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Alterations in Growth Related Genes (*GH-I*, *IGF-I* and *IGF-II*) Expression with Acute Copper Exposure in Rainbow Trout

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Abstract: In this study, the acute effects of copper ($CuSO_4.5H_2O$) on the expression of Growth Hormone-I (GH-I) and Insulin like Growth Factors I and II (IGF-I and IGF-II) were investigated in rainbow trout (*Oncorhynchus mykiss*) muscle tissue. For this purpose, rainbow trout were exposed to 100 μg L^{-1} (Sublethal concentration) copper sulfate in moderately hard freshwater. After 6, 12, 24 and 48 h of treatment, expression of white muscle GH-I, IGF-I and IGF-II mRNAs were measured by means of quantitative real-time PCR. During the exposure experiments, no mortalities occurred. All mRNA levels significantly decreased after copper administration ($p<0.05$). The results indicate that short term copper exposure suppresses the mRNA expression levels of GH-I, IGF-I and IGF-II in rainbow trout muscles.

Key words: Copper, growth hormone, insulin like growth factors, gene expression, multiplex real-time PCR, rainbow trout

INTRODUCTION

Natural water resources have become dirty and unusable because of increasing industry and industrialization. Water pollution is one of the most important current environment problems. Chemical pollution frequently results from usage of pesticides and chemicals in agriculture as well as from development of industry and industrialization especially, recently in waters.

Chemical elements are neither created nor destroyed but are redistributed in the environment. Some of the more toxic elements in this class are metals such as zinc, lead and copper (Di Giulio and Hinton, 2008). Copper is a trace element that plays a fundamental role in the biochemistry of all organisms. Aquatic organisms can take up copper directly from water. Despite the essential role of Cu^{2+} in a number of enzymatic processes, this metal has the potential to exert adverse toxicological effects (Heath, 1995; Cerqueira and Fernandes, 2002; Heerden *et al*., 2004). Copper is well known to be severely cytotoxic, generating oxidative stress and cellular damage if it accumulates beyond the chelating capacity of the cell (Gaetke and Chow, 2003). The typical copper concentrations found in polluted surface waters is around 4 pg L^{-1}. The LD50 for rainbow trout is around 100 μg L^{-1}. Chronic sublethal exposure to Cu^{2+} causes a series of cellular and physiological changes in fish that enable the animals to survive (Henczova *et al*., 2008). Moreover, copper is frequently used as a general biocide in the aquacultural industry (Schlenk *et al*., 1999). Fish compared to invertebrates are more sensitive to many pollutants and are convenient test-objects for water quality assessment. Especially, rainbow trout has been widely used in toxicity testing with fish (Di Giulio and Hinton, 2008). It is well known that fish growth is highly influenced by expression levels of various genes such as the GH-IGF axis (including GH, GH Receptor (GHR), IGF-I and IGF-II, IGF receptors and IGF binding proteins) after exposure to metals and pesticides (Erdogan *et al*., 2007, 2011; Aksakal *et al*., 2010; Ekinci *et al*., 2011).

Growth Hormone (GH) is a pluripotent hormone produced by the pituitary gland in teleosts as in other vertebrates (Reinecke *et al*., 2005). There are two genes for the 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).

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).

IGF-II is a single chain polypeptide of 67 amino acid residues that has amino acid homology with IGF-I and proinsulin1 (Humbel, 1990). Both IGF-I and IGF-II bind to

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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).

There are many studies with different organisms which evaluated expression levels of various genes after exposure to heavy metals (Schlenk *et al*., 1999; Gonzalez *et al*., 2006; Hook *et al*., 2006; Craig *et al*., 2007; Chen *et al*., 2007; Henczova *et al*., 2008; Davis *et al*., 2009). However, little data is available about the effects of Cu^{+2} exposure on the IGF/GH axis in rainbow trout muscles. Therefore, the objective of the present study was to determine time dependent influences of Cu^{+2} exposure on the expression of GH-I, IGF-I and IGF-II mRNAs in rainbow trout muscle.

MATERIALS AND METHODS

Fish husbandry and maintenance: About 1 year old Rainbow trout (100±10 g) were purchased from a local hatchery (Agricultural Faculty at Ataturk University, Erzurum, Turkey). 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 Pinar Yem Company, Izmir-Turkey. Feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association (ARENA) and all experiments were conducted in triplicate.

Prior to experiments, fish in each group were kept in 1×1.2 m (Wide-deep) fiber-glass tanks for adaptation during 1 month. Constant aeration and a 12 h light/dark photoperiod were provided. The average water temperature was 12°C during the tests. The water quality parameters were analyzed in the Laboratory of Erzurum Hygiene Institute. Total hardness was approximately 150 ppm as $CaCO_3$. Fiber-glass tanks contained fifteen fish in 800 L water. The water was renewed during adaptation period. The average dissolved oxygen level was adjusted to 8.6 ppm and maintained by means of a ventilation system. The tanks which did not contain copper were used as controls while the copper concentration in treatment tanks was adjusted to 100 μg L^{-1} $CuSO_4.5H_2O$. After metal treatment, white muscle samples were collected from five randomly selected fish from each group at 6, 12, 24 and 48 h post-treatment. All fish samples were frozen with liquid nitrogen and stored at -86°C until RNA isolation.

RNA isolation and cDNA synthesis: Total RNA was extracted from 500 mg of frozen white muscle tissues, collected from between the dorsal fin and lateral line of both metal treated and control fish using TRIzol reagent (Invitrogen). 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 First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's protocol. All cDNA was stored at -20°C until use.

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 (GenBank Accession Number NM_001124689.1) sequences and BLASTed to ensure specificity (Aksakal *et al*., 2010). GAPDH (Glyceraldehyde-3-phosphate Dehydrogenase) was used as a suitable reference gene since it was not affected by any of the treatments (GenBank Accession Number NM_001124246). In order to perform multiplex real time PCR, the TaqMan probe of the reference gene was conjugated with Cy5/Blackhole Quencher 2, the fluorophore and quencher, respectively whereas the probes for target genes were conjugated with FAM/TAMRA. The primer and probe sequences are shown in Table 1.

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

Primers and probes	Sequence (5'¬3')	Amplification length (bp)	GenBank Accession No.
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}		

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 of both target and reference TaqMan probes and 1X FastStart TaqMan Probe Master Mix (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase Uracil N-Glycosylase (UNG), dNTPs with dUTP and optimized buffer components. Amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: 50°C for 2 min for activation of 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 sec 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 treated groups relative to a 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 was calculated via Stratagene MxPro3000 software.

Statistical analysis: All experiments were conducted in triplicate. The statistical analysis was performed using SPSS (Version 17.0) software. Data were presented as mean±Standard Error of the Mean (SEM) and analyzed by one-way Analysis of Variance (ANOVA) after checking for normality. The significant means were compared by Duncan's multiple range tests at $p<0.05$.

RESULTS

Expression of GH-I: Copper exposure resulted in significant decreases in GH-I mRNA expression starting from 6 h ($p<0.05$) (Fig. 1a). However, there was no significant decrease between 12 and 48 h.

Expression of IGF-I: Treatment of rainbow trout with copper during 48 h resulted in decreased white muscle IGF-I mRNA expression level. A significant decrease took place between 6 and 48 h ($p<0.05$). However, there was no significant decrease between 12 and 24 h. The greatest decrease took place at 48 h (Fig. 1b).

Expression of IGF-II: Exposure of trout to copper resulted in a decrease in expression of IGF-II starting from 6 h.

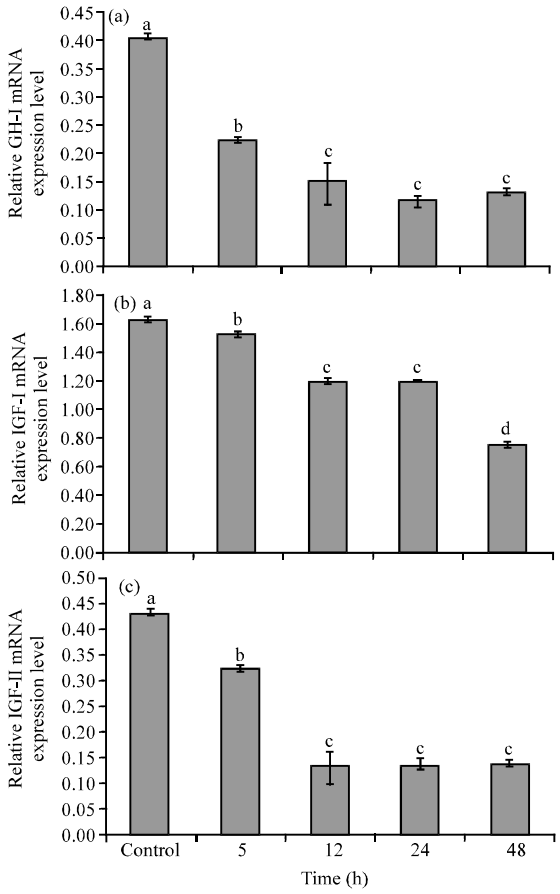

Fig. 1: Relative mRNA expression levels of GH-I, IGF-I and IGF-II (a, b and c, respectively) in the muscles of rainbow trout exposed to copper. GAPDH was used as the reference gene. a-d = Statistical differences at different time points

However, IGF-II mRNA expression was not suppressed from 12-48 h in terms of statistical significance ($p<0.05$). A decrease was seen between 6 and 12 h and the greatest decrease was seen only at 12 h (Fig. 1c).

DISCUSSION

The main goal of the present study was to investigate the effects of copper on growth-related gene expression. Although, relatively good information is available about the acute toxicity of copper to fish, little data is available on growth related gene expression in response to copper exposure.

There are a few investigations regarding alterations in expression levels of insulin-like growth factors and growth hormone in fish. For instance, o,p'-DDE (Dichlorodiphenyl Dichloroethene), heptachlor and 17

β-estradiol (Riley *et al*., 2004; Davis *et al*., 2009) as well as environmental temperature (Gabillard *et al*., 2003) were demonstrated to have influences on the 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 a time and dose dependent manner.

Craig *et al*. (2007) assessed the effects of Cu^{+2} exposure on zebra fish. In this study, exposure to sublethal Cu^{+2} concentrations (15 μg L^{-1}) for 48 h in soft water elicited significant changes in markers of oxidative damage, enzymes in the oxidative stress response pathways and altered mitochondrial properties. Acute Cu^{+2} exposure also induced changes in genes involved in oxidative stress response and in a key metal chaperone.

Also, the normal essential molecular functions of copper have been defined in zebra fish (Hernandez and Allende, 2008). According to this study, copper has a significant role in copper-exporting ATPase activity, high-affinity copper ion transmembrane transporter activity (Mackenzie *et al*., 2004), copper chaperone activity (Craig *et al*., 2007), copper-zinc superoxide dismutase activity (Gonzalez *et al*., 2006), cytochrome c oxidase (Broughton *et al*., 2001), divalent metal transporter (Donovan *et al*., 2002), copper ion binding (Chen *et al*., 2007) induction of metallothionein in response to metals (Chen *et al*., 2002), oxidoreductase activity calcium channel activity (Whitehead *et al*., 2005), copper ion binding, oxidoreductase activity (Hanumanthaiah *et al*., 2002), protein-lysine 6-oxidase activity (Gansner *et al*., 2007), dopamine beta-monooxygenase activity (Ryu *et al*., 2007; Lucas *et al*., 2006) and oxidoreductase activity (Yang and Johnson, 2006).

However, no reports could be found on the effects of copper exposure on the expression of IGF-I, IGF-II and GH mRNAs 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 Cu^{2+} exposure. The exposure generally evoked significant declines in the expression of these genes. Fish exposed to the sublethal dose of Cu^{+2} experienced a significant decreasing of the GH-I, IGF-I and IGF-II in white muscle tissue. Similar alterations have been described for other species contaminated with copper and other heavy metals such as Zn or Co (Ekinci *et al*., 2011).

Researchers also demonstrated that expression of the growth hormone-I and insuline like growth factor I-II genes are related to each other. Some of the actions of growth hormone are through Insulin-like Growth Factor I (IGF-I) (Sakamoto and McCormick, 2006).

CONCLUSION

The study showed that short term sublethal Cu^{+2} exposure suppressed growth related genes in fish muscle at a significant level. These suppressions might be due to interactions between metal binding proteins and these metals. Moreover, GH-I, IGF-I and IGF-II can be used as indicators for determination of metal effects. Each compound or metal generates a unique gene expression signature and these patterns can be determined by qRT PCR (Hook *et al*., 2006). Furthermore, because some genes with altered expression could be related to mode of toxic action, real time PCR data could be used to determine the potential impact of environmental toxicants. The previous research also support the results of the current study.

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