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Influence of Cobalt and Zinc Exposure on mRNA Expression Profiles of Metallothionein and Cytocrome P450 in Rainbow Trout

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Abstract The present research aims to evaluate the effects of cobalt and zinc exposure of rainbow trout (*Oncorhynchus mykiss*) on metallothioneins and cytocrome P450. Mature rainbow trouts were exposed to 10 mg/L $CoCl_2.6H_2O$ and 1 mg/L $ZnSO_4.7H_2O$. After 6, 12, 24, and 48 h of treatment, expressions of muscle MT-A, MT-B, and CYP P4501A1 mRNAs were measured by means of quantitative real-time polymerase chain reaction. During the exposure experiments, no mortalities occurred. We observed that expression levels of all genes increased with exposure time. Since the organism has not learned how to completely dispose of heavy metals and tends to bioaccumulate them, our results indicate that cobalt and zinc exposure may result in accumulation of the non-eliminated metals which may lead to fish death.

Keywords Gene expression · Metallothionein · Cytocrome P450 1A · Heavy metal

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Introduction

Fish are able to readily absorb heavy metals and their bodies regulate to accommodate their presence. If the fish is exposed to further contamination, these substances are easily stored in fatty tissue and tend to bioaccumulate, which means an increase in the concentration of a chemical in a biological organism over time compared with the chemical's concentration in the environment. In addition, heavy metals adjust many cellular pathways [1] and stimulate the expression of a number of genes including metallothioneins and cytochrome P450 [2].

Metallothioneins (MTs) are small proteins with a molecular weight of approximately 6 kDa that are induced by free cytosolic metal ions [3]. Because of a high content of cysteine, MT can also function as an antioxidant [4, 5]. These properties have implicated MT to be an important factor in metal resistance in aquatic vertebrates [5]. Metallothioneins are a class of metalloproteins first described by Margoshes and Vallee [6] in the horse kidney. There are two MT isoforms, MT-A and MT-B in rainbow trout [7]. MT-A is the isoform preferentially responding to metals, whereas MT-B is the constitutive isoform [8].

Cytochrome P450s (CYPs) constitute a superfamily of microsomal monooxygenases capable of catalyzing a variety of reactions generally leading to the oxidation of endogenous and exogenous substrates [9, 10]. Several genes of this superfamily are inducible by xenobiotics that bind to and activate specific intracellular receptors, consequently modulating gene expression [10, 11]. The CYP 4501A family is inducible by, and involved in, oxidative metabolism of exogenous chemicals including heavy metals [2, 12]. Teleost fish species show different inducibilities of CYP1A as measured by enzyme activity, immunological protein quantification, and mRNA levels [13]. An understanding of the regulation of CYP1A expression and its responsiveness in various fish species is an important factor in the interpretation of bio-monitoring information [13].

Heavy metals can reach into water from the mining industry, agricultural run-off, fields, industrial and consumer waste, volcanoes, or even from acidic rain breaking down soils and releasing them into streams, lakes, rivers, and groundwater. This may be hazardous for living systems, including specific enzymes, proteins, and genes [14, 15]. There are many studies with different organisms which evaluated expression levels of various genes including metallothioneins and cytochrome after exposure to heavy metals [2, 16–23]. However, lack of knowledge how short-term exposure to zinc and cobalt affects the gene expression levels in rainbow trout introduces importance of this study. For these reasons, it was aimed in this study to determine time-dependent influences of Co and Zn on metallothionein A, metallothionein B, and cytochrome P450 1A gene expression levels of rainbow trout muscle.

Materials and Methods

Fish Husbandry and Maintenance

Rainbow trout samples were obtained from the Fisheries Department of the Agricultural Faculty at Atatürk University in Erzurum, and weighed of 250±24 g. They were fed twice a day with a commercial pelleted trout feed at a daily ration of 1% of their wet body mass during the study. Trout feed was purchased from Pınar Yem Company, İzmir, Turkey. Feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association.

Prior to experiments, fish in each group were kept in 1×1.2 m (wide-deep) fiberglass tanks for 1 month. Aeration was provided along the experiments. The average water temperature was 9±2°C during the tests. The water quality parameters were analyzed in Laboratory of Erzurum Hygiene Institute. Fiberglass tanks contained 15 fish in 600 cm^3 water. The average dissolved oxygen level was adjusted to 8.8 ppm and maintained by means of ventilation system. One tank was used as control and did not contain any metal while others were added 0.1, 0.5, 1, 5, and 10 mg $CoCl_2.6H_2O$/L and 0.01, 0.05, 0.1, 0.5, and 1 mg $ZnSO_4.7H_2O$/L. The muscle samples were collected after the heavy metal treatment from each group fish at sixth, 12th, 24th, and 48th hours. Tree fish of each of treatments were killed with a sharp blow on the head; muscle tissues were excised and frozen immediately in liquid nitrogen. Samples were stored at −86°C until use.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from frozen muscle tissues of both heavy metal-treated and control fish using TRIzol reagent. RNA was treated with DNase in order to avoid genomic contamination. RNA concentrations and quality were verified by means of Nanodrop spectrophotometer and gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ real-time polymerase chain reaction (PCR) System for First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. All cDNA was stored at −20°C until use.

Primer and TaqMan Probe Design

Primers and TaqMan probes were designed in Primer3 software (v. 0.4.0; http://frodo.wi.mit.edu/) using rainbow trout MT-A (GenBank accession number M18103), MT-B (GenBank accession number M18104), and CYP-1A (GenBank accession number AF015660) sequences and BLASTed to ensure correct mRNA sequences. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene (GenBank accession number NM_001124246). In order to perform multiplex real-time PCR, TaqMan probe of the housekeeping gene was conjugated with Cy5/Blackhole Quencher 2, the fluorophore, and quencher molecules, whereas the probes for target genes were conjugated with FAM/TAMRA. The primer and probe sequences are provided in Table 1.

Multiplex Real-Time PCR

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler Stratagene MxPro3000. The PCR was carried out in a reaction volume of 50 μl containing template DNA, 900 nM of both target and reference forward and reverse primers, 250 nM both target and reference TaqMan probes, and 25 μl FastStart TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil *N*-glycosylase (UNG), dNTP with dUTP, and optimized buffer component. Amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: at 50°C for 2 min for activation of optical AmpErase UNG enzyme, 95°C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Real-time PCR data were analyzed using the ratio of the relative gene expression of a contaminated condition over that of the control group and

Table 1 Sequence of primers and probes used for multiplex real-time PCR

Primers/probes	Sequence 5′-3′
MT-A forward	ATCCTGCAAGTGCTCCAAC
MT-A reverse	ACAAAAGCTATGCTCAAGATGG
MT-A TaqMan probe	^{FAM}-TGCGTGTGCAAGGGCAAGACT-^{TAMRA}
MT-B forward	ATCCTGCAAGTGCTCAAACT
MT-B reverse	ATGACTGCATTGTCACGGTA
MT-B TaqMan probe	^{FAM}-AAAGTTGCTGCCCCTGCTGTCC-^{TAMRA}
CYP450 1A forward	AGTGATGAGTTTGGGCAGGT
CYP450 1A reverse	TCACGGATGTTGTCCTTGTC
CYP450 1A TaqMan probe	^{FAM}-TCGTTACCTGCCCAACCGCAC-^{TAMRA}
GAPDH forward	ATCAAAGGGGCTGTCAAGAA
GAPDH reverse	AGGAGTGGGTGTCTCCAATG
GAPDH TaqMan probe	^{Cy5}- CGCCGAAGGACCCATGAAGG -^{BQ2}

housekeeping gene GAPDH. Analytical sensitivity was confirmed by running standard curves. Amplification efficiency (e) was calculated based on the slopes of the curves (slope) using the formula $e=10^{(-1/\text{slope})}$ [24] and the slope value via Stratagene MxPro3000 software.

Statistical Analyses

All experiments were triplicated. Presented data, expressed as mean values±SE, show differences between control and treated groups. One-way analysis of variance was used to determine the toxic effects of heavy metals. The analyses were performed in Statistica 7.0 (StatSoft).

Results

In the present study, we investigated the effects of zinc and cobalt administration on MT-A, MT-B, and CYP 450 1A gene expression levels in rainbow trout muscle. The results are shown in Figs. 1 and 2. According to the data, we observed that both metals increased MT-A, MT-B, and CYP 450 1A gene expression levels time dependently compared with control group and reference gene GAPDH. The following results were obtained for $CoCl_2.6H_2O$ and $ZnSO_4.7H_2O$.

After cobalt exposure, MT-A expression level (0.516) is less than MT-B at 48th hour (8.440). While the difference in the increase at 48th hour for MT-B is significant ($p<0.05$), the differences between other time intervals are not strong. The difference between MT-A (1.468) and MT-B (3.200) is less in zinc administration. CYP 450 1A expression is higher in cobalt exposure as compared with zinc. For cobalt and zinc, relative gene expression values of CYP 450 1A were found as 6.501 and 0.103, respectively, at 48th hour. The measured water quality parameters were O_2=8.8 ppm, pH=7.9, Mg=8.83 ppm, Ca=31.13 ppm, Na=4.58 ppm, Cl=6.75 ppm, K=2.99 ppm, B=0.17 ppm, Fe = trace, SO_4^{-2}=10.33 ppm, CO_3^{-2}=120 ppm, HCO_3^{-}=146.4 ppm, PO_4^{-3}=trace, NO_3^{-}=3.45 ppm, NO_2^{-}=trace, conductivity=276 us/cm, and total hardness=114 ppm of $CaCO_3$.

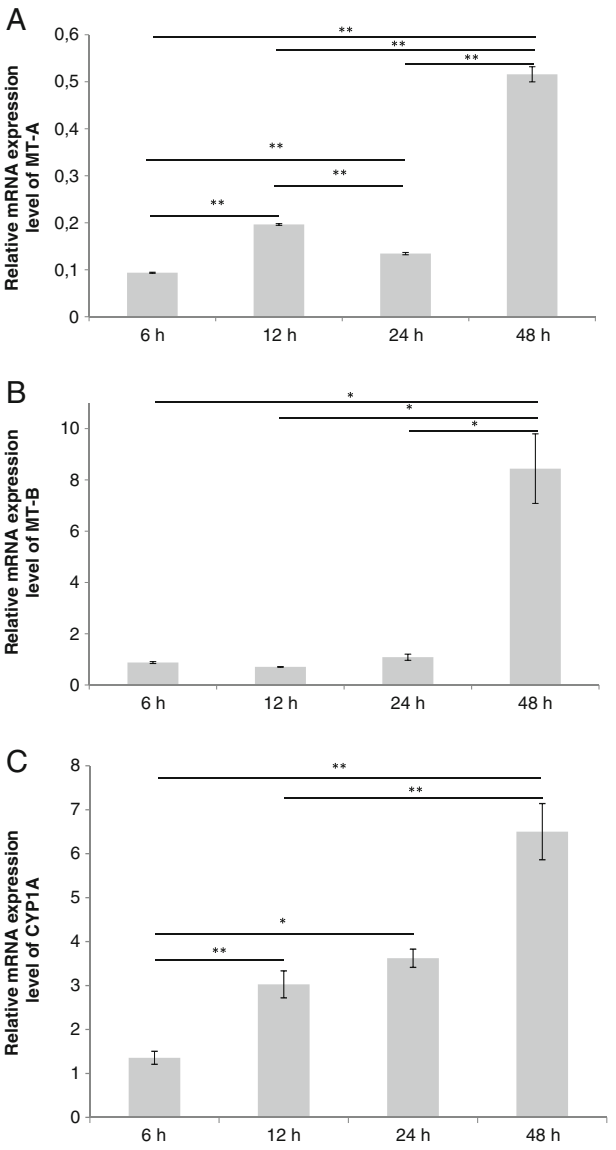


Fig. 1 Relative mRNA levels of metallothionein A (**a**), metallothionein B (**b**), and cytochrome P450 1A (**c**) in *Oncorhynchus mykiss* muscle after exposure to 10 mg $CoCl_2 6H_2O$/L (*p<005; **p<001; ***p<0001)

Discussion

A number of studies have addressed the hazardous impacts of various chemicals, drugs, metals, and anions on enzyme and gene expression levels of different organisms, and this issue has become very popular due to the deep need for environmental safety and ecotoxicology investigations [25–30]. Thus, it is important to perform further studies with various organisms in order to characterize the hazards and to explain mechanisms of actions.

It is interesting that small amounts of heavy metals are common in our environment and diet and are actually necessary for good health, but large amounts of any of them may cause

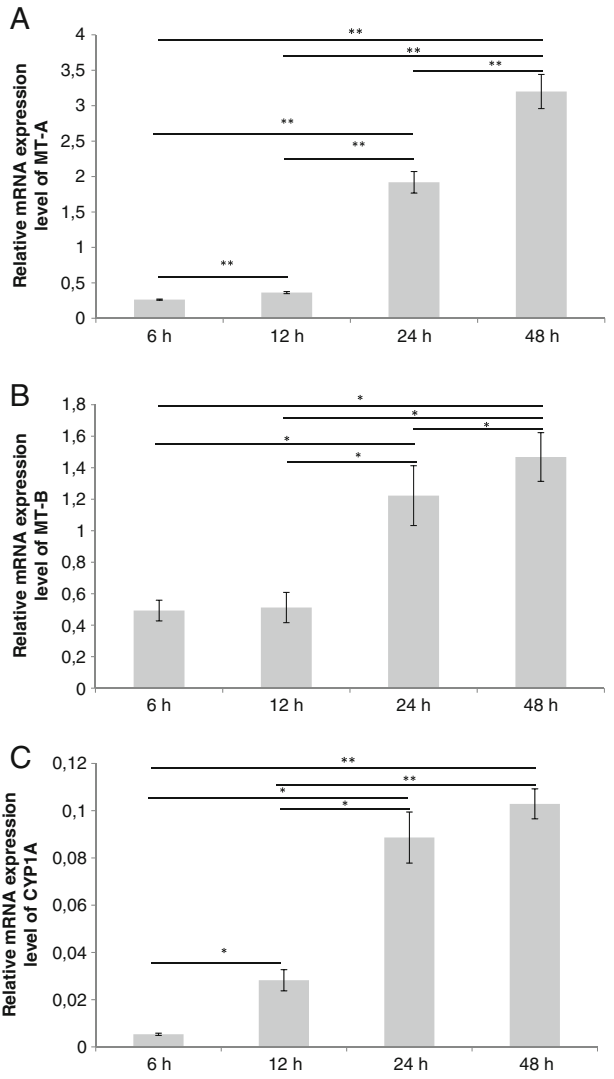


Fig. 2 Relative mRNA levels of metallothionein A (**a**), metallothionein B (**b**), and cytochrome P450 1A (**c**) in *O. mykiss* muscle after exposure to 1 mg $ZnSO_4 7H_2O$/L (*p<005; **p<001; ***p<0001)

acute or chronic toxicity. In general, heavy metal toxicity can result in damaged or reduced mental and central nervous function [31], lower energy levels, and damage to blood composition [32], gills [33], kidneys [34], liver [35], and other vital organs. Cobalt and its salts have high acute toxicity especially on aquatic life when it reached high concentrations [36]. Like cobalt, excess zinc can be harmful, and further cause zinc toxicity. The free zinc ion in solution is highly toxic to plants, invertebrates, and even vertebrate fish [37]. Furthermore, sublethal concentrations of Zn in water may delay growth, sexual maturity, and reproduction in fish [38]. The fish metabolism is more active in higher temperatures then in cooler environments, which allows toxins to act faster. Probably because calcium decreases the uptake across the gills [39], it tends to reduce the toxicity of heavy metals [40] including cobalt and zinc. Both cobalt and zinc and their salts are highly persistent in aquatic environment, with a half-life of greater than 200 days [36].

The organism has not learned how to completely dispose of heavy metals, even in very low concentrations, and tends to bioaccumulate them in the lymphatic and circulatory systems, vital organs such as the brain, the liver, and the muscle, and also in fatty tissues. This accumulation varies depending on species, age of fish, season, feeding habits, time, temperature, interacting agents, metabolic activity of fish, and biological half lives of metals [41]. Research indicated that metal accumulation is more rapid than metal elimination, probably due to the presence of metal binding proteins in tissues, and this has contributed to their deleterious effect [41]. This is because several factors influence the elimination of metals from the tissues of aquatic animals. Elimination routes of metals from fish are generally through gill, bile, urine, skin, and via mucus [41–43]. Most heavy metals form complexes upon inhalation or absorption through the gastrointestinal tract. These complexes then bind to essential amino acids, precipitate proteins, inhibit enzymes, or enter cells.

In light of above-mentioned information, the results of the present study indicate that even a short-term exposure of fish to cobalt and zinc may cause considerable changes in their metal detoxification mechanisms with increasing gene expression levels of metallothioneins and cytocrome P450 1A1 in the organism. Another point worth considering is the sensitivity of the mechanism exhibited by fish against heavy metals in such a short time as 6 h. This exposure may result in either fish death or accumulation of the non-eliminated metals.

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