Copper Toxicity and Calcium Supplementation on nkx2.5 and mt2 Gene Expression in Zebrafish Embryos

Sahib Mohammad Bakir [1*], Theodore B. Henry [2], Richard D. Handy [3]

Abstract

Background: Heavy metals are pervasive pollutants in aquatic ecosystems, posing significant threats to the survival and development of aquatic organisms, particularly during their early life stages. While copper is necessary for fish growth and maturation, elevated levels of dissolved copper in water can lead to adverse effects on early developmental stages. The aim of this study was to evaluate the expression pattern of the nkx2.5 gene and the potential of Mt2 expression as a biomarker for metal toxicity. Methods: The study assessed the expression levels of the nkx2.5 gene at different time points during embryonic development, focusing particularly on its peak expression time. Zebrafish embryos were exposed to dissolved copper, either alone or in combination with calcium (Cu+Ca), to examine the effects on nkx2.5 expression. The expression of Mt2, a potential marker for metal toxicity, was also analyzed under copper exposure conditions. Results: The results revealed that nkx2.5 gene expression peaked at 16 hours post-fertilization (hpf), a critical time period for heart formation and development in zebrafish embryos. Exposure to both copper and calcium (Cu+Ca) significantly increased nkx2.5 expression by nearly tenfold compared to control conditions or exposure to copper alone. Furthermore, the expression of Mt2 showed a substantial sixfold increase under copper exposure conditions in the absence of additional calcium (Ca2+), indicating metal-induced stress. Conclusion: The findings of this study determined the susceptibility of zebrafish embryos to metal exposure during early developmental stages, leading to disruptions in cellular biology and heart development.

Keywords: Zebrafish, Heavy metals, Embryonic development, nkx2.5 gene expression, Copper toxicity, Mt2 biomarker, Calcium.

Significance | This study determined how copper toxicity and calcium supplementation affect gene expression in early zebrafish embryos to inform aquatic environmental health and developmental biology.

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Introduction

Pollution has been widely recognized as a significant global issue (Calabrese et al., 1973; Sevcikova et al., 2013). Toxic metals are notable contaminants in the aquatic environment and can lead to decreased survival rates in the early life stages of aquatic animals. Unlike other contaminants, they are non-biodegradable (Wepener et al., 2004) and transform into stable hazardous chemicals (Rainbow and Luoma, 2011). These substances have the capability to be deposited and absorbed in water, accumulating in aquatic creatures at levels higher than naturally present in the environment (Adams et al., 2011).

The presence of metals in the aquatic environment is typically at low levels. However, these levels have risen due to the increasing activities of both natural and human-induced industrial processes (Wepener et al., 2004). Excessive metal accumulation in organisms

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can cause various health issues, such as weakening the immune system (Zelikoff, 1993; Betoulle et al., 2002), triggering stress proteins like metallothionein (Hogstrand et al., 1991), and causing oxidative stress (Hoyle et al., 2007). These chemicals can alter the osmoregulatory system, the balance of electrolytes, and the transcription of genes essential for organogenesis and embryonic development in juvenile fish.

The contamination of aquatic ecosystems by trace metals, including copper, zinc, iron, silver, cadmium, and lead, is well-documented (Hodson, 1988). However, small amounts of certain trace metals, such as copper, zinc, and iron, are crucial for the metabolic processes and proper growth of fish (Canli and Atli, 2003; Wohrle and Pomogailo, 2003). Increased concentrations of these metals can induce adverse consequences (Sanchwitez et al., 2005; Öner et al., 2009). Comprehensive studies have examined the notable effects of essential trace metals, such as copper and zinc, which serve as crucial cofactors for several enzymes (Hogstrand et al., 1996b). Nevertheless, higher amounts of these metals are harmful and poisonous to aquatic life, causing the formation of reactive oxygen species and leading to oxidative stress (Handy, 2003; Grosell et al., 2004; Qu et al., 2014).

Non-essential trace metals, such as cadmium, lack any biological purpose and pose significant threats as pollutants in ecosystems due to their adverse environmental impacts and hazardous nature (Basha and Rani, 2003; Vijayavel et al., 2007; Rainbow and Luoma, 2011; Lahman et al., 2015). Copper, although harmful, exhibits decreased toxicity when bound with water-soluble calcium and proteins such as metallothionein, which facilitate metal removal (Pagenkopf, 1983). Elevated levels of calcium, contributing to water hardness, effectively inhibit copper binding and reduce toxicity at the gills.

Metallothionein functions as an intracellular metal chelator in adult fish exposed to copper poisoning, but its role in developing embryos remains incompletely understood. During the early stages of zebrafish embryonic development, metallothionein is acquired from the mother, particularly from the yolk, with a subsequent decline in its levels during the gastrula stage (Riggio et al., 2003).

To circumvent ethical restrictions associated with mammalian models, zebrafish (Danio rerio) embryos, which share a wide genetic similarity to humans but with fewer legal and ethical constraints, are preferred models for studying gene development and environmental pollution's implications for human health due to chemicals and minerals (Lieschke et al., 2007; Fishman et al., 2001). Additionally, the short early life stage (ELS) of zebrafish provides an optimal means to assess acute and chronic toxicity from aquatic pollutants during the fish's early, sensitive developmental phases (Ali et al., 2011; Tom et al., 2005).

During vertebrate embryonic development, the heart emerges as the initial organ and orchestrates vital functions (Targoff et al., 2008). However, it is susceptible to metal toxicity (Cao et al., 2009; Barjhoux et al., 2012). Gene activation, particularly of nkx, MEF2, GATA, and Tbx, plays a crucial role in regulating heart growth timing and location (McGinnis, 1992). Nkx2.5 has been implicated in initiating cardiac cell differentiation in mammals and zebrafish embryos (Chen & Fishman, 1996). Zebrafish nkx2.5 gene expression is influenced by polycyclic aromatic hydrocarbons (Zhang et al., 2012). While substantial progress has been made in understanding heart formation in zebrafish at the molecular level, the impact of metal exposure remains uncertain, despite the relevance of copper balance to human congenital heart abnormalities (Hu et al., 2014).

This study aims to investigate how exposure to copper and silver affects the expression of gene transcripts for nkx2.5 and mt2 during embryonic development. Additionally, it aims to summarize calcium's protective effects against copper intoxication and its significance in heart function. The study's objective is to evaluate the influence of calcium supplementation on Cu-dependent reactions concerning these parameters.

Materials and Methods

Experimental animals

Adult male zebrafish (Danio rerio) ranging from 0.5 to 1.5 years old were obtained from the University of Plymouth's zebrafish research center. These adult zebrafish were used to generate embryos for experimental purposes. The facility was maintained at a temperature of 28°C with a 12-hour photoperiod. The adult fish were housed in glass aquaria containing dechlorinated City of Plymouth municipal water, with a daily 10% water replenishment through a recirculating system. The fish were fed flake food (Tetramin) twice a day and given Artemia nauplii once a day. Fully mature fish were bred, and fertilized embryos were promptly collected, thoroughly purified, and used for tests within two hours of fertilization.

Experimental design

The experiments were conducted with two objectives in mind.

Firstly, to establish any potential correlation between the stage of embryonic development and the expression of the nkx2.5 gene transcript.

Secondly, to observe the impact of copper toxicity, both in the presence and absence of calcium, on the transcription of the nkx2.5 and mt2 genes.

The impact of copper toxicity in the presence and absence of calcium supplementation

The experimental design involved varying copper concentrations (0, 100, 250, and 500 µg Cu L-1) in water while keeping calcium levels constant. The treatment group had matching Cu concentrations but received additional calcium supplementation (approximately 40 mg Ca2+ L-1) in freshwater, for up to 72 hours.

The exposure investigation was conducted in triplicate, using 400-mL glass beakers filled with 300 mL of exposure water. Each beaker contained 170 fertilized eggs, and exposure was concluded at 16 hours post-fertilization (hpf) after a duration of 2–16 hpf. From each beaker, 100 viable embryos were selected for gene expression analysis and preserved at -80°C until analysis.

***Nkx2.5* and *mt2* genes expression**

The correlation between nkx2.5 gene expression and embryonic development stage was investigated through two experiments. Each experiment utilized a cohort of embryos fertilized simultaneously (within a 15-minute window) from different combinations of male and female fish. Embryos younger than 1 hour post-fertilization (hpf) were distributed into eight 400-mL glass beakers, each containing 170 embryos, and filled with 300 mL of fish water. A random time post-fertilization (hpf) was assigned to each beaker, at which point all embryos within were sampled for gene expression analysis.

In the first experiment, sampling occurred at 5, 10, 13, 16, 19, 24, 28, and 34 hours post-fertilization (hpf). In the second experiment, samples were taken 16 hours post-fertilization (hpf) from embryos exposed to copper concentrations of (100, 250, and 500 µg l-1), both with and without calcium supplementation.

Preparation of embryo homogenate for gene expression

One hundred viable embryos were obtained through experimental methods and then transferred into 1.5 mL Eppendorf tubes for gene expression investigation. These tubes were placed in a container filled with crushed ice, and excess water was removed using a pipette. The tubes were briefly exposed to a temperature of -80°C before the thawed embryos underwent sonication using the Misonix Microson XL, an ultrasonic cell disrupter with 20 intensity levels manufactured in the United States.

The process began by adding 350 µl of RLT buffer to the frozen embryos. To maintain a low temperature during sonication, the Eppendorf tube was positioned within a 100-mL glass beaker filled with crushed ice. This precaution aimed to minimize potential damage to nucleic acids caused by elevated temperatures. Before sonication, the sonicator probe was sterilized with 70% ethanol and then carefully inserted into the center of the sample to ensure effective sonication.

Continuous sonication was carried out initially for 5 seconds at a burst strength ranging from 2 to 3. Subsequently, another round of sonication was performed to disintegrate fully developed, observable embryos. After each round of sonication, the samples were promptly returned to ice until processing of the remaining samples was completed.

Extraction of total RNA

The researchers utilized the Qiagen RNA Easy Mini Kit for Animal Tissue from the Center for Environmental Biotechnology (CEB) at the University of Tennessee, USA, to isolate total RNA from zebrafish embryos. Initially, 350 microliters of embryo homogenate were combined with RLT buffer and loaded onto a QIA shredding spin column. The spin column, placed in a 2 mL collecting tube, underwent centrifugation at 16200 g for 2 minutes. The resulting substance was transferred to a new 1.5 mL Eppendorf tube and purified with a 3-minute centrifugation step. Following this, 350 µl of 70% ethanol was added to the lysate, and 700 µl of the mixture was loaded onto the RNeasy minicolumn in a 2 mL collection tube. Centrifugation for 15 seconds at 16200 g resulted in RNA binding to the silica gel during elution flow collection.

DNA digestion

To purify the RNA sample and remove any DNA contamination, DNase treatment was performed. Initially, the contents of the RNeasy tiny column were cleansed by transferring 350 µl of buffer RW1 using a pipette. The column was then centrifuged at 16200 g for 15 seconds, and the separated liquid was discarded. Subsequently, 80 µl of each sample, comprising 10 µl of DNase I concentrated solution and 70 µl of RDD buffer, was carefully distributed onto the silica gel membrane of the RNeasy micro column using a pipette. The micro column was left on the laboratory bench at 20 ± 2 °C for 15 minutes. Following this, another 350 µl of RW1 was used to purify the RNA bound to the silica gel, and the waste liquid was discarded after centrifugation at 16200 g for 15 seconds. To ensure complete absence of contamination, the mini column was transferred to a fresh 2 mL collection tube. The method involved two consecutive injections of 500 µl of RPE buffer to thoroughly wash the microcolumn. The waste liquid was disposed of after undergoing centrifugation at a maximum force of 200 times the acceleration due to gravity for a duration of 15 seconds. To verify the absence of moisture in the RNeasy silica membrane gel, an extra centrifugation step was conducted on the small column. This phase lasted for a duration of 2 minutes, with a maximum velocity of 16200 g. Upon finishing the RNA extraction procedure, the column was transferred to a fresh 1.5-mL Eppendorf tube for collection. Subsequently, a volume of 30 µl of RNase-free water was immediately added to the RNeasy silica gel membrane. The column was subjected to a 2-minute resting time and thereafter underwent centrifugation at 16200 g for one minute to extract the RNA. Using a Nano Drop spectrophotometer, particularly the Labtech International Nano Drop ND-1000 Spectrophotometer, it was possible to measure the RNA concentration by measuring the absorbance at a wavelength of 260 nm. The optimal threshold for spectrophotometer quantification of RNA is below 100 ng/µl. The RNA/DNA absorbance ratio at 260:280 should be between 1.9 and 2.2. This ratio is used to check how pure the RNA is and find protein contamination. The 260/230 absorbance ratio for RNA and protein must be within the range of 1.9 to 2.0. RNA elute samples were stored at -80°C for subsequent procedures. Total RNA from zebrafish embryos was isolated using

the Qiagen RNA Easy Mini Kit for Animal Tissue. This kit was developed at the Center of Environmental Biotechnology (CEB), University of Tennessee, USA.

To begin the isolation process, 350 µl of embryo homogenate was pipetted into a QIA shredding spin column. The spin column was then placed into a 2 mL collection tube to start the extraction. After centrifugation at the highest level (16200 g) for 2 minutes using a Micro Centaur device, the liquid was carefully transferred to a new 1.5 mL Eppendorf tube.

Following this, another centrifugation step lasting 3 minutes was performed to enhance the purification of solid components by precipitating them. The lysate was then moved to a fresh 1.5 mL Eppendorf tube and mixed with 350 µl of 70% ethanol. This resulting mixture, totaling 700 µl, was transferred to the RNeasy minicolumn, positioned within a 2 mL collection tube.

The sample underwent centrifugation at a maximum acceleration of 16200 g for 15 seconds. Subsequently, the flow-through was discarded, leaving the RNA attached to the silica gel.

Reverse transcription of RNA to generate cDNA

The ImProm IITM Reverse Transcription System, manufactured by Promega and distributed by Sigma-Aldrich, was used to generate complementary DNA (cDNA) from whole RNA. A substantial amount of cDNA was produced, facilitating the creation of duplicate samples for quantitative polymerase chain reaction (Q-PCR) analysis. For cDNA synthesis, 800 nanograms (equivalent to 8 microliters) of RNA were employed per sample. The cDNA was then transferred into sterile and nuclease-free Bilatec 0.2 ml PCR tubes using a pipette, with 16 microliters of RT-Mix added to each tube. The RT-Mix consisted of 6.6 µL of nuclease-free water, 4 µL of ImProm-IITM 5X reaction buffer water, 2.4 µL of MgCl2 (25 mm), 1 µL of dNtp mix (10 mM per dNTP), 1 µL of hexa nucleotide primers, and 1 µL of ImProm-IITM Reverse Transcriptase. Each tube was securely sealed and handled carefully to ensure preservation, with ice strategically placed to minimize contamination and evaporation. The cDNA synthesis was conducted using a thermal cycler instrument (GeneAmp® PCR System, 9700, Applied Biosystems), involving annealing at 25 °C for 5 minutes, followed by extension at 42 °C for 60 minutes. Inactivation of the transcriptase was achieved by incubating all tubes at 70 °C for 15 minutes. The cDNA samples were stored at -80 °C until required for q-RT-PCR analysis of gene expression.

Quantitative reverse transcriptase PCR (qRT- PCR)

The Primer Blast tool was employed to choose the primers for the genes nkx2.5, mt2, and the housekeeping gene (β actin) from the GenBank database of the National Center for Biotechnology Information (NCBI). The primer combinations used for nkx2.5 were as follows: the forward primer sequence was 5'-AGTTCTCTTCTCTCAGGCGCAGG-3', while the reverse primer sequence was 5'-TGGCACAGAGATGCGTCTCGGA-3'. The forward primer sequence for Mt2 was 5'-CTGCGAATGTGCCAAGACTGGAAC-3', and the reverse primer sequence was 5'-GCGATGCAAAACGCAGACGT-3'. The primers utilized for the housekeeping gene β actin were as follows: the forward primer sequence was 5-ACACAGCCATGGATGAGGAAATCG-3, and the reverse primer sequence was 5-TCACTCCCTGATGTCTGGGTCGT-3. Table 1 contains the specific information about the primers.

We used the Sigma SYBR® Green StartTM Taq Ready MixTM technique to evaluate how much the nkx2.5, mt2, and β actin genes were expressed. The freeze-dried primers (Eurofins MWG Operon, Ebensburg, Germany) were mixed with nuclease-free water at first until the concentration reached 100 µmol L-1. After completing the previous step, the SYBR Green JumpStart Taq Ready Mix was introduced into the initial mixture. The reaction volume was adjusted to 20 µl, and the concentration was set at 375 nmol. The master mixture for each sample was made as follows: The solution comprises 12.5 µl of SYBR Green JumpStart Taq ReadyMix, in addition to 0.75 µl of forward primer and 0.75 µl of reverse primer. The qRT-PCR experiment employed Applied BiosystemsTM MicroAmp® Fast 96-well plates obtained from Thermo Fisher Scientific in China. The sample was diluted at a 1:10 ratio, and then, 6 µl of the diluted sample was combined with 16 µl of Ready Mix. Furthermore, a negative control was generated by employing 20 liters of water that is devoid of nuclease activity. A no template control was prepared by combining 6 liters of nuclease-free water with 16 liters of Ready Mix. Three replicates were performed for each of the target gene samples, negative controls, no template controls, and β-actin. The fluorescence was measured using the StepOne Real-Time PCR System from Applied Biosystems for a total of 40 cycles. The heat qPCR reaction technique consisted of the following stages: The technique consists of two stages: the holding stage, which involves incubating at a temperature of 94 °C for a period of 2 minutes, and the cycling stage, which necessitates 40 cycles. The cycle begins with a denaturation phase at a temperature of 94 °C for a length of 15 seconds. Next, primer annealing occurs at a temperature of 55 °C for 1 minute, followed by an extension phase at 72 °C for 1 minute. Fluorescence is measured at several points during this operation. Plate normalization was accomplished by performing the cDNA standard curve on the identical plate.

The qRT-PCR studies were performed using a cycle threshold of 25,000. The fold changes (2-ΔΔCt) were calculated by comparing the expression levels of the target genes with those of the reference control. In 2009, Henry et al. proposed the use of the comparative quantification approach (2-Ct) to assess the fold-changes of the nkx2.5 and Mt2 gene transcripts. The qRT-PCR experiment was conducted using Applied Biosystems™ MicroAmp® Fast 96-well plates from Thermo Fisher Scientific, China.

Table 1. The Zebrafish (Danio rerio) gene-specific primers. The names of the primers (nkx2.5, mt2, and β-actin), the NCBI reference sequence numbers, and the length of the product in base pairs.

Gene	Ref.seq.no.	Forward (5'-3')	Reverse (5'-3')	Product (bp)	Annealing Temp.(°C)
Nkx 2.5	NM_131421.1	AGTTCTCTTCTCTCAGGCGCAG	TGGCACAGAGATGCGTCTCGGA	223	58
Mt2	NM_001131053.2	CTGCGAATGTGCCAAGACTGGAAC	GCGATGCAAAACGCAGACGT	243	
B-actin	NM-131031.1	ACACAGCCATGGATGAGGAAATCG	TCACTCCCTGATGTCTGGGTCGT	138	55

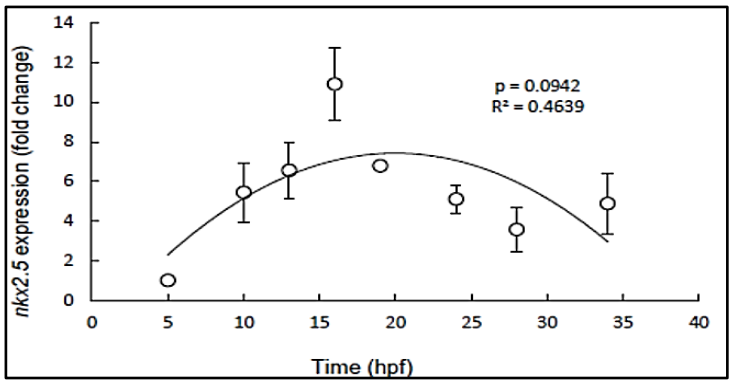

Figure 1. The fold change in nkx2.5 expression was measured in two separate experiments based on the stage of development of the zebrafish embryo. Embryos were collected at different times after fertilization, and the level of nkx2.5 expression was measured using the delta-delta Ct method. β-actin was used as a standard for comparison. Each data point represents the proportional alteration in gene expression for a single sample of 100 embryos. The disparity in concentrations was evaluated through the execution of a one-way ANOVA, yielding a p-value greater than 0.05.

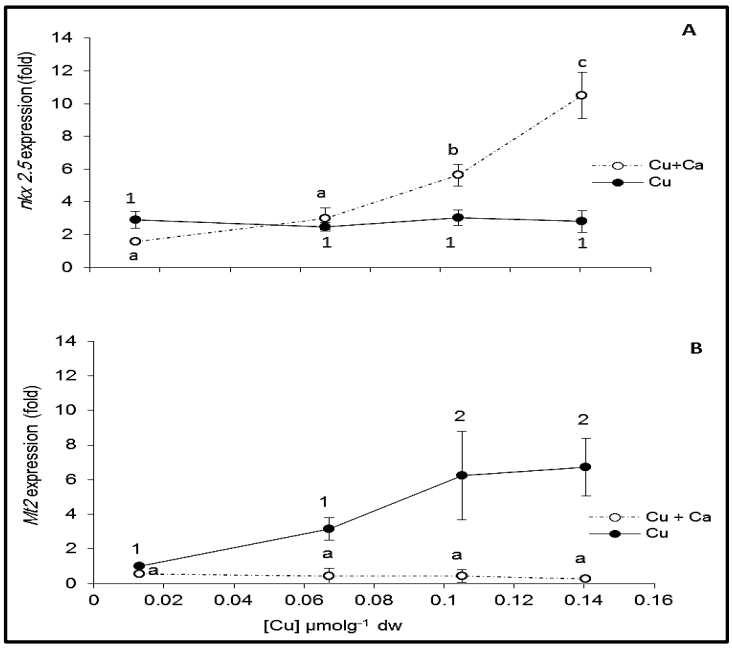

Figure 2 displays the expression of the nkx2.5 (A) and mt2 (B) genes, which are associated with the buildup of copper in living zebrafish embryos. Embryos were collected at 16 hours post-fertilization. The fold changes in gene expression were determined using the 2ΔΔCt technique, with β-actin serving as the reference gene for normalization.

The sample was diluted at a ratio of 1:10, and then 6 µl of the diluted sample was mixed with 16 µl of Ready Mix. A negative control was created by utilizing 20 µl of nuclease-free water, while a no template control was generated by combining 6 µl of nuclease-free water with 16 µl of Ready Mix. Each of the target gene samples, negative controls, no template controls, and β-actin were subjected to three replicates.

The Zebrafish (Danio rerio) gene-specific primers are listed in Table 1, which includes the names of the primers (nkx2.5, mt2, and β-actin), the NCBI reference sequence numbers, and the length of the product in base pairs.

Statistical analysis

The data were analyzed using Stat Graphics Plus version 5.1. The effect of copper, with and without additional calcium, was assessed at the end of the experiment (16 hours after fertilization) through one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) test was employed to detect differences among treatment means. The validity of each ANOVA was assessed using Bartlett's test. Additionally, a two-way ANOVA was conducted to examine differences in the outcomes of copper exposure therapy with or without calcium. Results are presented as mean values ± standard deviation. Gene expression levels of nkx2.5 and mt2 were measured using the 2-Ct technique, normalized to the β-actin reference gene, as described by Henry et al. (2009).

Results

***Nkx2.5* expression**

The nkx2.5 gene transcripts were scarcely observed during the initial phases of embryonic development, particularly at 5 hours post-fertilization (hpf). Subsequently, there was a gradual increase observed between 10 and 16 hours post-fertilization. However, in the later stages of development, notably between 18 and 34 hours post-fertilization, there was a significant decrease in the expression of nkx2.5 gene transcripts (Figure 1). The degree of nkx2.5 expression exhibited significant variability ($R^2 = 0.49$) throughout embryonic development. The quadratic equation showed a statistically insignificant correlation (ANOVA, $P > 0.05$), with the highest level of expression at 16 hours post-fertilization.

Gene expression

The study involved targeted gene expression analysis to assess the impact of calcium regulation on copper toxicity. Elevated calcium levels and increased copper concentrations led to an upregulation of the nkx2.5 gene. However, embryos exposed to copper at typical calcium concentrations found in Plymouth water showed no discernible impact on nkx2.5 expression. Conversely, mt2 expression in embryos increased concomitantly with copper exposure under normal calcium levels in Plymouth water, but additional calcium did not produce any noticeable effect on mt2 expression despite the increased calcium content in the water.

The data presented are average values ± standard error of the mean (SEM) based on a sample size of 3 for each treatment. Two methods were used to expose the embryos to copper: one with calcium addition (solid circles) and the other without (open circles). The presence of copper, with and without calcium inclusion, significantly affected distinct letters and numerals compared to the control group (ANOVA, $P < 0.05$). However, a p-value greater than 0.05 in the one-way ANOVA analysis indicates no significant effect of identical letters and numbers on the treatments.

The study revealed a correlation between nkx2.5 gene transcripts and copper levels in the presence of additional calcium. Nonetheless, the presence of copper alone, without calcium inclusion, did not result in any noticeable effect on nkx2.5 expression in the embryos.

Discussion

Previous studies have identified Nkx2.5 and the zinc-finger factor Gata4 as early markers crucial for precardiac cell development (Durocher et al., 1997; Zhang et al., 2012). Nkx2.5 is known to play pivotal roles in myocardial cell fate and initiation of cardiogenic differentiation (Zhang et al., 2012; Balci & Akdemir, 2011). Loss of Nkx2.5 in mice results in impaired cardiac looping and disrupted gene expression associated with cardiac muscles, while lack of Gata4 leads to disruptions in late cardiac morphogenetic movements (Jamali et al., 2001; Holtzinger & Evans, 2005).

In this study, we observed an increase in nkx2.5 expression in embryos compared to specific stages of embryonic development. The highest expression levels were noted during the segmentation phase, particularly between 10.33 and 24 hours after fertilization, peaking at exactly 16 hours after fertilization. Embryos exposed to copper without additional calcium did not show significant changes in nkx2.5 expression within 16 hours post-fertilization (hpf). However, supplementation with calcium resulted in a significant upregulation of the nkx2.5 gene, accompanied by an increase in copper accumulation within the embryos.

Copper (Cu) and calcium (Ca) compete for binding to the Cys2-His2 zinc finger protein, which activates nkx genes (Lee et al., 1998), thereby enhancing nkx2.5 gene expression. Calcium ions (Ca2+), acting as second messengers, can modulate gene expression (Hardingham & Bading, 1999). Both extracellular and intracellular calcium contribute to increased nkx2.5 expression by activating RNA-binding proteins (Ikura et al., 2002), which play a significant role in stimulating specific RNAs (Blech-Hermoni & Ladd, 2013).

The expression of nkx2.5 may be enhanced to accelerate the differentiation of myocardial myocytes during early embryonic development while also compensating for potential reductions in gene expression due to toxicity. Previous research has shown that exposing zebrafish embryos to hexabromocyclododecane (HBCD) for 72 hours leads to increased heart rates and cardiac arrhythmia.

This increase in heart rate is accompanied by elevated nkx2.5 levels. It is hypothesized that excessive HBCD can disrupt the zinc binding site or interact with the thiol group of cysteine and histidine (C2H2) zinc finger proteins, thereby hindering the catalytic action of zinc and disrupting gene expression. Unlike embryos at 24 hours old (Fig. 4.7B), those that were 72 hours old or had already hatched did not express nkx2.5, suggesting the gene is no longer essential as cardiac myocyte differentiation is complete.

Research has shown that copper increases the activity of metallothionein genes in fish embryos during the gastrulation and segmentation stages, while maternal metallothionein levels decline (Riggio et al., 2003). Metallothionein 2 (Mt2) is a well-known biomarker for metal toxicity and provides protection against various stressors, including metal toxicity and temperature-induced shock. Different metallothionein genes, such as mt1 and mt2 mRNA, are more highly expressed in common carp under stress (Hermeszs et al., 2001). In a previous study on Danio rerio, cadmium exposure increased the expression of both mt2 and mt1 genes (Gonzalez et al., 2007). Our study demonstrated that initial exposure of zebrafish embryos to copper resulted in an upregulation of mt2 gene expression. Subsequently, the addition of calcium led to a downregulation of mt2 expression. This phenomenon can be attributed to the competition between calcium (Ca) and copper (Cu) ions for binding to the active site of metal response elements (MREs) in the zebrafish genome (Cheuk et al., 2008).

Conclusion

The expression of nkx2.5 genes remained unchanged when exposed to copper (Cu) alone, but showed sensitivity to Cu in the presence of additional calcium (Ca). This suggests that the interaction between Cu and Ca may influence nkx2.5 gene expression. However, further research is needed to explore this interaction more thoroughly and to understand its effects on nkx2.5 gene expression in the heart. Such studies could provide deeper insights into the molecular mechanisms involved and potential implications for cardiac health.

Author contributions

S.M.B., T.B.H., R.D.H. conceptualized, literature review, data analysis, edited and reviewed the manuscript. All authors read and approved the final version of the review.

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Competing financial interests

The authors have no conflict of interest.

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