different species by many authors, in the present work, the purified IgM was revealed a single perception against specific polyvalent antiserum to fish immunoglobulin, t has clearly been proved (Jones *et al*., 1971) that the effect of Cd on the immune system depends on concentrations and exposure period. The great reduction in circulating IgM titer after one week of exposure indicates that, initially, Cd seriously impairs the immune system of tilapia. However, the subsequent increase in IgM titer suggests that, after initial inhibition of immune functions, fish started to activate some protective or compensatory mechanism. This may be correlated to the capacity of lemphocytes to synthesize metallothioneins in response to Cd exposure (Harley *et al*., 1989).

Haematological parameters have been considered as important indicators of fish health (Chen *et al*., 2004, and Martins *et al*., 2004). Regarding to haematological profile of Cd-exposed fish, RBCs, Hb, and Ht values decreased. Studies demonstrated that the reduction in the number of erythrocytes in the blood and in Ht may be signs of bacterial infection (McNulty *et al.,* 2003, Benli and Yildiz 2004 and Shoemaker *et al,*. 2006). In contrast, the erythrocyte number of another fish species, *Scyliorchinus canicula,* was increased when the fish was exposed to 15 mg Cd l^{-1} for a period of 24-96 hours (Tort and Torres, 1988). While, O'Connor and Fromm (1975) reported a decrease in RBCs number and Ht, due to hemolysis as a consequence of Hg toxicity. On the other hand, other authors (Benfey and Biron 2000; Affonso *et al*., 2002 and Sampaio *et al*., 2007) suggested that in experiment of toxicity a lowered Ht level could be related to the conditions of confinement or stress induced by the lack of food. The decrease in blood parameters is accompanied by an increase in MCV and MCH and stable MCHC which may be due to haemolytic action which led to fluid loss to the tissue with subsequent decrease in plasma volume (Swift, 1981 and Svoboda *et al.,* 2001). Significant increases in the Hb value and the number of the Ht were found in *Carassius auratus gibelio* and they were attributed to the toxic effects of textile dyes (Al-Sabti, 2000). MCV levels increased in Nile tilapia after the 4th week and MCH levels increased after the 3rd week of Cd exposure. These results are in agreement with Ruperalia *et al*. (1990).

Total protein is a relevant parameter for evaluating the physiological status and condition of fish, and thus an important diagnostic aid (Svetina *et al.,* 2002). This parameter was hardly stable in this study. An important function of serum protein is the maintenance of osmotic balance between the circulating blood and the tissue spaces (Heath, 1995). Therefore, serum protein levels can be used as a diagnostic tool and a valuable test for evaluating the general physiological state. The study revealed that all studied functional and immune-physiological parameters changed under Cd ions impact. However, the directions of these changes were different in blood and other organs. This reflects the contributions of each organ in hemopoiesis and their sensitivity to toxicant.

This study provides significant evidence that Cd concentration cause anemia and suppress immunity in exposed fish. It could be concluded that Cd toxicity induced deleterious effect on fish which were reflected on the concentration of blood IgM, haematological parameters, tissue Cd residual and growth rate of Nile tilapia. Moreover, immune suppression could play an important role in predisposing for further infections conditions.

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Table 1. The growth parameters rate and survival (Mean±SE) of Nile tilapia exposed to different Cd concentrations for 4 weeks

Items	Initial weight (g)	Final weight (g)	Weight gain (g)	Survival rate %
Treatments (mg l^{-1})				
Control	65.3±0.6	78.4^{a}±1.2	13.1^{a}±0.57	98.3^{a}±1.6
0.05 (T1)	66.2±0.7	75.2^{a}±1.4	9.0^{b}±0.25	90.2^{b}±2.5
0.10 (T2)	65.4±0.5	73.3^{b}±1.2	7.9^{b}±0.26	86.1^{b}±2.4
0.15 (T3)	64.8±0.8	72.8^{b}±1.4	8.0^{b}±0.50	85.4^{c}±2.6

Means in the same column having different letters are significantly different at P<0.05.

Table 2. Changes of Cd residues in different organs (ug/g dry weight) (Mean±SE) of Nile tilapia exposed to different Cd concentrations for different periods

Items	Gills	Liver	Muscles
Treatments (mg l^{-1})			
1st week of exposure			
Control	0.011^{d}±0.00	0.18^{d}±0.02	ND*
0.05 (T1)	0.148^{c}±0.003	28.24^{c}±2.25	0.212^{c}±0.009
0.10 (T2)	0.316^{b}±0.005	34.21^{b}±2.12	0.521^{b}±0.011
0.15 (T3)	3.219^{a}±0.015	59.54^{a}±4.23	0.719^{a}±0.019
3rd week of exposure			
Control	0.013^{d}±0.002	0.21^{d}±003	ND*
0.05 (T1)	0.251^{c}±0.005	40.31^{c}±2.22	0.425^{c}±0.018
0.10 (T2)	0.486^{b}±0.009	58.41^{b}±4.52	0.931^{b}±0.035
0.15 (T3)	6.421^{a}±0.321	92.41^{a}±5.95	1.512^{a}±0.052
4th week of exposure			
Control	0.016^{d}±0.002	0.25^{d}±0.02	ND*
0.05 (T1)	0.341^{c}±0.009	65.22^{c}±6.23	0.518^{c}±0.017
0.10 (T2)	0.562^{b}±0.012	86.32^{b}±7.29	1.212^{b}±0.042
0.15 (T3)	10.317^{a}±0.952	112.21^{a}±9.53	2.217^{a}±0.089

Means in the same column within each classification bearing different letters are significantly (P<0.05) different.
*ND: not detected

Table 3. Changes in serum protein, albumin, and globulin (Mean±SE) of Nile tilapia exposed to different Cd concentrations for different periods

Items	Protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})
Treatments (mg l^{-1})			
1st week of exposure			
Control	4.22±0.52	1.15^{a}±0.11	3.07±0.21
0.05 (T1)	4.12±0.32	1.01^{a}±0.21	3.11±0.23
0.10 (T2)	4.01±0.41	0.94^{a}±0.04	3.07±0.24
0.15 (T3)	3.91±0.25	0.82^{b}±0.05	3.09±0.31
3rd week of exposure			
Control	4.13±0.32	1.03±0.06	3.10±0.24
0.05 (T1)	4.03±0.29	0.98±0.07	3.05±0.27
0.10 (T2)	3.92±0.31	0.86±0.08	3.06±0.31
0.15 (T3)	3.82±0.27	0.82±0.07	3.00±0.22
4th week of exposure			
Control	4.21±0.31	1.11^{a}±0.21	3.10±0.22
0.05 (T1)	4.03±0.28	0.92^{a}±0.06	3.11±0.21
0.10 (T2)	3.92±0.21	0.81^{a}±0.05	3.11±0.19
0.15 (T3)	3.82±0.22	0.74^{b}±0.04	3.08±0.16

Means in the same column within each classification bearing different letters are significantly (P<0.05) different.

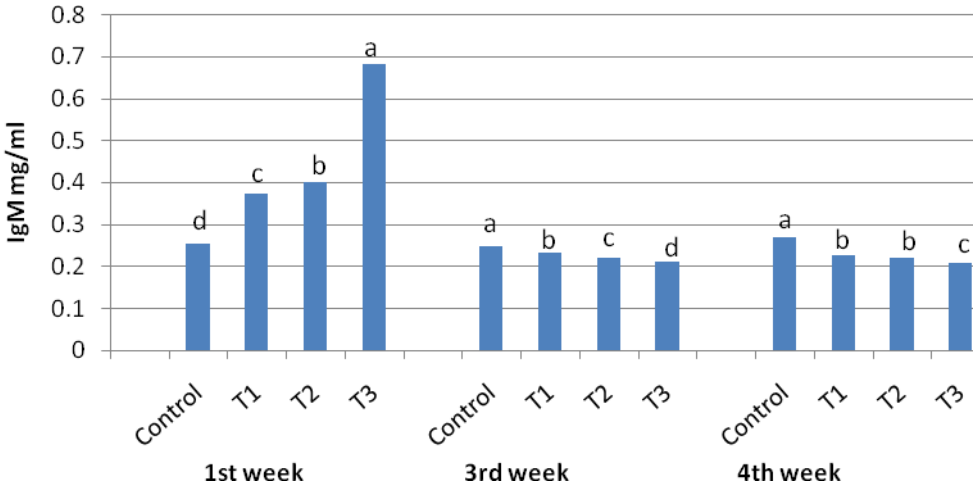

Figure 1. Changes in IgM (mg ml $^{-1}$) in Nile tilapia exposed to different Cd concentrations for different periods.

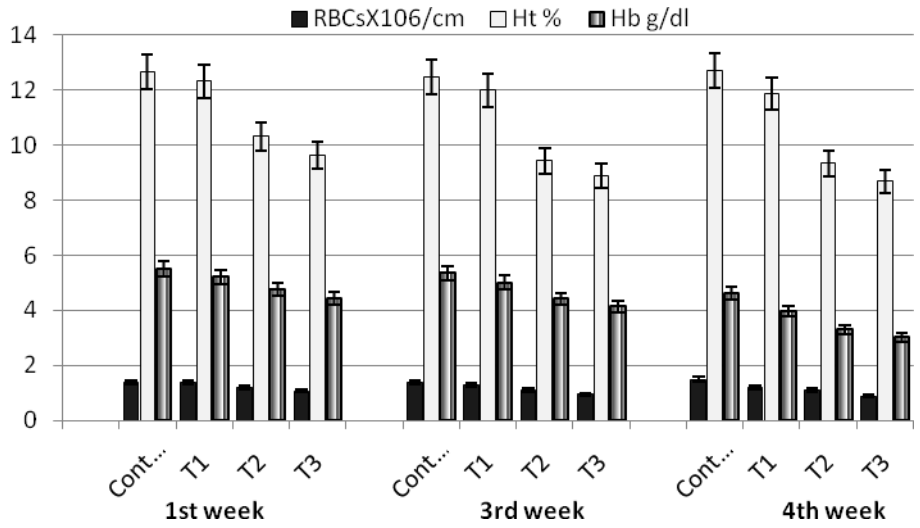

Figure 2. Changes in RBCs (x10^6/cm), Ht (%), and Hb (g dl^{-1}) in Nile tilapia exposed to different Cd concentrations for different periods.

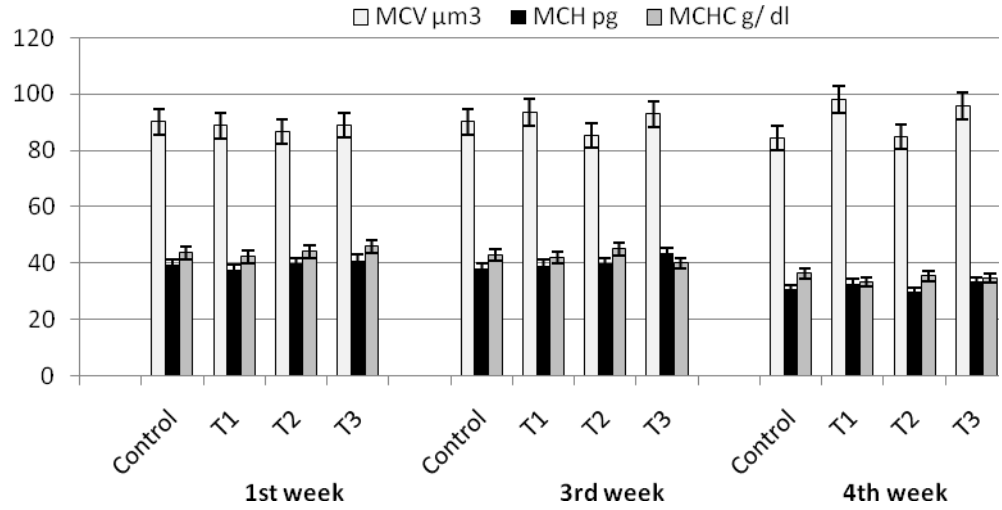

Figure 3. Changes in MCV (µm^3), MCH (pg) and MCHC (g dl^{-1}) in Nile tilapia exposed to different Cd concentrations for different periods.

تأثير الكادميوم المنقول عن طريق المياه على النمو و مدلولات المناعة الفسيولوجية للبلطي النيلي

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وزعت اسماك البلطي النيلي عشوائيا (وزن ٦٥.٢ ± ٢.٢ جم) على ١٢ حوض زجاجي تعبر عن اربع معاملات (٣ مكررات/ معاملة) استمرت التجربة لمدة ٢٨ يوم وتم تجميع الدم مباشرة بعد تخدير الاسماك بعد الاسبوع الاول, الثالث والرابع من التعرض لكلوريد الكادميوم عند التركيزات ٠.٠٥ (م١), ٠.١٠ (م٢) , ٠.١٥ مجم/لتر (م٣). حدث تأثير لوزن السمك بعد الاسبوع الرابع وقلت نسبة البقاء معنويا بزيادة تركيزات الكادميوم في نهاية التجربة. تم تقدير تراكم الكادميوم في الخياشيم والكبد والعضلات لاسماك البطي النيلي حيث زادت هذه القيم معنويا بزيادة تركيزات الكادميوم بعد الاسبوع الثالث والرابع من التعرض. زادت نسبة الجلوبيولين المناعي معنويا بزيادة تركيزات الكادميوم بعد اسبوع واحد من التعرض, بينما بعد الاسبوع الثالث والرابع قلت نسبة الجلوبيولين المناعي معنويا بزيادة تركيزات الكادميوم وزيادة الفترة التي تم التعرض لها. تأثرت عدد كرات الدم الحمراء ونسبة الهيماتوكريت معنويا بالتعرض لتركيزات الكادميوم, كانت اعلى قيم تم الحصول عليها لعدد كرات الدم الحمراء ونسبة الهيماتوكريت في المجموعة المقارنة وقلت معنويا بزيادة تركيزات الكادميوم في نهاية الاسبوع الاول, الثالث والرابع من التعرض. سجل الهيموجلوبين اقل قيم عند الجرعة الاعلى للكادميوم ٠.١٢ مجم/لتر في نهاية كل فترة من فترات التعرض. لم يحدث أي تأثير معنوي لمقياس متوسط كمية الهيموجلوبين في كريات الدم الحمراء MCH , قياس تركيز الهيموجلوبين في كريات الدم الحمراء MCHC عند التعرض لتركيزات الكادميوم. بينما زادت قيم قياس متوسط حجم كريات الدم الحمراء MCV معنويا مع زيادة تركيزات الكادميوم. لم يتأثر البروتين الكلي في سيرم الدم وكذلك الجلوبيولين بزيادة تركيزات الكادميوم في الفترة الكلية للتجربة بينما تأثر الالبومين معنويا عند المعاملة الثالثة بعد الاسبوع الاول والرابع من التعرض. يمكن استنتاج ان التعرض للكادميوم المحمول عن طريق المياه كان له تأثير ضار على الاسماك والذي انعكس ذلك على معدل النمو في البلطي, الكادميوم المتبقي في الانسجة, تركيز الجلوبيولين المناعي ومكونات الدم. علاوة على ان نقص المناعة تلعب دور هام في التهيئة لمزيد من الاصابات.