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IGF and GH mRNA levels are suppressed upon exposure to micromolar concentrations of cobalt and zinc in rainbow trout white muscle

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ABSTRACT

The objective of this study was to assess the effects of cobalt and zinc exposure of rainbow trout (*Oncorhynchus mykiss*) on insulin like growth factors (IGF) and growth hormone (GH). Mature rainbow trouts were exposed to 0.42, 2.1, 4.2, 21 and 42 μmol/L Co^{2+} (added as $CoCl_2 \cdot 6H_2O$) and 0.34, 1.7, 3.4, 17 and 34 μmol/L Zn^{2+} (added as $ZnSO_4i \cdot 7H_2O$). After 6, 12, 24 and 48 h of treatment, expressions of white muscle IGF-I, IGF-II and GH mRNAs were measured by means of quantitative Real Time PCR. During the exposure experiments, no mortalities occurred. The most effective metal concentrations, which caused significant alterations, were determined to be 42 μmol/L Co^{2+} (10 mg $CoCl_2 \cdot 6H_2O$/L) and 3.4 μmol/L Zn^{+2} (1 mg $ZnSO_4 \cdot 7H_2O$/L). The following results were obtained for these concentrations. Expression of IGF-I did not change at 6 h in zinc treatment while the decrease ($p<0.05$) was observed at 12 h and 24 h, and this decrease became stronger at 48 h. Cobalt exposure caused a decrease in IGF-I mRNA level at 6 h, 12 h, 24 h and 48 h ($p<0.05$). Both zinc and cobalt exposure resulted in significant decreases in GH expression at 6 h. Exposure of trout to Zn resulted in a decrease in expression of IGF-II starting from 6 h whereas the significant decrease started at 6 h in cobalt exposure and this decrease elevated at 24 h. The results indicate that micromolar cobalt and zinc exposure causes significant attenuation in the expressions of these three genes' time dependently. Our findings show that IGF-I is the most resistant and GH is the most sensitive component against cobalt and zinc exposure. We conclude that IGF/GH axis might be strongly affected by the short term exposure to low micromolar concentrations of zinc and cobalt due to alterations of these genes.

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1. Introduction

Insulin-like growth factor-I (IGF-I), a single chain polypeptide with 70 amino acid residues, plays a central role in regulating somatic growth in all vertebrates via the modulation of growth hormone (GH) (Mathews et al., 1986). Growth hormone also stimulates the release of insulin-like growth factor (IGF) and these initiate an array of direct and indirect effects on the developing fish (Moksness et al., 2004). GH increases the abundance of IGF-I mRNA by enhancing the transcription of the IGF-I gene, as well as affecting the processing of IGF-I mRNA (Daughaday and Rotwein, 1989). There are two genes for growth hormone (GH-I and GH-II) in rainbow trout which have minor differences. However, in both trout and salmon, GH-I mRNA is predominant (Rentier-Delrue et al., 1989).

It is well known that animal growth is controlled by genetical and environmental factors in vertebrates. Animal growth is also affected by water temperature, photo periods, food availability, metamorphosis (flatfishes, eels) or smoltification (salmonids), sexual maturation and spawning. In most vertebrates, however, nutritional deprivation, which causes growth arrest in juvenile animals, leads to an increase rather than a decrease in circulating GH levels. This phenomenon has been documented in many vertebrate species including humans, sheep, dogs, chicken and fish. These observations indicated that the primary cause of the growth arrest is resistant to GH action at the tissue level and that this mechanism is common in most vertebrates (Duan, 1998).

IGF-II is a single chain polypeptide of 67 amino acid residues that has amino acid homology with IGF-I and proinsulin 1 (Humbel, 1990). Both IGF-I and IGF-II bind to the IGF-I receptor with high affinity, which initiates mitogenic and anti-apoptotic responses in the cell (Osborne et al., 1989; Daughaday and Rotwein, 1989; Perdue et al., 1991; Baserga, 1995). Increased expression of IGFs in a variety of cancer cells and various tumors has been reported (Daughaday,

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1990). Experimental evidence suggests that IGF-II may act as an autocrine growth factor in the pathogenesis not only of childhood cancers but also of several types of adult cancers (Osborne et al., 1989; Daughaday, 1990; Cullen et al., 1992; Christofori et al., 1994; Rogler et al., 1994; Bates et al., 1995; Fichera et al., 2000).

Cobalt is an element that occurs naturally in the environment in air, water, soil, rocks, plants and animals. Likewise, cobalt compounds have been also found to occur naturally in seawater, surface water, spring water and groundwater (Smith and Carson, 1981). Cobalt is beneficial for the body because it is a part of vitamin B_{12}, which is essential for health. It is also used to treat anaemia with pregnant women, because it stimulates the production of red blood cells. In the big aquatic environment, cobalt is needed by blue-green algae and other nitrogen fixing organisms. However, too high concentrations of cobalt may damage aquatic life. In aquatic ambiance, the cobalt can be bound to sediments permanently unless there is a major change in the chemistry of the overlying waters (Bubb and Lester, 1996) and this may be hazardous for organisms including fish. Cobalt can enter the aquatic environment from a number of sources, both natural and anthropogenic. The cobalt is not found as a free metal and is generally found in the form of ores. Natural sources include weathering of rocks and soil, seawater spray, volcanoes, forest fires, decomposition of plant waste, and continental and marine biogenic emissions. It may also enter air and water and settle on land through wind-blown dust and enter surface water through run-off when rainwater runs through soil and rock containing cobalt. One of the main human related releases into the aquatic environment is from sewage due to the advanctage in modern sewage treatment. Although the average level of cobalt in soils is 8 ppm, there are soils with as little as 0.1 ppm and others with as much as 70 ppm. It has been reported that the average cobalt levels of water ranged 2 to 107 ppb (ATSDR, 1992). Because cobalt is capable of bioaccumulating or concentrating in fish tissues, this can cause serious effects even mass fish deaths.

As for zinc, it occurs naturally in air, water, soil and in organisms, but zinc concentrations are rising unnaturally due to the addition of zinc through human activities. Zinc plays an important role in cell division and growth and it is essential for protein and DNA synthesis. It also helps in liver function and proper insulin activity. In addition, zinc is essential for the proper functioning of the sexual organs, found in abundance in semen and a component of many enzymes that help in the metabolism of lipids, carbohydrates and proteins. However, excess zinc can be harmful, and even causes zinc toxicity. The free zinc ion in solution is highly toxic to plants, invertebrates, and even vertebrate fish (Fosmire, 1990). Furthermore, sublethal concentrations of Zn in water may delay growth, sexual maturity and reproduction in fish (Pierson, 1981). Most zinc is added during industrial activities, such as mining, coal and waste combustion and steel processing. In tap water, the zinc concentration can be much higher as a result of the leaching of zinc from piping and fittings (Elinder, 1986; Nriagu, 1980). Some soils are heavily contaminated with zinc, and these are to be found in areas where zinc has to be mined or refined. Water is also polluted with zinc due to the presence of large quantities of zinc in the wastewater of industrial plants, which is not purified satisfactorily. Soluble species of zinc are readily available for biological reactions, therefore considered as the most toxic.

It has been well known that the metabolism and growth is highly influenced by environmental conditions (Ekinci and Beydemir, 2010a, 2010b; Ceyhun et al., 2010; Ekinci et al., 2007a, 2007b; Coban et al., 2007, 2008; Alici et al., 2008; Gulcin et al., 2008) and nutrition through the modulation of the GH-IGF axis (including GH, GH receptor (GHR), IGF-I and IGF-II, IGF receptors and IGF binding proteins) (Duan, 1998; Aksakal et al., 2010). Thus, the modulators of these parameters should be well characterized (Ekinci and Beydemir, 2009a, 2009b; Ekinci et al., 2010).

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 bio-accumulate which means an increase in the concentration of a chemical in a biological organism over time, compared to the chemical's concentration in the environment.

Heavy metals may be hazardous for living systems, including specific enzymes, proteins and genes (Ekinci et al., 2007a, 2007b; Ekinci and Beydemir, 2010b). There are many studies with different organisms which evaluated expression levels of various genes after exposure to heavy metals (Cheung et al., 2004; Wong et al., 2000; Peters et al., 1997; Machala et al., 1997; Hogstrand et al., 1996; Olsson et al., 1990; George, 1986). However, the lack of knowledge on how exposure to metals, particularly zinc and cobalt, affects the IGF/GH axis in rainbow trout introduces the importance of this study. For these reasons, it was aimed in this study to determine time dependent influences of Co and Zn exposure on the expressions of IGF-I, IGF-II and GH in rainbow trout white muscle.

2. Materials and methods

2.1. Fish husbandry and maintenance

One year old rainbow trout samples (mixed sex) were obtained from the Fisheries Department of the Agricultural Faculty at Atatürk University in Erzurum and weighed 150 ± 10 g in autumn, 2009. 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. The trout feed was purchased from Pınar Yem Company, İzmir-Turkey. The feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association (ARENA) (2002) and all experiments were triplicated.

Prior to experiments, the fish in each group were kept in 1×1.2 m (wide-deep) fiber-glass tanks for adaptation during one month. Aeration was provided along the experiments. The average water temperature was 9 °C during the tests. The water quality parameters were analyzed in the Laboratory of Erzurum Hygiene Institute. Total hardness was approximately 140 ppm as $CaCO_3$. Fiber-glass tanks contained fifteen fish in 600 l water. The average dissolved oxygen level was adjusted to 8.8 ppm and maintained by means of ventilation system. The tanks, which did not contain cobalt or zinc, were used as control while others were added 0.42, 2.1, 4.2, 21 and 42 μmol/L Co^{2+} (added as $CoCl_2 \cdot 6H_2O$) and 0.34, 1.7, 3.4, 17 and 34 μmol/L Zn^{2+} (added as $ZnSO_4 \cdot 7H_2O$)(three tanks for each concentration). After metal treatment, white muscle samples were collected from the randomly selected three fish from each group at 6 h, 12 h, 24 h, and 48 h. All fish samples were frozen with liquid nitrogen and stored at −86 °C until RNA isolation.

2.2. RNA Isolation and cDNA Synthesis

Total RNA was isolated from 500 mg of frozen white muscle tissue, between the dorsal fin and lateral line, of both 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 spectrophotometer (Nanodrop) and RNA gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for the First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. All cDNA was stored at −20 °C.

2.3. TaqMan probe and primer design

Primers and TaqMan probes were designed in Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/) using rainbow trout IGF-I (GenBank accession number M95183.1), IGF-II (GenBank accession number

M95184.1) and GH (NM_001124689.1) sequences and BLASTed to ensure correct mRNA sequences. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a suitable reference gene since it was not affected by any of the treatments (NM_001124246). In order to perform multiplex Real Time PCR, TaqMan probe of the reference 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.

2.4. Real-Time PCR

Quantification of gene expression by Real-Time PCR analysis was performed using a thermal cycler Stratagene MxPro3000. The Real-Time 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: 50 °C for 2 min for the 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 efficiency (e)$^{(-\Delta\Delta Ct)}$ method (Pfaffl, 2001), which is used to determine mRNA levels in gene expression against control group and reference 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 $e = 10^{(-1/slope)}$ (Pfaffl, 2001), and the slope value via Stratagene MxPro3000 software.

2.5. Statistical analysis

All experiments were triplicated. Following arc-sine transformation, one-way ANOVA and Duncan's multiple range test via SPSS 9.0 was used to determine the toxic effects of the metals and $p<0.05$ was considered statistically significant.

3. Results

3.1. Expression of IGF-I

Mature rainbow trouts were exposed to 0.42, 2.1, 4.2, 21 and 42 μmol/L Co^{2+} and 0.34, 1.7, 3.4, 17 and 34 μmol/L Zn^{2+}. However, the most effective attenuations were observed for 10 mg/L $CoCl_2 \cdot 6H_2O$ and 1 mg/L $ZnSO_4 \cdot 7H_2O$. Although little dose-dependent alterations were observed, the following data is for the most effective concentrations for the three genes.

The treatment of trout with Zn for 6 h did not result in a decreased white muscle IGF-I mRNA level. However, a significant decrease took place between 12 h and 24 h ($p<0.05$). At the 48 h, strong suppression was observed (Fig. 1.) In cobalt exposure, IGF-I expression was suppressed at 6 h. The decrease in IGF-I mRNA became significant between 12 h and 24 h. The highest decrease took place at 48 h (Fig. 2).

3.2. Expression of GH

Both zinc and cobalt exposure resulted in significant decreases in GH expression starting from 6 h ($p<0.05$) (Figs. 1 and 2).

3.3. Expression of IGF-II

The exposure of trout to Zn resulted in a decrease in the expression of IGF-II starting from 6 h. As for Co treatment, a decrease was seen between 6 h and 12 h ($p<0.05$) and the highest decrease was seen only at 24 h (Figs. 1 and 2).

4. Discussion

There are a lot of substances affecting living systems including heavy metals (Senturk et al., 2009; Ekinci and Beydemir, 2009a, 2009b; Coban et al., 2009). The toxicity of heavy metals depends on a number of factors (Ekinci et al., 2007a, 2007b; Ekinci and Beydemir, 2010a, 2010b). These factors vary according to the metal in question, water hardness and temperature, calcium content of water, the total dose absorbed, type and origin of contamination, permanence in contaminated area and whether the exposure is acute or chronic. For instance, fish can tolerate 10 times the amount of heavy metals at 18° hardness as compared with 1°. The fish's 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 (Comhaire et al., 1994), it tends to reduce the toxicity of heavy metals (Janssen, 2000) including cobalt and zinc. Both cobalt and zinc and their salts are highly persistent in aquatic environment, with a half-life of longer than 200 days (Vincoli, 1996).

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 (Joel et al., 2009). The research indicated that metal accumulation is more rapid than metal elimination, probably due to the presence of metal binding proteins

Table 1
Sequence, amplification length, genbank accession number of primers and probes used for real-time PCR.

Primers and probes	Sequence (3′–5′)	Amplification length (bp)	GenBank accession number
GH-I Forward	AATGGTCAGAAATGCCAACC	201	NM_001124689.1
GH-I Reverse	AAGCAAGCCAACAACTCGTAG		
GH-I Probe	^{FAM}-CATCAACCTGCTCATCACGGGG-^{TAMRA}		
IGF-I Forward	ATGTGCTGTGTCTCCTGTACCC	149	M95183.1
IGF-I Reverse	TAAAAGCCTCTCTCTCCACACA		
IGF-I Probe	^{FAM}-TAACCCTGACTTCGGCGGCA-^{TAMRA}		
IGF-II Forward	GAAGGTCAAGATGATGTCTTCG	108	M95184.1
IGF-II Reverse	AGTTCTCCTCCACATAGCGTTT		
IGF-II Probe	^{FAM}-TCGAGTGCTGGTCATTGCGC-^{TAMRA}		
GAPDH Forward	ATCAAAGGGGCTGTCAAGAA	106	NM_001124246
GAPDH Reverse	AGGAGTGGGTGTCTCCAATG		
GAPDH Probe	^{Cy5}-CGCCGAAGGACCCATGAAGG-^{BQ2}		

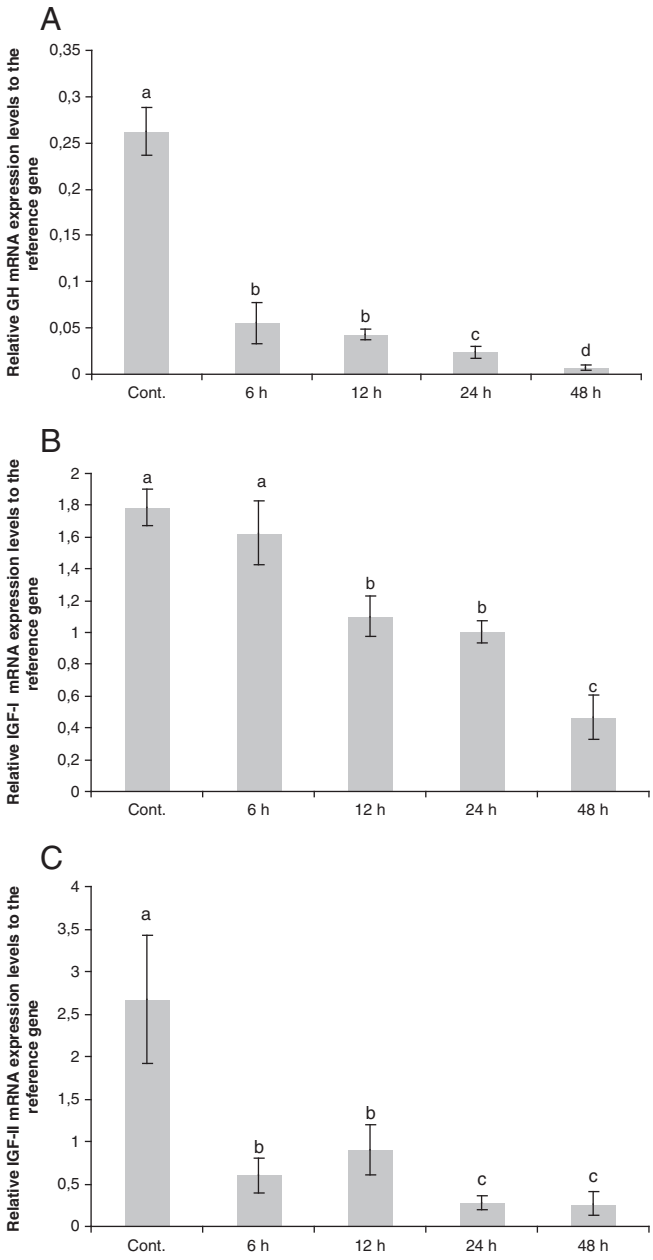


Fig. 1. Relative mRNA levels of GH (A), IGF-I (B) and IGF-II (C) to reference gene in *Oncorhynchus mykiss* white muscle after exposure to zinc. GAPDH was used as the reference gene. Presented data show time dependent alterations in mRNA expressions. a, b, c, d: statistical signifigance between each values in the columns. $p<0.05$ was considered as significant.

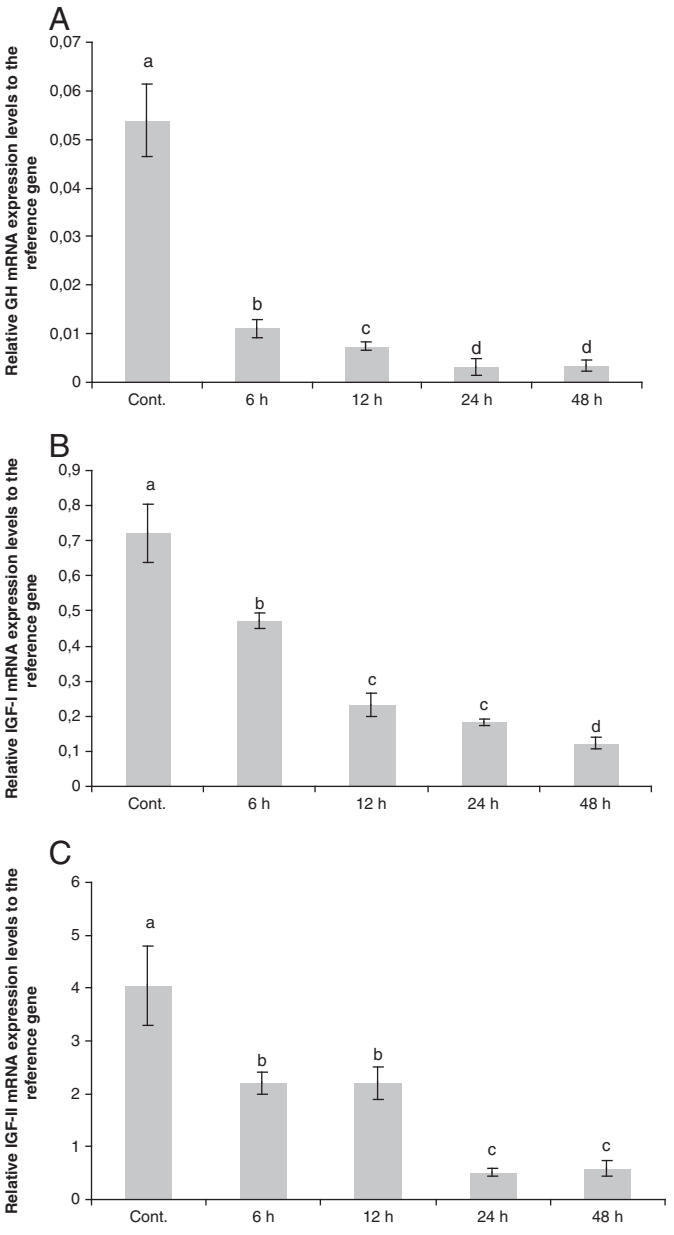


Fig. 2. Relative mRNA levels of GH (A), IGF-I (B) and IGF-II (C) to reference gene in *Oncorhynchus mykiss* white muscle after exposure to cobalt. GAPDH was used as the reference gene. Presented data show time dependent alterations in mRNA expressions. a, b, c, d: statistical signifigance between each values in the columns. $p<0.05$ was considered as significant.

in tissues and this has contributed to their deleterious effect (Joel and Amajuoyi, 2009). This is because several factors influence the elimination of metals from the tissues of aquatic animals.

Heavy metals can enter cells by different routes, such as membrane transport or passive diffusion of neutral chemical species (Delnomdedieu et al., 1992; Gonzalez et al., 2005). Besides, bivalent heavy metals such as Co^{+2} and Zn^{+2} can enter the cells via Ca^{2+} channels promoting the release of cytosolic calcium (Pourahmad and O'Brien, 2000), which may be responsible for an enhancement of cell aggregation and, indirectly for, speeding up larval settlement (Cebrian and Uriz, 2007). In cells, heavy metals can increase reactive oxygen species formation and thus generate an oxidative stress (Stohs and Bagchi, 1995) leading to lipid peroxidation in mitochondria and the release of cytochrome, which constitutes one of the last steps of cell apoptosis (Konigsberg et al., 2001; Gonzalez et al., 2005). To protect themselves against oxidative stress, cells have antioxidant defenses involving enzymes such as catalase, superoxide dismutase, or glutathione peroxidase. It is known that oxidative stress attenuates IGF/GH axis and the suppression of the genes by cobalt and zinc in our study might be due to the oxidative stress caused by the metals.

Lange et al. (2002) assessed the effects of Cd and Zn exposure of rainbow trout (*Oncorhynchus mykiss*) on hepatic glutathione (GSH) levels; and hepatic and branchial metallothionein (MT) mRNA expression. In the study, juvenile rainbow trouts were exposed to waterborne Cd (nominal concentrations: 1.5 or 10 μg Cd L^{-1}), Zn (150 or 1000 μg Zn L^{-1}) or Cd/Zn mixtures (1.5 μg Cd L^{-1} with 200 μg Zn L^{-1} or 10 μg Cd L^{-1} with 1000 μg Zn L^{-1}). They observed that hepatic GSH but not MT mRNA levels were significantly altered in Zn-exposed fish. Cd, on the contrary, affected mainly the MT response but not GSH. Also tissue specific differences in the regulation of the two thiol pools were expressed. Their results indicate that cellular

thiol pools show different reaction patterns with respect to specific metals and metal mixtures.

There are a few investigations regarding alterations in the expression levels of insulin-like growth factors and growth hormone in fish. For instance, chlorpyrifos (CP) and esfenvalerate (EV) (Eder et al., 2008), hexazinone and atrazine (Nieves-Puigdoller et al., 2007), temperature (Luckenbach et al., 2007), cyclic AMP and cortisol (Yada et al., 2005) somatostatins (Very et al., 2008; Poppinga et al., 2007) were demonstrated to have influences on IGF/GH axis. Deltamethrin was also reported to have genotoxic effects on the expression of IGF-I, IGF-II and GH in rainbow trout muscles (Aksakal et al., 2010). It significantly decreased the expression of these genes in time and dose dependent manner.

However, no reports could be determined on the effects of zinc and cobalt exposure on the expressions of IGF-I, IGF-II and GH in rainbow trout. Therefore, we investigated in this study the time dependent alterations in mRNA expression levels of IGF-I, IGF-II and GH in response to Co and Zn exposure. The exposures generally evoked significant declines in the expression of the genes. It is interesting that two metals exhibited similar effects although the cobalt concentration is more than ten times greater than zinc concentration. Another very interesting result is that IGF-I mRNA level is almost the same as the control at 6 h of zinc exposure while it is significantly different at 12 h and very different at 48 h. This demonstrates time dependent influence of the metal because zinc is already efficient at 6 h. From the results, it seems clear that the most sensitive component of the axis to both metals is GH because both Zn and Co caused significant suppression in GH levels starting from 6th hour. Also, IGF-II is very sensitive to zinc exposure as it was extremely attenuated at 6th hour. Overall findings suggest that IGF-I is the most resistant component against zinc and cobalt exposure.

We analyzed single isoforms for each species of mRNA. Indeed, teleosts have undergone whole genome duplication (WGD) in their evolutionary history, and salmonids another WGD. These WGD events are reflected in the number of expressed isoforms of nearly all genes. Thus, gene isoforms of the corresponding hormones in rainbow trout merits further investigation.

5. Conclusion

In many aquatic systems, metal concentrations are elevated over natural background levels due to a continuous release of metals from industrial and agricultural sources. Frequently, metals are present as mixtures in the environment because of their concomitant release from mining activities or industrial uses. Studies on the toxicity of metals for fish have been focused on the effects of short term exposures to single metals at relatively high concentrations rather than investigating the toxic impact of long-term exposures to metal mixtures at environmentally realistic concentrations (Lange et al., 2002). The growth hormone and insulin-like growth factors have gained considerable focus over recent years Here we report on the influences of zinc and cobalt exposure on the expressing levels of IGF-I, IGF-II and GH in rainbow trout. Our findings indicate that short term exposure to these metals result in significant suppression of the corresponding genes. This suppressions might be due to the interactions between metal binding proteins and these metals.

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