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Histopathological and Biochemical Effects of Humic Acid Against Cadmium Toxicity in Brown Trout Gills and Muscles

Gonca Alak[1,*], Muhammed Atamanalp[2], Ahmet Topal[2], Harun Arslan[2], Ertan Oruç[3], Serdar Altun[3]

[1] Ataturk University, Agriculture Faculty, 25240 Erzurum, Turkey.
[2] Ataturk University, Fisheries Faculty, 25240 Erzurum, Turkey.
[2] Ataturk University, Veterinary Faculty, 25240 Erzurum, Turkey.

* Corresponding Author: Tel.: +90.442 2360958; Fax: +90.442 2360958;
E-mail: galak@atauni.edu.tr

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Abstract

It was biochemically and histopathologically investigated whether humic acid (HA) has protective effects on cadmium (Cd) toxicity on muscle and gills of brown trout (Salmo trutta fario Linnaeus, 1792). The brown trout were exposed to cadmium (2 ppm) and/or humic acid (5 ppm). For this purpose, levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA) was investigated in muscle and gills tissues of brown trout. The activities of GPx and SOD in the tissues of fish exposed to Cd was significantly lower than the control groups ($P<0.05$). MDA levels did not increase in the groups exposed to cadmium ($P>0.05$). Hovewer, humic acid did not affect biochemical damage in cadmium group. Cd caused a significant increase in histopathological changes in muscle and gills tissues, but histopathological changes were lower in the muscle tissue of Cd+HA group. These results suggest that humic acid may counteract the cadmium toxicity in muscles tissue in histopathologic aspect.

Keywords: Toxicity, antioxidant enzyme, brown trout, histopathology, humic acid.

Humik Asitin Kahverengi Alabalık Solungaç ve Kaslarında Kadmiyum Toksisitesine Karşı Histopatolojik ve Biyokimyasal Etkisi

Özet

Kadmiyum toksisitesine karşı humik asitin koruyucu etkisi biyokimyasal ve histopatolojik olarak kahverengi alabalık (*Salmo trutta fario* Linnaeus, 1792) solungaç ve kas dokularında araştırılmıştır. Balıklar kadmiyum ve/veya humik asite (2ppm Cd, 5 ppm humik asit) maruz bırakılmışlardır. Bu amaçla kahverengi alabalıkların kas ve solungaç dokularında glutatyon peroksidaz (GP_X), süperoksit dismutaz (SOD), malondialdehit (MDA) düzeyleri incelenmiştir. Cd'a maruz kalan gruplarda GP_X ve SOD enzim aktiviteleri kontrol grubuna kıyasla düşük değerler vermiştir ($P<0.05$). MDA seviyesi kadmiyuma maruz kalan gruplarda artmamıştır ($P>0.05$). Bununla beraber kadmiyum uygulanan gruplarda humik asit biyokimyasal zararı önlemede etkili olmamıştır. Cd kas ve solungaç dokularında histopatolojik olarak önemli artışlara neden olurken, Cd+HA'in birlikte uygulandığı grupta kas dokusu daha az etkilenmiştir. Bu sonuçlara göre, humik asit kas dokusunda histopatolojik olarak koruyucu etki gösterebilir

Anahtar Kelimeler: Toksisite, antioksidan enzim, kahverengi alabalık, histopatoloji, humik asit.

Introduction

Cadmium is a potentially toxic heavy metals element; its concentration has been increasing in the air, water, soil, and plants since the beginning of the last century (Amdur *et al.*, 1991). After entering into the aquatic animals through food or water, cadmium binds to albumins and erythrocytes in the blood and then is transferred into tissues and organs, where it is bound to proteins of low molecular mass producing metallothioneins by the induction of metallothionein mRNA synthesis (George and Wright 1996; Karadeniz *et al.*, 2009). Accumulation of cadmium in living organisms is a major ecological concern especially because of its ability to accumulate very quickly (Okocha and Adedeji, 2011). In fish, cadmium can cause a number of structural and pathomorphological changes in various organs (Besirovic *et al.*, 2010). The highest cadmium levels were detected in the kidneys and liver of fish (Thophon *et al.*, 2003). The interactions between specific enzyme systems and different drugs, metal ions and chemicals have been extensively studied in the recent years (Cankaya *et al.*, 2007; Ekinci *et al.*,

2007; Ceyhun *et al.*, 2011). Many environmental pollutants are capable of inducing oxidative stress in aquatic animals (Ceyhun *et al.*, 2010). Chemical toxic pollutants are important sources of ROS in biological systems and inhibit the activity of some enzymes of the antioxidative defense system (Pruel and Engelhardt, 1980; Zikic *et al.*, 1997). Oxidative stress and damage to fundamental biomolecules and to antioxidant defenses of organisms is an established field in environmental toxicology and ecotoxicology (Talas *et al.*, 2008). ROS, which cause tissue damage, are decreased by antioxidant enzymes such as endogen glutathione (GSH), superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) (Halliwell *et al.*, 1995; Anderson, 1996).

Humic acid is ubiquitous in the environment and have been found to influence physiological functions of aquatic organisms (Andersson *et al.*, 2010). Humic acid has been used as an antidiarrheal, analgesic, immunostimulatory, and antimicrobial agent in veterinary practices in Europe (Rath *et al.*, 2006). There are literature states that it has growth related effect as well as health protection capacity by changing some physiology and developing immunity in different species of animal (Islam *et al.*, 2005).

The aim of this study was to investigate a possible protective effect of humic acid against cadmium toxicity in brown trout. Toxicity was assessed histopathologically and levels of the muscle and gills antioxidants GPx, SOD and MDA were measured.

Materials and Methods

Experimental Design

Brown trout, *Salmo trutta fario*, L. were obtained from Ataturk University, Faculty of Fisheries and Inland water Fish Breeding and Research Center. The research was arranged in the Fish Toxicology Laboratory. The experiments were performed according to the approved ethical rules. Fish were fed for 15 days in a stock pond to provide their acclimatisation to the environmental conditions. After the adaptation period, ten fish were placed in 400 lt volume fiberglass water tanks. The tested fish had an average 203.31 ± 8.09 g weight and 22.21 ± 0.49 cm length. Physico-chemical properties of tank water are: temperature (10-12^{0}C); pH (7.4-8.0); dissolved oxygen (7.52 ± 0.50 ppm); water hardness $CaCO_3$ (164.1 ± 4.17 ppm). Humic acid obtained from Farmavet Medicine. The stock solutions of cadmium chloride (Sigma) and humic acid were used and the final concentration was achieved. The concentrations of the Cd and HA in the stock solutions were that the exposure concentrations (ppm) was Cd 2ppm, while HA was 5ppm (Talas *et al.*, 2008; Kamunde and MacPhail, 2011).

Fish were divided into four groups as group I control group, group II cadmium group, group III humic acid group and group IV cadmium+humic acid group each containing 10 fish. Fish in group II were given a single dose of 2 ppm concentration of cadmium chloride ($CdCl_2$). This dose was selected as it has been previously reported to induce toxicity in rainbow trout (Talas *et al.*, 2008). Fish in group III were exposed to a single dose of 5 ppm concentration of humic acid. Fish in group IV were exposed to a mixture dose of 2 ppm cadmium chloride and 5 ppm humic acid. These doses were administered to fish for seven days.

Biochemical Analysis

Glutathione peroxidase (GPx), superoxide dismutase (SOD), and malondialdehyde (MDA) levels in brown trout tissues analyzed according to Alak *et al* (2013). Extracts from each tissue were prepared from each individual in according to Wiegand *et al.* (2001) with a little modification. To prepare the tissue homogenates, tissues were ground with liquid nitrogen in a mortar. The samples were homogenized by KH_2PO_4 (30mM, pH=7.3) buffer. And then homogenates were centrifuged at 13000 rpm, 2 hours at 4 °C. These supernatants were used for the determination of the enzymatic activities. All results were referred to the protein content in the samples. MDA levels of fish tissues were estimated according to Gülcin *et al.*, (2009). 200 µl hemolysate, 800 µl phosphate buffer (50 mM, pH 7.4), 25 µl BHT and 500 µl of 30% TCA were added mixed fast and incubated at -20 °C for 2 hours, than centrifuged at 2000 rpm for 15 min. 1.0mL supernatant was separated. Afterwards 75 µl EDTA-Na_2H_2O, 250 µl TBA were added to each sample and control. Then they were placed in a boiling water bath for 15 min, cooled to room temperature and measured at 532 nm. Total thiobarbituric acid-reactive materials are expressed as MDA, using a molar extinction coefficient for MDA of 1.56 x 10^5 $cm^{-1}M^{-1}$ protein content of each homogenate was measured according to Bradford (1976) with Coomassie Brilliant Blue G-250 using bovine serum albumin as a standard (Aydın *et al.*, 2012).

Histopathological Examination

The tissue samples for light microscopic examination were fixed in 10% formaldehyde 24 h, dehydrated in a graded alcohol series, and cleared in xylol. After dehydration, specimens were embedded in melt paraffin. Sections were cut using a microtome (Leica, Germany). Each paraffin block was serially cut into 5 µm-thick sections. The sections were stained with hematoxylin-eosin (H-E) for light microscopic examination (Olympus BX52 with DP72 camera system) (Presnell and Schreibman, 1997). All histopathological alterations were estimated with an image processing system (Olympus, DP2-BSW). The scores were derived semi-quantitatively using light

microscopy on the preparations and were reported as follows:
none: -, mild: +, moderate: ++, and severe: +++.

Statistical Analysis

All values were expressed as mean±S.E. Statistical analysis of data was performed using a one-way analysis of variance (ANOVA) and Duncan test. A value of $P<0.05$ was considered statistically significant. Data were analyzed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA) software (Bingöl ve Kocamış, 2010).

Results

Biochemical Results

Cadmium administration significantly decreased the levels of SOD and GPx in the fish muscle and gill tissues compared with the control group ($P < 0.05$) (Tables 1, 2). HA treatment with Cd did not produce a significant increase in the level of GPx and SOD in the muscle and gill. There were not statistically significant increases in MDA levels in the muscle and gill of brown trout exposed to Cd ($P>0.05$). Also it was observed that there were no significant ($P>0.05$) convergences in comparison to the control group values for MDA activity in gill tissues of brown trout (Tables 1, 2).

Histopathological Results

There were no histopathological alterations in gill (Figure 1a) and muscle (Figure 2a) tissues of control group. Similarly, no histopathological alterations were observed in humic acid group (Figure 1b and 2b). Prominent changes were observed in experimental cadmium group.

Hydropic degeneration, vacuolative degeneration, hyperplasia of epithelial cells in secondary lamella and destruction of some secondary lamella (Figure 1c) were observed in gill sections of cadmium treated group. In addition to these findings there were interstitial mononuclear cell infiltrations in muscle tissues (Figure 2c) in cadmium group. Similar histopathological alterations were compared to cadmium+humic acid group. There were congestion of blood vessels, cellular degeneration and desquamations were observed in gill section (Figure 1d). While, inflammatory cells infiltration was observed in low degree in cadmium+humic acid group (Figure 2d). The intensity and severity of histopathological alterations were displayed in Table 3.

Table 1. The effects on GPx, SOD activity and MDA levels in the muscle tissue of humic acid (HA) administration to fishes with or without cadmium (Cd)

Treatment	GPx (µmol/mg prot.)	SOD (U/mg prot.)	MDA (nmol/mg prot.)
Control	4.54±3.13^{a}	2.16±0.22^{a}	0.06±0.01^{a}
HA	3.15±0.07^{b}	1.97±0.35^{a}	0.05±0.01^{a}
Cd	1.86±2.33^{c}	0.83±0.21^{b}	0.07±0.01^{a}
Cd+HA	2.09±0.23^{c}	0.75±0.20^{b}	0.05±0.02^{a}

All data points are the average of n = 8 ± SD, Different superscript letters indicate statistically significant differences ($P < 0.05$)

Table 2. The effects on GPx, SOD activity and MDA levels in the gill tissue of humic acid (HA) administration to fishes with or without cadmium (Cd)

Treatment	GPx(µmol/mg prot.)	SOD (U/mg prot.)	MDA(nmol/mg prot.)
Control	3.55 ±1.33^{a}	0.38 ±0.10^{a}	0.38±0.16^{a}
HA	2.01 ±0.06^{b}	0.72±0.09^{b}	0.31±0.41^{a}
Cd	1.00 ±1.23^{c}	0.17±0.20^{c}	0.45±0.09^{a}
Cd+HA	0.93 ±1.05^{c}	0.19±0.61^{c}	0.35±0.26^{a}

All data points are the average of n = 8 ± SD, Different superscript letters indicate statistically significant differences ($P < 0.05$)

Table 3. The intensity and severity of histopathological alterations in gill and muscles tissues

Histopathologic lesion	Control group	HA group	Cd group	Cd+HA group
Gill				
Congestion	-	-	++	++
Cytoplasmic vacuols	-	-	++	++
Desquamation	-	-	++	++
Hyperplasia	-	-	++	++
Muscles				
Congestion	-	-	+	-
Inflammatory cells infiltration	-	-	++	+

-: none, +: mild, ++: moderate, +++: severe.

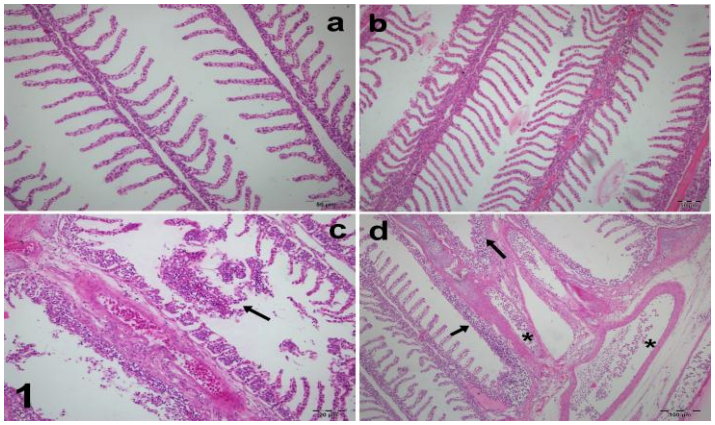

Figure 1. Normal histologic appearance of gill tissue in control (a) and HA (b) groups. Severe degeneration and desquamation of gill epithelium in Cd group (c-arrow) and similar epithelial loss in Cd+HA group (d-arrow). Many congestive vessel (*) in Cd+HA group.

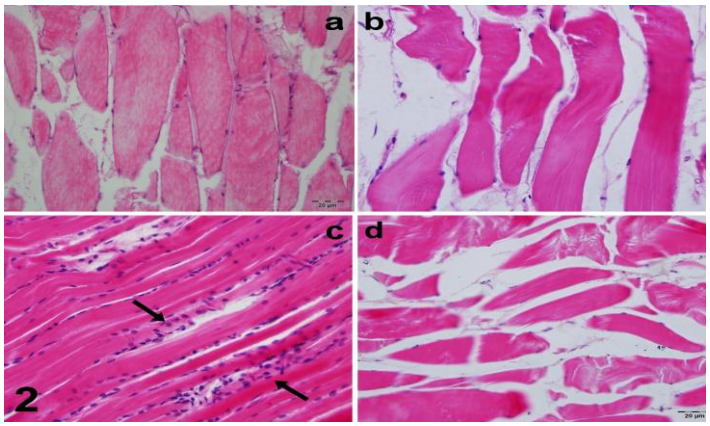

Figure 2. Normal histologic appearance of muscle tissue in control (a) and HA (b) groups. Inflammatory mononuclear cell infiltrations in Cd group (c-arrows). There is no inflammatory cells in Cd+HA group (d).

Discussion

The purpose of this study was to investigate the toxic effects of cadmium in brown trout fish and the possible prevention of them by humic acid. Free radicals and other reactive oxygen species (ROS) have been recently incriminated in the pathogenesis of various metal toxicities (Senapati *et al.*, 2001; Kumar, 2010). It has been reported that alterations in free radicals production and antioxidant defense system of the body after cadmium exposure (Oner *et al.*, 1995). Several studies revealed that exposure to pollutions, including Cd, in aquatic ecosystems can enhance the intracellular formation of ROS which could cause oxidative damage to biological systems (Ercal *et al.*, 2001; Dabas *et al.*, 2012). In this sense, it has been showed that Cd can compete with essential metals in protein-binding sites, triggering a release of Fe^{2+} and Cu^{2+} ions and causing increased generation of ROS (Pruski and Dixon, 2002).

Therefor, the level of antioxidant enzyme is a good indicator for the impacts of pollutants like heavy metals. (Ates *et al*., 2008). Antioxidant enzymes such as glutathione peroxidase and superoxide dismutase that prevent oxidative stress (Stajn *et al*., 1997; Ognjanovic *et al*., 2008). In our study, enzymes activities (GPx and SOD) were significantly decreased in muscle and gill of brown touts in cadmium group ($P<0.05$). Our results clearly indicated that SOD and GPx may play a role in the suppression of oxygen free radical formation in muscle and gill tissue. Similar results showed that SOD and GSH-Px levels were decreased by affected heavy metal toxication in the fish. Heavy metal toxicity led to free radicals and oxidative damage on tissue. (Talas *et al.*, 2008). It has been reported that cadmium cause defects in cells and tissues by damaged to mitochondrial enzymes (Lacroix and Hontela, 2004).

Generally metals induce oxidative stress directly through redox cycling or indirectly through interference with the enzymatic and/or non-enzymatic oxidative stress defense systems (Kamunde and MacPhail, 2011). We therefore evaluated the utility of MDA, a product of membrane lipid peroxidation, as a biomarker of oxidative toxicity for the detection of early cellular damage due to metallic exposure. The results indicated that MDA concentrations in gills and

muscle did not change in Cd and Cd+HA groups. Also, HA treatment with Cd did not produce a significant increase in the level of GPx and SOD in the muscle and gill ($P > 0.05$).

It has been reported that the humic acid increased the acute and chronic toxicity of Cd and there was no effect of HA on the bioaccumulation of either metal (Winner, 1984), but in another study, it has been showed that humic acid consistently reduced Cd accumulation in all the tissues/organs (Kamunde and MacPhail, 2011). It had been reported that waterborne Cd exposure caused significant accumulation of Cd in whole body, muscle and aquatic vertebrae. In the same study, muscle Cd levels recorded in experiment were sufficiently high to cause concern effect (Liu *et al.,* 2011). Histopathological alterations were lower in Cd+HA group (Figure 2d). This result may show that HA may reduce cadmium toxicity in muscle.

The heavy metal damage is an important factor in many pathological and toxicological processes (Ates *et al.,* 2008). In fish, cadmium can cause a number of structural and pathomorphological changes in various organs (Thophon *et al.,* 2003). Gill is an important tissue because of its direct contact with water and any effect or agency has to go through it to come into the fish body. The lamella epithelial lining reacts to dissolved lead creating tissue osmoregulatory imbalance (Jana and Bandopadhyaya, 1987). Gills are reported to act as storehouse of cadmium in experimental studies (Allen, 1995; Okocha and Adedeji, 2011).

Wong and Wong (2000) reported that morphological and biochemical changes caused in the gills of Tilapia *(Oreochromis mossambicus)* after experimental cadmium exposure. The hyperactivity of fish when exposed to toxicants may be due to hypoxia faced by the fish due to gill damage by the irritant and the increase in haemoglobin content may be to compensate for impaired respiratory efficiency (Remyla *et al.,* 2008). It had been previously reported to induce toxicity in *Leuciscus cephalus* of Cd toxicity (Yılmaz *et al.,* 2011). Our research results in this respect were similar with the findings of Yılmaz *et al.* (2011). We can say that this histopathological alteration was caused by cadmium poisoning.

In conclusion, Cd treatment induces gills and muscle damage as indicated by the elevation of histopathological alterations, the decline of the antioxidant activity. HA did not have much effect against damage induced by cadmium, but HA was found to decrease the Cd- induced histopathological alterations in muscle tissues. Therefore, humic acid may mediate the cadmium toxicity in muscle tissues because of reducting histopathological effects.

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