Carbonic Anhydrase Activity Responses and Histopathological Changes in Gill and Liver Tissues after Acute Exposure to Chromium in Brown Trout Juveniles

Kahverengi Alabalık Yavrularında Kroma Akut Maruz Kaldıktan Sonra Solungaç ve Karaciğer Dokularında Histolojik Değişiklikler ve Karbonik Anhidraz Aktivitesi Yanıtları

Research Article

Yeliz Demir[1], Ertan Oruç[2], Ahmet Topal[3]*

[1]Atatürk University, Faculty of Science, Department of Chemistry, Erzurum, Turkey.
[2]Atatürk University, Faculty of Veterinary, Department of Pathology, Erzurum, Turkey.
[3]Atatürk University, Faculty of Fisheries, Department of Basic Sciences, Erzurum, Turkey.

ABSTRACT

Heavy metals are major pollutants in ground, air, marine and fresh water and they cause toxic effects in fish tissues. This study was conducted to determine histopathological effects and changes in carbonic anhydrase (CA) enzyme activity in liver and gill tissues of brown trout juveniles exposed to chromium (Cr^{3+}). Fish were exposed to chromium concentrations (1 mg/L and 2 mg/L) for 24, 48 and 96h. It was found that CA was significantly inhibited at 48 and 96h of two concentrations ($p<0.05$). Besides, it was observed that the most common liver changes produced by Cr^{3+} were hyperemia and hepatocyte degeneration. The gill tissues of fish exposed to Cr^{3+} were characterized by lamellar hyperemia, lamellar edema, clumping, epithelial degeneration and lamellar atrophy. The present study indicated that Cr^{3+} inhibits carbonic anhydrase enzyme and causes histopathological damages in gill and liver tissues.

Key Words
Carbonic anhydrase, histopathology, liver, gills, chromium.

ÖZ

Ağır metaller toprak, hava, deniz ve tatlı sularda önemli kirleticilerdir. Balık dokularında toksik etkilere neden olurlar. Bu çalışma, kroma (Cr^{3+}) maruz kalmış kahverengi yavru alabalık karaciğer ve solungaç dokularında karbonik anhidraz (CA) enzim aktivitesindeki değişiklikleri ve histolojik etkileri belirlemek amacıyla yapıldı. Balık 24, 48 ve 96 saat için (1 mg/L ve 2 mg/L) derişimlerde kroma maruz bırakıldı. CA iki derişimde, 48 ve 96 saat'ta önemli bir şekilde inhibe olduğu bulundu ($p<0,05$). Ayrıca, krom tarafından üretilen en yaygın karaciğer değişiklikleri kan göllenmesi ve hepatosit dejenerasyonu görülmüştür. Kroma maruz kalan balıkların solungaç dokularında katmanlı kan göllenmesi, katmanlı ödem, topaklanma, epitel dejenerasyonu ve katmanlı atrofi ile karakterize edildi. Bu çalışmada Cr^{3+} karbonik anhidraz enzimini inhibe eder solungaç ile karaciğer dokularında histopatolojik zararlara neden olduğu belirtildi.

Anahtar Kelimeler
Karbonik anhidraz, histopatoloji, karaciğer, solungaçlar, krom.

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Correspondence to: A. Topal, Atatürk University, Faculty of Fisheries, Department of Basic Sciences, Erzurum, Turkey.
Tel: +90 442 231 7053 Fax: +90 442 231 7065 E-Mail: drahmettopal@hotmail.com

INTRODUCTION

The contamination of aquatic environment by pollutants has become a matter of great concern over the last few decades not only because of the threat to public water supplies but also with the damage caused to the aquatic life [1]. Heavy metals such as chromium and cadmium reach the aquatic systems as a consequence of industrial, agricultural, sewage disposal, soil leaching and rainfall, thus aquatic organisms are exposed to a significant amount of these pollutants in aquatic environments [2,3]. It has been reported that heavy metals cause toxicity in aquatic environments and the heavy metal damage is an important factor in many pathological and toxicological processes. Several authors reported that heavy metals can alter enzymatic activities by binding to functional groups, such as sulfhydryl, carboxyl and imidazole, or by displacing the metal associated with the enzyme [4,5]. Also, heavy metals may promote oxidative damage by directly increasing the cellular concentration of reactive oxygen species and by reducing the cellular antioxidant capacity [3]. Chromium (Cr) is a heavy metal that used in industrial processes, and is released into aquatic environments by electroplating, steel production, leather tanning and textile industries [6,7]. It has been reported that Cr produces toxic effects in aquatic organisms. The high deposition of Cr in fish gills causes to tissue damage, including hyperplasia, clubbing of lamellae and necrosis [8]. Velma and Tchounwou (2010) indicated that Cr caused biochemical, genotoxic and histopathologic effects in liver and kidney tissues of goldfish [9].

Liver is an important point for storing of metals. In the studies conducted on various species of fish, when the compared to other organs, metal bioaccumulation has been found higher in the liver [10]. The gill is the first tissue contacting with the contaminants in the water. Due to its large surface area and the small diffusion distance between the water and blood, the gills are primarily affected by contaminants such as metals. In general, the gill cells respond rapidly to various chemicals to overcome physiological impairment or tissue damage, and chemicals may have a negative effect on the overall gill function, enhancing fish susceptibility to toxic compounds and potentially leading to fish mortality [11,12].

It is well known that almost all chemicals and drugs show their effects on various enzymes in the metabolism. Particularly, some enzymes are target for this substances such as glucose 6 phosphate dehydrogenase, paraoxonase including carbonic anhydrases [13-23]. Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1 (CA)) is expressed almost all tissues. The zinc enzyme catalyzes the reversible reactions of carbon dioxide hydration and bicarbonate dehydration, physiologically.

$$CO_2 + H_2O \quad H_2CO_3^- \leftrightarrow H^+ + HCO_3^-$$

This reaction is the main role of CA enzymes in physiological conditions. Carbonic anhydrase is important in the osmotic and acid–base regulation in the fish. The gills play the most vital role in acid-base relevant ion transfer, which is the transfer of H^+ and/or HCO_3^-. CA has been purified from many tissues, and kinetic properties determined. Also, CA^- metal interactions have been investigated on a variety of organisms, including fish, crabs and humans *in vitro* and *in vivo* for a long time [24-30]. In our study, we tried to determine the *in vivo* inhibition of chromium and also, evaluated histopathological levels in liver and gill tissues after acute exposure concentrations of the chromium in brown trout juveniles.

MATERIALS AND METHOD

Materials

CNBr-activated Sepharose 4B, protein assay reagents, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Para-aminobenzene sulfonamide and L-tyrosine were from Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt Germany). All other chemicals were analytical grade and obtained from either Sigma-Aldrich or Merck.

Fish Husbandry and Maintenance

Brown trout juveniles (Salmo trutta fario) weighing 5-6 g were provided by Ataturk University, Faculty of Fisheries, and Inland water Fish Breeding and Research Center. The water quality parameters were determined in all glass aquarium (temperature 10°C, pH 7.0, dissolved oxygen 8.8 mg/L, SO_4^{-2} = 0.35 mg/L, PO_4^{-3} = trace, NO_3^- = 1.52 mg/L and NO_2^- = trace). Fish were acclimated for 15 days under the laboratory conditions and they were fed twice in a day with a commercial pelleted trout feed (at 1% body weight).

***In vivo* inhibition assays**

In this study, sublethal concentrations of chromium (1.0 and 2.0 mg/L) was used. Because it was lower than lethal concentrations. The LC_{50} value for trout of chromium was 4.4 mg/L and it had been previously reported to induce toxicity in rainbow trout [31]. Stock solution of chromium was prepared by dissolving in distilled water. Throughout the experiments, glass aquarium contained 15 fish in 25 cm^3 dechlorinated tap water. One aquarium was used as control and did not contain metal while others were added 1.0 and 2.0 mg/L Cr(III), respectively. Fish were exposed to chromium concentrations (1 mg/L and 2 mg/L) for 24, 48 and 96 h. At the end of exposure period, control and all treatment fish were chosen from each aquarium randomly and were sampled at 24 h, 48 h and 96 h. In each hour, fish were killed by cervical section. Liver and gill tissues were immediately removed. Tissues of each group were placed in 10% formalin solution for histopathological examination and were stored at -20°C until analysis carbonic anhydrase enzyme activity.

Determination of carbonic anhydrase enzyme activity

Liver and gill tissue samples were washed three times with 0.9% NaCl. Each of tissues were homogenized with buffer 25 mM Tris-HCl + 0.1 M Na_2SO_4 (pH 8.7) by homogenizer and supernatant was centrifuged at 4°C, 15000g for 60 min. Enzyme activity was assayed by following the hydration of CO_2 according to the protocol established by Wilbur and Anderson 1948 [32]. CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation (to-tc/tc) where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Histopathological process

Tissue samples were fixed in 10% buffered formalin solution. After the routine histopathology process, paraffin sections in 5 μm were stained with hematoxylin and eosine (HE). Histopathological lesions were semi-quantitatively assessed under the light microscope (Olympus BX51 with DP72 camera attachment system). The scores were derived as semi-quantitatively according to the severity and extent of and were reported as follows: none: –, mild: +, moderate: ++ and severe: +++.

Statistical Analyses

All data were presented as mean±SEM. Statistical analysis of data was done using a one-way analysis of variance (ANOVA) and LSD test and analyzed using SPSS version 10.0 software. A value of $p< 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Heavy metals have been found in increasing concentrations in aquatic environments due to factors such as rapid population growth, agricultural activities and industrial organizations. Therefore, aquatic organisms, including fish, are affected by increasing concentrations of heavy metals [33,34]. Heavy metals accumulate in fish tissues and organs [35-37]. Histological techniques are used to assess toxic effect of pollutants including heavy metals and pesticides in the aquatic environment [38]. Gills and liver are suitable organs for histopathological examination to determine the effects of pollution [39,40]. It is noticed that they are the organs most affected by contaminants in the aquatic environment [41]. In liver tissues exposed to chromium were observed hyperaemia, hepatocytes degeneration. These changes were common in treatment groups. It has been reported that Cr caused histopathological effects such as degeneration and necrosis in the liver tissues of goldfish [9]. Our results have shown similar results with other heavy metals in different fish species. For example, Van Dyk et al. (2007) reported lesions such as hyalinization,

Table 1. Concentration dependent decreases in brown troutliver CA activity (U/mg protein)

	1 mg/L	2 mg/L
24 h	147.56 ±1.47	130.44 ± 4.10
48 h	130.64 ± 0.61*	125.97 ± 0.76*
96 h	109.71 ± 1.90*	88.136 ± 5.39*
Control	140.55 ± 3.57	141.47 ± 2.24

* Significantly different from control fish at $p < 0.05$.

Table 2. Concentration dependent decreases in brown trout gills CA activity (U/mg protein).

	1 mg/L	2 mg/L
24 h	90.56 ± 0.32	88.09 ± 0.13
48 h	85.46 ± 0.31*	80.04 ± 0.09*
96 h	73.86 ± 1.43*	58.78 ± 1.99*
Control	91.71 ± 1.47	90.71 ± 1.99

* Significantly different from control fish at $p < 0.05$.

hepatocyte vacuolation, cellular swelling and congestion in the liver tissues of Oreochromis mossambicus exposed to zinc and cadmium [42]. In another study, Suiçmez et al. (2006) observed vacuolation, tubular degeneration and necrosis in the liver tissues of rainbow trout exposed to lead heavy metals [43].

Gills are used in the assessment of aquatic pollutants and they are the target organs which expose to aquatic pollutants [39]. Therefore, toxic pollutants can cause histopathological damage in the gill tissues by disrupting the osmoregulatory function and reducing the oxygen consumption of aquatic species [44,45]. In our study, lamellar hyperemia, epithelial degenerations, edema, lamellar degenerations and atrophy were observed in the gill tissues. These results showed that histopathological damages can be direct responses against chromium. The defense responses will take place at the expense of the respiratory efficiency of the gills and eventually, the respiratory impairment must outweigh any protective effect against pollution uptake [46]. The high deposition of Cr in fish gills causes to tissue damage, including hyperplasia, clubbing of lamellae and necrosis, and the impairment of the ability to osmoregulate and respire [8]. Similar histopathological responses have previously been reported in the gills of fish after exposed to different heavy metals such as lead and cadmium [43,47]. Suiçmez et al. (2006) observed epithelial rupture, fusion, shortening in secondary lamellae in the gill tissues of rainbow trout after exposed to lead heavy metal [43]. Alak et al. (2013) reported hydropic degeneration, vacuolative degeneration, hyperplasia of epithelial cells in secondary lamella and destruction of some secondary lamella in the gill tissues of brown trout exposed to cadmium [47]. The toxicological effects of heavy metals are usually enzyme inhibition and denaturation [48]. CA has previously been purified and characterized from many living organisms including animals [49-52]. Its activity is virtually ubiquitous in nature and involved in a wide variety of physiological processes [53,54]. Heavy metals are known to affect metabolism by decreasing enzyme activities at relatively low doses.

In vivo studies demonstrated to effect of different doses of Cr(III) (1.0 and 2.0 mg/L) exposure at 24, 48 and 96 h on brown trout liver and gill CA activity. There was time dependent decrease in enzyme activity at 1.0 and 2.0 mg/L concentrations after exposure of Cr(III), but no significant difference was observed at 24h ($p>0.05$). CA was significantly inhibited at two concentrations (1.0 and 2.0 mg/L) at 48 and 96 h ($p<0.05$). (Table 1, Table 2). Typical histopathologic lesion was not observed in gill and

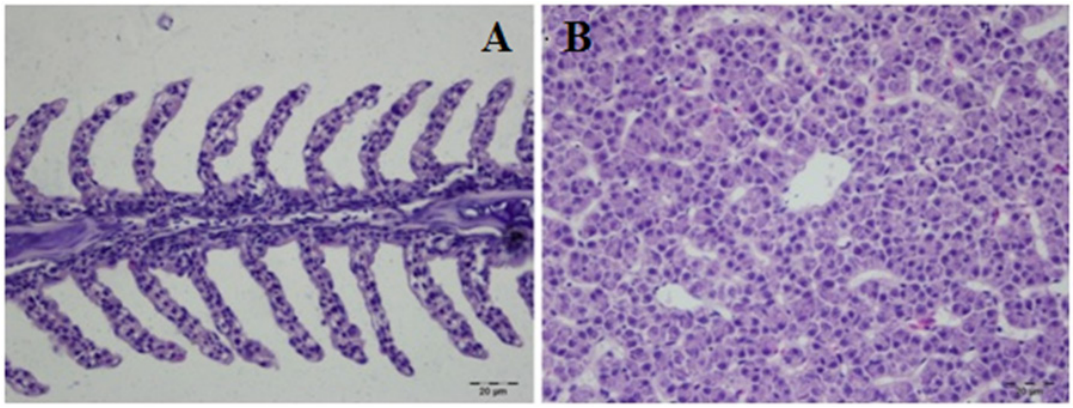

Figure 1. Normal architecture of gill (A) and liver (B) sections from control group, HE.

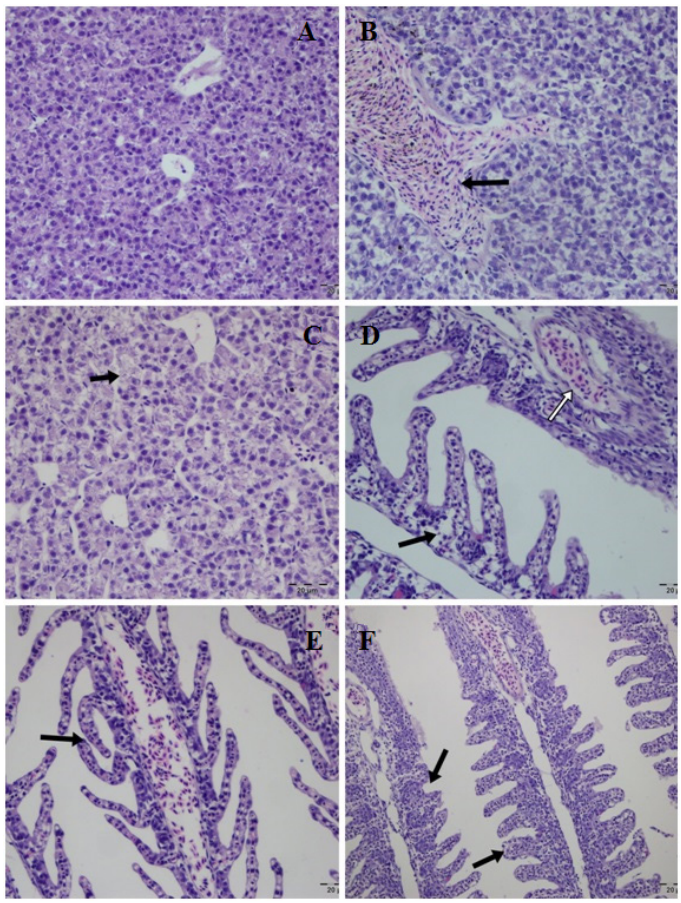

Figure 2. No typical changes at 24 h (A), severe hyperemia at 48 h (B) and (C) degenerative hepatocytes at 96 h in liver of fish exposed to 1 mg/L. Hyperaemic lamellas (white arrow in D) and edema at 24 h (black arrow in D), hyperemia and plumping in lamellas at 48 h (arrow in E) and swelling of lamellas at 96 h (arrows in F) in gill tissue of fish exposed to 1 mg/L. HE.

liver tissues of control animals (Figure 1A and 1B). In the examination of liver tissues, there was no histopathological change in 24 h of 1mg/L (Figure 2A). Hyperaemia in the central veins at 48 h and degenerative hepatocytes at 96 h of fish exposed to 1 mg/L were observed (Figure 2B and 3C). Hyperaemia within the sinusoids and central veins were more severe at 24-48 h of 2 mg/L (Figure 3A and 3B). Degenerative hepatocytes in moderate degree were detected at 96 h of 2 mg/L (Figure 3C). In gill tissues, lamellar hyperemia (Figure 2D and 2E), edema, epithelial degenerations and clumping were observed at 24 h of fish exposed to 1 mg/L. Cellular infiltration were more

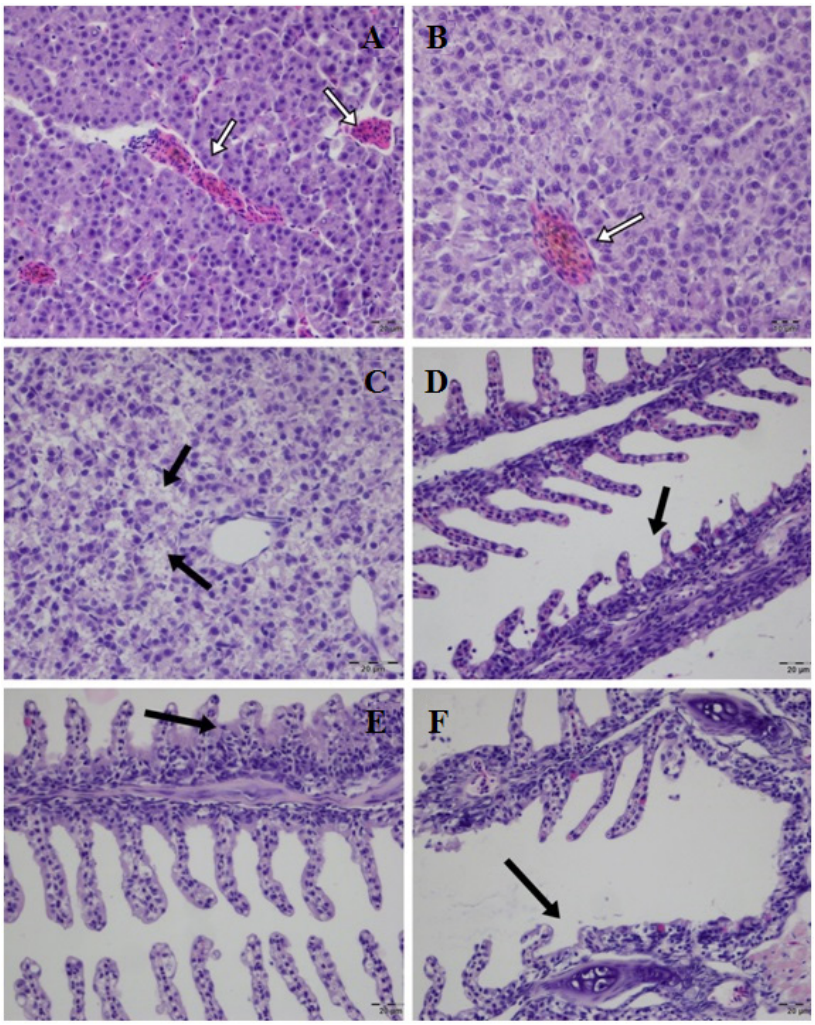

Figure 3. Severe hyperemia between 24 h and 48 h (arrows in A and B) and degenerative hepatocytes at 96 h in liver of fish exposed to 2 mg/L. Shortened lamellae between 24 and 48 h and lamellar loss at 96 h (arrow in F) in gill tissue of fish exposed to 2 mg/L. HE.

prominent at 96 h of same dose (Figure 2F). Lamellar degenerations and atrophy in varying degree were detected at 24-96 h of fish exposed to 2 mg/L (Figure 3D and 3F). Histopathological comparison of groups were displayed in Table 3.

According to the results, we observed that inhibition effect increases and gets faster together with increasing inhibitor concentrations. Thus, Cr^{3+} is potent inhibitors for brown trout liver and gill CA enzyme activity. The results obtained in our study are similar to data reported by many researchers. For example, Soyut et al. (2012) determined the inhibition order as Co^{2+} > Zn^{2+} > Cu^{2+} > Ag+ for muscle tissue [18]. The effects of Co^{2+}, Cu^{2+}, Zn^{2+} and Ag+ were investigated, and the inhibitory effects of Cd^{2+} on brain and kidney tissue were also evaluated. The inhibition rank was Ag+ > Co^{2+} > Cu^{2+} > Zn^{2+} for liver tissue, Co^{2+} > Zn^{2+}> Cu^{2+}> Cd^{2+} > Ag^{+} for brain tissue and Co^{2+} > Zn^{2+} > Cu^{2+} > Ag^{+} > Cd^{2+} for kidney tissue [14,19,55].

CONCLUSION

In our study, Cr^{3+} inhibits the enzyme at very low doses and causes histopathological damages. It indicates that fish are susceptible to Cr^{3+} contamination in natural and cultural environments. This contamination may result in collective fish deaths. This situation may lead disruption of ecological balance. Moreover, it may affect other living organisms including human beings, directly or indirectly.

Table 3. Histopathological comparision of control and experimental groups. none: –, mild: +, moderate: ++ and severe: +++.

Lesion	Control			1 mg/L			2 mg/L		
	24 h	48 h	96 h	24 h	48 h	96 h	24 h	48 h	96 h
					Liver				
Hyperaemia	-	-	-	-	+	-	++	++	-
Hepatocyte degeneration	-	-	-	-	+	+	-	++	++
					Gill				
Lamellar edema	-	-	-	+	++	+	+	++	-
Lamellar hyperemia	-	-	-	++	++	+	++	++	-
Epithelial degeneration	-	-	-	-	-	++	+	++	+++
Lamellar atrophy	-	-	-	-	-	++	+	++	++
Celluary infiltration	-	-	-	-	-	++	-	++	-
Clamping	-	-	-	-	++	-	-	+	-

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